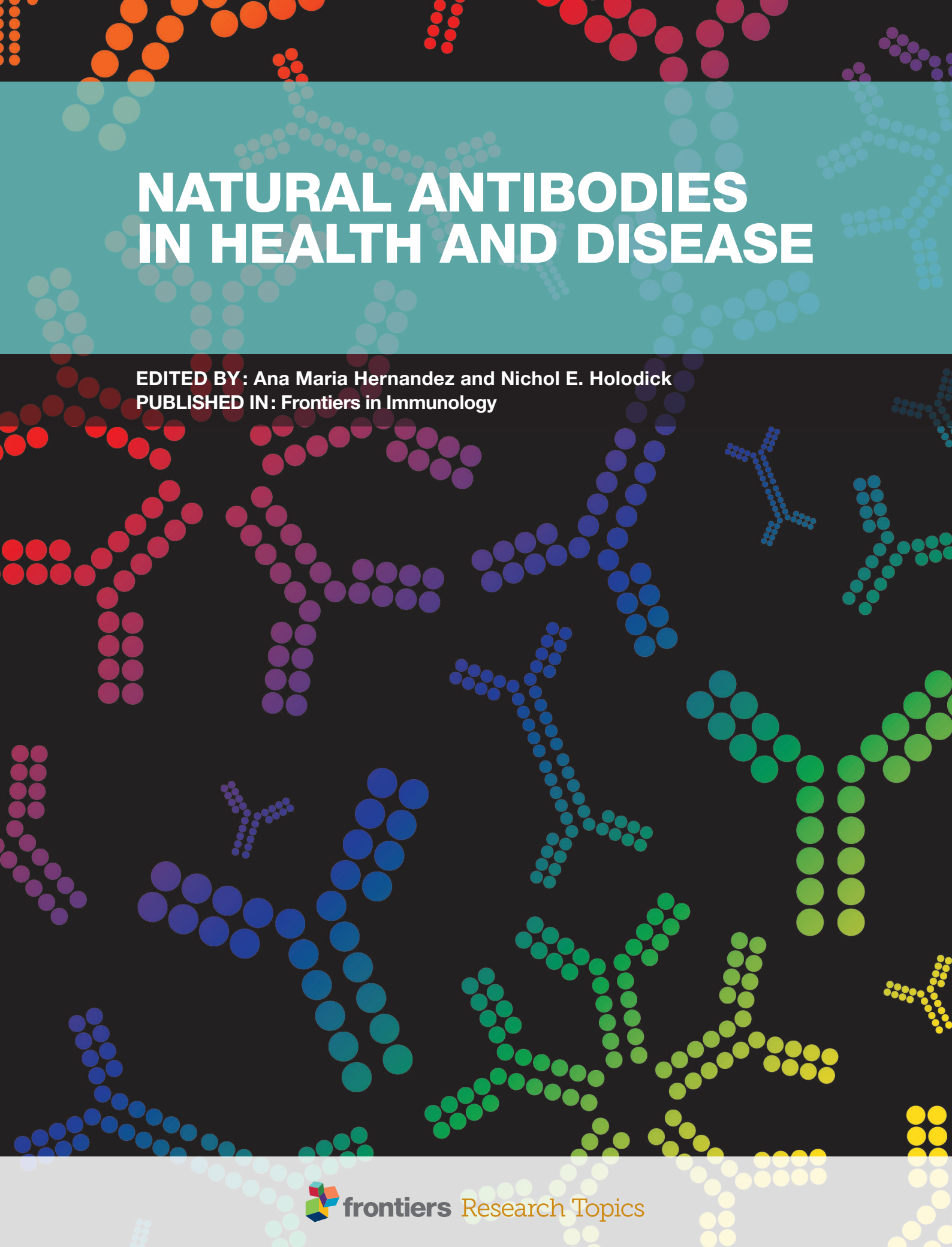
NATURAL ANTIBODIES IN HEALTH AND DISEASE

EDITED BY: Ana Maria Hernandez and Nichol E. Holodick
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-405-1
DOI 10.3389/978-2-88945-405-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

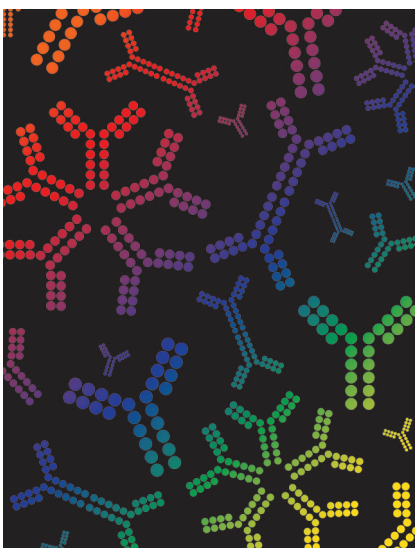
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

NATURAL ANTIBODIES IN HEALTH AND DISEASE

Topic Editors:
Ana Maria Hernandez, Center of Molecular Immunology, Cuba
Nichol E. Holodick, Western Michigan University Homer Stryker M.D. School of Medicine, United States



Natural Antibodies are essential for providing a first line of defense against pathogens; however, their functions go beyond protection from infection. These functions are as varied as their isotypes and other characteristics that define these important antibodies.

Cover image: designed by Selest El-Nashef.

Natural antibodies (NAbs) are found in normal individuals in the absence of exogenous antigenic stimulation. Natural antibodies rapidly recognize and protect against pathogens that have not been previously encountered. NAbs also cross-react with several self-antigens, which, besides their role as a first line of defense against pathogens, affords them the ability to perform important housekeeping functions in healthy organisms. Such housekeeping functions include the clearance of oxidized damaged structures and/or apoptotic cells, which prevents the induction of pro-inflammatory effects. In addition, NAbs play a role in preventing the expansion of specific auto-reactive clones, thereby behaving as regulatory elements in acute or chronic inflammation. To maintain the non-pathogenic balance between the dual pathogen/self-antigen cross-reactivities of NAbs, a strict regulation in NAb secretion and function is necessary to avoid autoimmune disease. Actually, some of the NAbs related auto-reactivities, such as anti-DNA and anti-MOG, have been associated with autoimmunity. Furthermore, NAbs have been shown to bind to 'neo-self' carbohydrate antigens on glycolipids and glycoproteins found on malignant but not normal cells, which suggests NAbs may take part in tumor immunosurveillance.

Many aspects regarding NAbs have yet to be studied in more detail: the reactivity and function of NAbs in health and disease, the behavior of the NAb repertoire with increasing age, the regulation of natural antibody production and auto-reactivity, the ways to specifically activate NAbs producing cells with desired specificities, the characteristics of human NAbs, among

others. This special topics eBook consists of a number of articles exploring the cells that produce NAbs as well as the characteristics, function, specificity, and/or the role of natural antibodies in health and disease.

Citation: Hernandez, A. M., Holodick, N. E., eds. (2018). Natural Antibodies in Health and Disease. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-405-1

Table of Contents

frontiers in Immunology

EDITORIAL
published: 13 December 2017
doi: 10.3389/fimmu.2017.01795

Editorial: Natural Antibodies in Health and Disease

Ana Maria Hernandez[1]* *and Nichol E. Holodick*[2]*

[1] Natural Antibodies Group, Tumor Immunology Division, Center of Molecular Immunology, Havana, Cuba, [2] Department of Biomedical Sciences, Center for Immunobiology, Western Michigan University Homer Stryker MD School of Medicine, Kalamazoo, MI, United States

Keywords: natural antibodies, B cells, B-1 cells, antibodies, natural antibodies repertoire

Editorial on the Research Topic

Natural Antibodies in Health and Disease

Natural antibodies (NAbs) are most commonly defined as immunoglobulins present in the absence of exogenous antigen stimulation. In fact, numerous groups have demonstrated the presence of NAbs in both specific pathogen-free and germ-free mice (1–3). These NAbs provide immediate protection against infection while the adaptive arm of the immune system mounts a specific and long-lasting response. Beyond immediate protection from infection, NAbs have been shown to play various functional roles in the immune system, which include clearance of apoptotic debris (Gronwall et al.), suppression of allergic responses (4, 5), regulation of B cell responses (6), selection of the B cell repertoire (7, 8), protection from cancer (9, 10), regulation of B cell development [Baumgarth; (7, 11)], and protection against atherosclerosis (12–15). These various functions of NAbs are afforded by their reactivity, which is broad, cross-reactive, and shown to recognize evolutionarily fixed epitopes present in foreign antigens [Gronwall et al.; (16–21)]. Furthermore, NAbs have unique characteristics that also contribute to their functional roles and set them apart from antigen-specific antibodies. Such characteristics include germline structure (lacking non-templated nucleotides and little to no somatic hypermutation) and a restricted repertoire (16, 22–24).

Determining and subsequently examining the B cells producing NAbs have been the subject of intense investigation since the early 1980s despite NAbs being studied since the late 1960s. NAb producing B-1a cells were first identified in mice and characterized by surface expression of $CD5^+$, IgM^{high}, IgD^{low}, $CD19^{high}$, $B220^{low}$, $CD23^-$, and $CD43^+$ (25), which contrasts with the surface phenotype of follicular B-2 cells: $CD5^-$, IgM^{low}, IgD^{high}, $CD19^+$, $B220^+$, $CD23^+$, and $CD43^-$. Studies have demonstrated that B-1a cells are found in the peritoneal cavity, pleural cavity, spleen, bone marrow, lymph nodes, and blood of mice (26). Furthermore, various subsets of B-1a cells have been identified and include those expressing PD-L2 ($PD\text{-}L2^{+/-}$) (27, 28), CD25 ($CD25^{+/-}$) (Tumang et al.), CD73 ($CD73^{hi/lo}$) (29), and PC-1 ($PC\text{-}1^{hi/lo}$). Throughout the many years of B-1a cell investigation, it has been shown that not all subsets of murine B-1a cells secrete NAbs. This has important implications when investigating the source of protective and/or pathogenic NAbs.

More recently, focus has been given in determining the subset of B cells in humans capable of producing NAbs. Early studies in humans focused on $CD5^+$ peripheral B cells (30–33) and CD5-CD45RAlo B cells (34). More recently, attempts to refine the human NAb producing B cell subset have generated a new phenotypic definition: $CD20^+CD27^+CD43^+CD70^-CD38^{mod}$ (35, 36). Interestingly, most of these cells express CD5. Further investigation is still required in the human system to determine whether other subsets of NAb producing B cells exist and the location of such subsets beyond peripheral blood.

Many aspects of NAbs and the cells generating them have yet to be studied in great detail: the reactivity and function of NAbs in health and different diseases, the behavior of the NAb repertoire

OPEN ACCESS

Edited by:
Harry W. Schroeder, University of Alabama at Birmingham, United States

Reviewed by:
Raul M. Torres, University of Colorado Denver, United States
Christoph J. Binder, Medical University of Vienna, Austria

***Correspondence:**
Ana Maria Hernandez
anita@cim.sld.cu;
Nichol E. Holodick
nichol.holodick@med.wmich.edu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *07 November 2017*
Accepted: *30 November 2017*
Published: *13 December 2017*

Citation:
Hernandez AM and Holodick NE (2017) Editorial: Natural Antibodies in Health and Disease. Front. Immunol. 8:1795. doi: 10.3389/fimmu.2017.01795

with increasing age, the regulation of NAb production and autoreactivity, the ways to specifically activate NAbs producing B-1 cells with desired specificities, and the characteristics of human NAbs, among others. This Frontiers research topic aimed to further investigate how NAbs are regulated, the cells that generate NAbs, and the roles NAbs play in maintaining health and/or leading to disease.

The 16 articles presented in this research topic explore a wide range of topics pertaining to NAbs (and the cells that produce them) in health and disease. These papers investigate the specificity of NAbs [Cruz-Leal et al.; Vale et al.; Zhang et al.], the function of NAbs [Pedersen et al.; Kohler et al.; Rothstein; Saha et al.], the cells producing NAb [Baumgarth; Popi et al.; Kaku et al.], and/or the role NAbs and/or NAb producing cells play in leading to disease [Wang et al.; Holodick et al.; Lobo; Wolfram et al.; Zhu et al.]. In addition, we include a perspective article aiming to start discussion and investigation into the definition of NAbs (Holodick et al.). With the plethora of established and new data on NAbs and NAb producing cells, it is clear our traditional definition of such antibodies might need to be refined or bolstered. Overall, this collection of articles adds to the NAb literature in a thoughtful and hopefully thought-provoking way. We thank all of the authors for contributing their work to this ebook, which will inspire many new lines of investigation into the structure, generation, and function of NAbs in health and disease.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the writing of this editorial.

ACKNOWLEDGMENTS

We thank Dr. Thomas L. Rothstein for his helpful review of this editorial and discussions on the subject of natural antibodies.

REFERENCES

1. Bos NA, Meeuwsen CG, Wostmann BS, Pleasants JR, Benner R. The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. *Cell Immunol* (1988) 112:371–80. doi:10.1016/0008-8749(88)90306-1
2. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *Eur J Immunol* (1997) 27:1557–63. doi:10.1002/eji.1830270635
3. Savage HP, Yenson VM, Sawhney SS, Mousseau BJ, Lund FE, Baumgarth N. Blimp-1-dependent and -independent natural antibody production by B-1 and B-1-derived plasma cells. *J Exp Med* (2017) 214:2777–94. doi:10.1084/jem.20161122
4. Kearney JF, Patel P, Stefanov EK, King RG. Natural antibody repertoires: development and functional role in inhibiting allergic airway disease. *Annu Rev Immunol* (2015) 33:475–504. doi:10.1146/annurev-immunol-032713-120140
5. Patel PS, Kearney JF. Neonatal exposure to pneumococcal phosphorylcholine modulates the development of house dust mite allergy during adult life. *J Immunol* (2015) 194:5838–50. doi:10.4049/jimmunol.1500251
6. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* (1998) 160:4776–87.
7. Nguyen TT, Elsner RA, Baumgarth N. Natural IgM prevents autoimmunity by enforcing B cell central tolerance induction. *J Immunol* (2015) 194:1489–502. doi:10.4049/jimmunol.1401880
8. Freitas AA, Viale AC, Sundblad A, Heusser C, Coutinho A. Normal serum immunoglobulins participate in the selection of peripheral B-cell repertoires. *Proc Natl Acad Sci U S A* (1991) 88:5640–4. doi:10.1073/pnas.88.13.5640
9. Vollmers HP, Brandlein S. Natural antibodies and cancer. *N Biotechnol* (2009) 25:294–8. doi:10.1016/j.nbt.2009.03.016
10. Madi A, Bransburg-Zabary S, Maayan-Metzger A, Dar G, Ben-Jacob E, Cohen IR. Tumor-associated and disease-associated autoantibody repertoires in healthy colostrum and maternal and newborn cord sera. *J Immunol* (2015) 194:5272–81. doi:10.4049/jimmunol.1402771
11. Nguyen TT, Klasener K, Zurn C, Castillo PA, Brust-Mascher I, Imai DM, et al. The IgM receptor FcmuR limits tonic BCR signaling by regulating expression of the IgM BCR. *Nat Immunol* (2017) 18:321–33. doi:10.1038/ni.3677
12. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B cells and humoral immunity in atherosclerosis. *Circ Res* (2014) 114:1743–56. doi:10.1161/CIRCRESAHA.113.301145
13. Zhang M, Alicot EM, Chiu I, Li J, Verna N, Vorup-Jensen T, et al. Identification of the target self-antigens in reperfusion injury. *J Exp Med* (2006) 203:141–52. doi:10.1084/jem.20050390
14. Binder CJ, Shaw PX, Chang MK, Boullier A, Hartvigsen K, Horkko S, et al. The role of natural antibodies in atherogenesis. *J Lipid Res* (2005) 46:1353–63. doi:10.1194/jlr.R500005-JLR200
15. Binder CJ, Silverman GJ. Natural antibodies and the autoimmunity of atherosclerosis. *Springer Semin Immunopathol* (2005) 26:385–404. doi:10.1007/s00281-004-0185-z
16. Feeney AJ. Predominance of the prototypic T15 anti-phosphorylcholine junctional sequence in neonatal pre-B cells. *J Immunol* (1991) 147:4343–50.
17. Brundish DE, Baddiley J. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. *Biochem J* (1968) 110:573–82. doi:10.1042/bj1100573
18. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* (2000) 105:1731–40. doi:10.1172/JCI8472
19. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* (2003) 9:736–43. doi:10.1038/nm876
20. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* (2009) 182:6031–43. doi:10.4049/jimmunol.0804191
21. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* (2009) 119:1335–49. doi:10.1172/JCI36800
22. Tornberg UC, Holmberg D. B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. *EMBO J* (1995) 14:1680–9.
23. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* (1997) 158:1175–86.
24. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G. Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J Exp Med* (1988) 168:687–98. doi:10.1084/jem.168.2.687
25. Baumgarth N. B-cell immunophenotyping. *Methods Cell Biol* (2004) 75:643–62. doi:10.1016/S0091-679X(04)75027-X
26. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) 11:34–46. doi:10.1038/nri2901
27. Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. *Eur J Immunol* (2007) 37:2405–10. doi:10.1002/eji.200737461

28. Zhong X, Lau S, Bai C, Degauque N, Holodick NE, Steven SJ, et al. A novel subpopulation of B-1 cells is enriched with autoreactivity in normal and lupus-prone mice. *Arthritis Rheum* (2009) 60:3734–43. doi:10.1002/art.25015
29. Kaku H, Cheng KF, Al-Abed Y, Rothstein TL. A novel mechanism of B cell-mediated immune suppression through CD73 expression and adenosine production. *J Immunol* (2014) 193:5904–13. doi:10.4049/jimmunol.1400336
30. Casali P, Notkins AL. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. *Immunol Today* (1989) 10:364–8. doi:10.1016/0167-5699(89)90268-5
31. Antin JH, Emerson SG, Martin P, Gadol N, Ault KA. Leu-1+ (CD5+) B cells. A major lymphoid subpopulation in human fetal spleen: phenotypic and functional studies. *J Immunol* (1986) 136:505–10.
32. Kipps TJ, Fong S, Tomhave E, Chen PP, Goldfien RD, Carson DA. High-frequency expression of a conserved kappa light-chain variable-region gene in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* (1987) 84:2916–20. doi:10.1073/pnas.84.9.2916
33. Casali P, Burastero SE, Nakamura M, Inghirami G, Notkins AL. Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. *Science* (1987) 236:77–81. doi:10.1126/science.3105056
34. Kasaian MT, Ikematsu H, Casali P. Identification and analysis of a novel human surface CD5- B lymphocyte subset producing natural antibodies. *J Immunol* (1992) 148:2690–702.
35. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* (2011) 208:67–80. doi:10.1084/jem.201014992011113c
36. Quach TD, Rodriguez-Zhurbenko N, Hopkins TJ, Guo X, Hernandez AM, Li W, et al. Distinctions among circulating antibody-secreting cell populations, including B-1 cells, in human adult peripheral blood. *J Immunol* (2016) 196:1060–9. doi:10.4049/jimmunol.1501843

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 September 2016
doi: 10.3389/fimmu.2016.00374

Phosphocholine-Specific Antibodies Improve T-Dependent Antibody Responses against OVA Encapsulated into Phosphatidylcholine-Containing Liposomes

Yoelys Cruz-Leal[1], Alejandro López-Requena[2†], Isbel Lopetegui-González[3], Yoan Machado[4†], Carlos Alvarez[1], Rolando Pérez[2] and María E. Lanio[1]**

[1] Laboratory of Toxins and Liposomes, Center for Protein Studies (CEP), Faculty of Biology, University of Havana, Havana, Cuba, [2] Immunobiology Direction, Center of Molecular Immunology (CIM), Havana, Cuba, [3] Biochemistry Department, Faculty of Biology, University of Havana, Havana, Cuba, [4] Development Direction, Center of Molecular Immunology (CIM), Havana, Cuba

Liposomes containing phosphatidylcholine have been widely used as adjuvants. Recently, we demonstrated that B-1 cells produce dipalmitoyl-phosphatidylcholine (DPPC)-specific IgM upon immunization of BALB/c mice with DPPC-liposomes encapsulating ovalbumin (OVA). Although this preparation enhanced the OVA-specific humoral response, the contribution of anti-DPPC antibodies to this effect was unclear. Here, we demonstrate that these antibodies are secreted by B-1 cells independently of the presence of OVA in the formulation. We also confirm that these antibodies are specific for phosphocholine. The anti-OVA humoral response was partially restored in B-1 cells-deficient BALB/*xid* mice by immunization with the liposomes opsonized with the serum total immunoglobulin (Ig) fraction containing anti-phosphocholine antibodies, generated in wild-type animals. This result could be related to the increased phagocytosis by peritoneal macrophages of the particles opsonized with the serum total Ig or IgM fractions, both containing anti-phosphocholine antibodies. In conclusion, in the present work, it has been demonstrated that phosphocholine-specific antibodies improve T-dependent antibody responses against OVA carried by DPPC-liposomes.

Keywords: B-1 cells, liposomes, phosphocholine-specific antibodies, peritoneal macrophages, humoral response

OPEN ACCESS

Edited by:
Nichol E. Holodick, The Feinstein Institute for Medical Research, USA

Reviewed by:
Wenxia Song, University of Maryland, USA
Frances E. Lee, University of Rochester Medical Center, USA

****Correspondence:***
María E. Lanio
mlanio@fbio.uh.cu;
Rolando Pérez
rolando@cim.sld.cu

†Present address:
Alejandro López-Requena, Laboratory of Clinical Immunology, Department of Microbiology and Immunology, KU Leuven, Leuven, Belgium;
Yoan Machado, Department of Molecular Biology, Division of Allergy and Immunology, University of Salzburg, Salzburg, Austria

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *04 December 2015*
Accepted: *07 September 2016*
Published: *22 September 2016*

Citation:
Cruz-Leal Y, López-Requena A, Lopetegui-González I, Machado Y, Alvarez C, Pérez R and Lanio ME (2016) Phosphocholine-Specific Antibodies Improve T-Dependent Antibody Responses against OVA Encapsulated into Phosphatidylcholine-Containing Liposomes. Front. Immunol. 7:374. doi: 10.3389/fimmu.2016.00374

Abbreviations: APC, allophycocyanin; Chol, cholesterol; CW-PSC, cell wall polysaccharide; DMPC, dimyristoyl-phosphatidylcholine; DOPC, dioleoyl-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine; DPPG, dipalmitoyl-phosphatidylglycerol; DRV, dehydration and rehydration vesicles; DSPC, distearoyl-phosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; i.p., intraperitoneal; Lp DPPC, DPPC and chol-containing liposomes; Lp DPPG, DPPG and chol-containing liposomes; Lp DPPC/OVA, DPPC and chol-containing liposomes encapsulating OVA; LPM, large peritoneal macrophages; OVA, ovalbumin; OVA-FITC, ovalbumin labeled with fluorescein isothiocyanate; PA, phosphatidic acid; PB, phosphate buffer; PerC, peritoneal cavity; PE Cy5.5, cyanine dye (Cy5.5) combined with phycoerythrin; PE, phycoerythrin; PEt, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; SPM, small peritoneal macrophages; SUV, small unilamellar vesicles; Xid, X-linked immunodeficient.

INTRODUCTION

IgM is the first antibody isotype to appear during ontogeny and the only isotype produced by all species of vertebrates (1, 2). It is also the first isotype produced during an immune response and plays a crucial role in front-line host defense against pathogens. Secreted IgM plays important roles in the early phases of the adaptive immune response, as it concentrates antigen into secondary lymphoid organs, initiates antibody responses and germinal center formation, and accelerates affinity maturation in immune responses to thymus-dependent antigens (1, 3, 4).

Up to 80% of circulating IgM in the mouse derives from B-1 cells (5). B-1 cells represent the main B cell population of the peritoneal and pleural cavities in mice (6) and differ from conventional B lymphocytes (B-2) in surface markers, antibody repertoire, developmental pathway, and B-cell receptor (BCR) signaling (7). CD5 expression splits B-1 cells into two subsets: $CD5^+$ B-1a and $CD5^-$ B-1b cells, which exhibit different functions in the immune system (8, 9). The natural repertoire of peritoneal B-1 cells contains phosphocholine and phosphatidylcholine-specific antibodies, and hence they might interact with liposomes composed by this lipid (10–12). The so-called natural antibodies, mainly produced by these cells, are present in circulation without any evident antigenic challenge (13). The most studied natural antibodies bind to phosphorylcholine-containing antigens, which are present and accessible on apoptotic cell membranes and in oxidized low density lipoproteins, and also constitutes the immunodominant epitope in the pneumococcal cell wall polysaccharide (CW-PSC) (14–16). There is a distinct set of natural antibodies that bind to determinants that arise on erythrocytes during their senescence or after enzymatic treatment with bromelain (10, 17–19). These anti-red cell antibodies are reported to recognize determinants that involve the entire phosphatidylcholine molecule in the outer cell membrane, but not other phosphocholine-containing antigens.

Recently, we demonstrated the contribution of B-1 cells to the adjuvant properties of dipalmitoyl-phosphatidylcholine (DPPC) and cholesterol (Chol)-containing liposomes (Lp DPPC) encapsulating ovalbumin (OVA) (Lp DPPC/OVA) (20). BALB/X-linked immunodeficient (*xid*) mice, which exhibit defects in the B cell compartment, particularly in the B-1 cell population (21–23), showed quantitative and qualitative differences in the anti-OVA antibody response compared with wild-type animals upon immunization with this preparation. The direct participation of B-1 cells was evidenced by the restoration of the immunostimulatory properties of Lp DPPC in BALB/*xid* mice adoptively transferred with B-1 cells purified from BALB/c animals; the internalization of these particles by B-1 cells; and the migration of B-1 cells from the peritoneal cavity (PerC) to the spleen. These cells were able to produce both *in vitro* and *in vivo* DPPC-specific antibodies upon stimulation with Lp DPPC (20). These antibodies recognized sphingomyelin (SM) but not dipalmitoyl-phosphatidylglycerol (DPPG), suggesting their phosphocholine specificity. However, the precise contribution of these antibodies to the enhancement of the OVA-specific antibody response promoted by Lp DPPC encapsulating this antigen was not elucidated. In the present work, we characterized the anti-lipid antibody response induced by this liposomal preparation, its specificity, and the influence of the presence of the antigen. The presence of OVA in the formulation did not increase the anti-DPPC IgM response. These antibodies also recognized the CW-PSC from *Streptococcus pneumoniae*, corroborating their specificity for phosphocholine. The opsonization of Lp DPPC/OVA with these antibodies enhanced the anti-OVA humoral response in B-1 cells-deficient BALB/*xid* mice, although without reaching the levels obtained in wild-type animals. The particles opsonized with serum total immunoglobulin (Ig)- or IgM-containing phosphocholine-specific antibodies were efficiently phagocyted by peritoneal macrophages, suggesting a role for these cells in the adjuvant properties of Lp DPPC.

MATERIALS AND METHODS

Reagents

OVA grade V, used as model antigen in immunization protocols in soluble form or encapsulated into liposomes and OVA grade II, used to coat ELISA plates, were purchased from Sigma–Aldrich (St. Louis, MO, USA). CW-PSC from *S. pneumoniae* used to coat ELISA plates was purchased from Statens Seruminstitut (Copenhagen, Denmark). DPPC, DPPG, and Chol, used to generate liposomes and to coat ELISA plates, were purchased from Northern Lipids (Alabaster, AL, USA). Dimyristoyl-phosphatidylcholine (DMPC), distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), SM, phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylethanolamine (PEt), used to coat ELISA plates, were purchased from Avantis Polar Lipids, Inc., Alabaster, AL, USA. Fluorescein isothiocyanate (FITC) from Sigma–Aldrich was used to label OVA. Dephosphorylated 18C polysaccharide from *S. pneumoniae* (dephos 18C PSC), used in the competitive ELISA, was generously provided by Dr. Janoi Chang from the Finlay Institute, Havana, Cuba.

Mice

Female BALB/c mice, 6 to 8 weeks of age, were purchased from the Center for Laboratory Animal Production (Havana, Cuba). Female and male BALB/*xid* mice, which carry a Bruton's tyrosine kinase mutation and have a severely diminished B-1 cell population (13, 23), were bred at the Center of Molecular Immunology (CIM; Havana, Cuba). All animals were specific pathogens-free and were maintained under standard animal house conditions with free access to water and standard rodent pellets.

Ethics Statement

All procedures were performed in compliance with the protocols approved by the Institutional Committee for the Care and Use of Laboratory Animals of the CIM (CICUAL, 0017/2008). Animals were sacrificed by cervical dislocation, minimizing their suffering.

Encapsulation of OVA into Liposomes

Liposomes encapsulating OVA were obtained by a procedure based on dehydration and rehydration of vesicles (DRV)

developed by Kirby and Gregoriadis (24). To obtain OVA-encapsulating liposomes, small unilamellar vesicles (SUV) composed of DPPC and an equimolar quantity of Chol were generated by ultrasonication and then mixed with OVA. After freezing at −70°C, the liposome and OVA mixture was lyophilized in an Edwards freezer dryer (Aaron Equipment Company, Bensenville, IL, USA) for 24 h. The rehydration step was carried out with a small volume of distilled water (1 µL water/0.2 µmol of lipids) at 45°C, above the phase transition temperature of DPPC. After incubating for 30 min at 45°C, 0.5 mL of phosphate-buffered saline (PBS), pH 7.4, was added. Separation of non-encapsulated OVA was performed by centrifugation at 100,000 *g* for 30 min (Centrifuge 5415 R, Eppendorf AG, Hamburg, Germany). Empty liposomes comprised of DPPG and Chol (Lp DPPG), DPPC and Chol (Lp DPPC), or DPPG, PA, and Chol in a ratio 0.25:0.75:1 (Lp DPPG:PA:Chol) (Lp DPPG:PA) were prepared following the same procedure, but in the absence of OVA.

Binding of Antibodies Induced by DPPC-Containing Liposomes to CW-PSC

The recognition of CW-PSC by antibodies induced by Lp DPPC was tested by ELISA. 96-well polystyrene flat-bottom high binding microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 10 µg mL^{-1} of CW-PSC from *S. pneumoniae* diluted in PBS, pH 7.2, overnight at 4°C. The plates were blocked with 5% (w/v) skim milk (Merck, Darmstadt, Germany) in PBS/0.05% Tween 20 (PBS/T) (v/v) (block solution I) for 1 h at 37°C. Serial dilutions of preimmune and immune serum samples were incubated overnight at 4°C. Bound antibodies were detected with a biotinylated goat anti-mouse IgM antibody (AbD Serotec, Oxford, UK) followed by alkaline phosphatase-conjugated streptavidin (Sigma–Aldrich). A serum from a human donor immunized with the 7-valent pneumococcal polysaccharide–protein conjugate vaccine (PCV7; Prevnar®, Wyeth Lederle Vaccines) was used as positive control of CW-PSC recognition, and binding detected with a biotinylated goat anti-human IgM antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) followed by alkaline phosphatase-conjugated streptavidin (Sigma–Aldrich). Chromogen *p*-nitrophenylphosphate diluted in diethanolamine/$MgCl_2$ buffer, pH 5, was added as substrate solution and optical density read at 405 nm ($OD_{405\ nm}$) in a plate reader (ELISA Ledial01, Wiener Neudorf, Austria). Wells without coating and wells coated with CW-PSC and incubated only with the secondary antibodies were used as background controls.

For the competitive ELISA, plates coated with 10 µg mL^{-1} of CW-PSC from *S. pneumoniae* were incubated with immune sera from mice immunized with Lp DPPC previously mixed with different concentrations of Lp DPPC as competitor molecule. CW-PSC was used as positive control and Lp DPPG:PA and dephos 18C PSC as negative controls. Solutions of PSC at 50 and 12 µg mL^{-1} and the liposomes at 80 and 20 µg mL^{-1} were mixed with the immune mouse sera diluted 1:100 in a ratio 1:1 (v/v) and incubated for 2 h at 37°C. Sera with or without competitor molecule were added to the plate and incubated overnight at 4°C. After three washes, bound antibodies were detected with a biotinylated goat anti-mouse IgM antibody (AbD Serotec) followed by alkaline phosphatase-conjugated streptavidin (Sigma–Aldrich). The reaction was developed as describe above. The percentage of binding of immune sera in the presence of competitor molecule was determined with respect to signal in the absence of competitor molecule.

IgM Purification from Sera of Mice Immunized with Empty Liposomes

To purify IgM fractions from sera of BALB/c mice immunized with empty Lp DPPC or Lp DPPG, Ig fractions were precipitated with NH_4SO_4 and applied into a column of agarose with covalently attached goat anti-mouse IgM (µ-chain-specific) IgG fraction (Sigma–Aldrich). After washing the column with 0.01M sodium phosphate buffer, pH 7.2, containing 0.5M NaCl (PB), the elution step was carried out with 0.1M glycine with 0.15M NaCl, pH 2.4. Finally, the IgG contaminant was eliminated using a Hi-Trap protein G column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The unbound fraction (IgM) was collected by washing the column with PB and the bound fraction (IgG) by eluting with 0.1M glycine–HCl buffer, pH 2.7. Both chromatographic steps were performed at a flow rate of 1 mL min^{-1}. Eluted fractions were neutralized using 200 µL of 1M Tris–HCl, pH 9.0. The protein concentration was estimated by absorbance at 280 nm. Polyacrylamide gel electrophoresis (SDS-PAGE) (25) and Western blotting analysis were performed to assess the purity of samples, using alkaline phosphatase-conjugated goat anti-mouse IgM (µ-chain-specific) and anti-mouse IgG (whole molecule) antibodies (Jackson ImmunoResearch), respectively. Molecular weight markers (Precision Plus Protein™ All Blue Standards) and IgM/IgG standards were purchased from Bio-RAD (Waltham, MA, USA) and Sigma–Aldrich, respectively. In addition, IgM and IgG concentration was estimated by ELISA. The specificity of the IgM fraction for DPPC was also checked by ELISA.

IgM and IgG Quantification by ELISA

To quantify the IgM fraction, 96-well polystyrene flat-bottom high binding microtiter plates (Corning™ Costar™, Thermo Fisher, Toronto, ON, Canada) were coated with a goat anti-mouse IgM (µ-chain-specific) antibody (Sigma–Aldrich), diluted 1:3500 in 0.05M sodium carbonate buffer, pH 9.6 (coating buffer). The plates were incubated overnight at 4°C and blocked with 1% (w/v) of bovine serum albumin diluted in PBS (block solution II) for 30 min at 37°C. The samples diluted in block solution II were added and incubated for 2 h at 4°C. Serial dilutions (1:2) of an irrelevant mouse IgM (Sigma–Aldrich) were used as standard curve. Bound antibodies were detected with an alkaline phosphatase-conjugated goat anti-mouse IgM (µ chain-specific) antibody (Jackson ImmunoResearch), diluted 1:10,000 in block solution II after incubation for 1 h at 4°C. The reaction was developed as described above.

The IgG fraction was quantified using a similar assay, with a goat anti-mouse IgG (whole molecule) antibody (Sigma–Aldrich) diluted 1:333 as capture antibody, and samples diluted in PBS-Tween 20 (0.05%) with fetal bovine serum (5%) (PBS-FBS).

Serial dilutions (1:2) of an irrelevant mouse IgG, produced at CIM (Havana, Cuba) were used as standard curve. After 1 h of incubation at 37°C, bound antibodies were detected using an alkaline phosphatase-conjugated goat anti-mouse IgG (Fcγ fragment-specific) (Jackson ImmunoResearch) diluted 1:10,000 in PBS-FBS.

Liposome Opsonization by Phosphocholine-Specific Antibodies

The opsonization assay was carried out by incubating Lp DPPC/OVA with serum total Ig and IgM fractions for 2 h at 37°C. Bound antibodies were detected by flow cytometry using a PE-conjugated goat anti-mouse Ig antibody and PE-conjugated goat anti-mouse IgM, respectively (eBioscience, San Diego, CA, USA). To opsonize the amount of Lp DPPC/OVA corresponding to one immunization dose, molar ratios antibodies:lipids of 0.01:0.792 and 0.0001:0.792 were used for serum total Ig and IgM fractions, respectively.

Immunization Protocols

The schedule followed in all intraperitoneal (i.p.) immunization protocols was one injection at day 0 and a booster after 14 days. Animals were bled at day 0 and 7 days after the booster. Anti-OVA and anti-lipid antibody responses were evaluated by ELISA. BALB/*xid* mice were also immunized with OVA labeled with FITC (FITC-OVA) encapsulated into Lp DPPC (Lp DPPC/FITC-OVA) opsonized or not with anti-phosphocholine antibodies-containing serum total Ig fraction.

Determination of Serum Antibodies Specific for OVA and Lipids

To detect OVA-specific antibodies, 96-well polystyrene flat-bottom high binding microtiter plates (Greiner-bio-one, Frickenhausen, Germany) were coated with 10 μg mL^{-1} of OVA diluted in coating buffer, overnight at 4°C. The plates were blocked with block solution I for 1 h at 37°C. Serial dilutions of serum samples were incubated for 2 h at 37°C. Bound antibodies were detected with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma–Aldrich) or biotinylated goat anti-mouse IgG1 or IgG2a antibodies (AbD Serotec) followed by alkaline phosphatase-conjugated streptavidin (Sigma–Aldrich). Serum dilutions giving signals corresponding to twice the value with the preimmune sera were considered as antibody titers.

To evaluate the presence of phosphocholine-specific antibodies, 96-well polystyrene flat-bottom microtiter plates (Maxisorp; Nunc) were coated with 4 μg of DPPC, DSPC, DOPC, DMPC, DPPG, SM, PA, PS, or PEt diluted in n-hexane and incubated at 37°C until drying. The plates were blocked with 5% (w/v) skim milk (Merck) in PBS (blocking solution III) for 1 h at 37°C. Serum samples were diluted in blocking solution III, and plates were incubated overnight at 4°C. Bound antibodies were detected with a biotinylated goat anti-mouse IgM antibody (AbD Serotec) or a biotinylated goat anti-mouse IgG antibody (Sigma–Aldrich) followed by alkaline phosphatase-conjugated streptavidin (Sigma–Aldrich).

In both anti-OVA and anti-lipid determinations, the reaction was developed as described above.

Evaluation of Opsonized Lp DPPC/OVA Uptake by Peritoneal Macrophages *In Vivo*

BALB/*xid* mice ($n = 3$) were immunized i.p. with Lp DPPC/FITC-OVA opsonized or not with anti-phosphocholine antibodies-containing serum total Ig fraction (Lp DPPC/FITC-OVA + Ab and Lp DPPC/FITC-OVA, respectively). The IgM fraction from sera of BALB/c mice immunized with empty Lp DPPC (IgM_{DPPC}) or Lp DPPG (IgM_{DPPG}) and a commercial irrelevant IgM ($IgM_{irrelev}$) (Sigma–Aldrich) were also used to opsonize Lp DPPC/FITC-OVA. One hour later, cells were collected from the PerC by repeated washing with RPMI 1640 medium (Sigma–Aldrich) and labeled with the following goat anti-mouse antibodies combinations: PE-conjugated anti-F4/80 (PE-F4/80); cyanine dye (Cy5.5) combined with PE-conjugated anti-CD11b (PE Cy5.5-CD11b) and eFluor700-conjugated anti-CD19 (eFluor700-CD19) or PE-conjugated anti-CD11b (PE-CD11b); PE cy-chrome 5 (Cy5)-conjugated anti-F4/80 (PE Cy5-F4/80), and allophycocyanin-conjugated anti-B220 (APC-B220). Macrophage populations were identified by flow cytometry from total cells as $CD19^{-}CD11b^{+}$ and $F4/80^{+}$ ($F4/80^{Low}$ and $F4/80^{High}$) (Figure S1 in Supplementary Material). Cells from non-immunized BALB/*xid* mice were used as negative control.

Flow Cytometry Analysis

For phenotype characterization, cell suspensions were pre-incubated with an anti-CD16/CD32 mAb (BD Biosciences Pharmingen, San Diego, CA, USA) to block Fcγ II/III receptors before staining with fluorochrome-conjugated antibodies. Cells were stained with different combinations of goat anti-mouse antibodies: PE-F4/80, PE-CD11b, PE-Cy5-F4/80, PE Cy5.5-CD11b, APC-B220, and eFluor700-CD19 using standard protocols. Cells were acquired using a Gallios flow cytometer (Beckman Coulter, Miami, FL, USA). The analysis was performed using the FlowJo 7.2.2 software (Tree Star Ashland, OR, USA). Total number of macrophages was estimated by total cell number in the PerC counted in a Neubauer chamber.

Statistical Analysis

Statistical analysis was performed using the SPSS software version 16.0 (SPSS). The Kolmogorov–Smirnov test was used to verify normal distribution of data and the Levene test to determine the homogeneity of variance. Data with normal distribution and equality of variance were analyzed with one-way variance analysis (ANOVA) simple classification, with Tukey as *post hoc* test to assess statistical significance between the means of more than two groups. Data not normally distributed or without equality of variance, even after scale transformation, were analyzed using the Kruskal–Wallis non-parametric test with Dunn as *post hoc* test and the Friedman test with Dunn as *post hoc* test for matched data. For comparing the means of two independent groups, the Mann–Whitney *U* test or the Wilcoxon signed-rank test were used.

RESULTS

B-1 Cells Produce Anti-Liposomal DPPC Antibodies with Specificity for Phosphocholine

To characterize the immune response induced by liposomal lipid DPPC, BALB/c mice were immunized with Lp DPPC with or without encapsulated OVA. As shown in **Figure 1A**, Lp DPPC, in the absence of antigen, induced similar DPPC-specific IgM response in BALB/c mice to those liposomes encapsulating OVA, used as control group. There were no differences in IgM titer after one or two administrations (**Figure 1B**), and no IgG antibodies were detected after immunization with empty liposomes (data not shown). Thus, liposomal DPPC induced a primary antibody response that was significantly impaired in B-1 cells-deficient BALB/*xid* mice (**Figure 1C**), indicating the crucial role of this B cell population, as had been demonstrated for Lp DPPC-containing OVA in our previously published experiments (20) and now confirmed with both empty and antigen-encapsulating particles.

The reactivity of sera from mice immunized with empty Lp DPPC against a panel of lipids, containing or not phosphocholine in their structure, was evaluated. As shown in **Figure 2A**, the signal ratio of immune sera over preimmune sera was only significantly higher than background in the cases of lipids containing the phosphocholine group (DSPC, DOPC, DPPC, DMPC, and SM). As expected, the immune sera did not recognize the lipids without phosphocholine (DPPG, PA, PS, and PEt). In addition, to evaluate the ability of these sera to recognize phosphocholine in a non-lipid molecule, we tested reactivity with CW-PSC from *S. pneumoniae*, a polysaccharidic structure that only shares with DPPC the presence of the phosphocholine group (**Figures 2B,C**). As shown in **Figure 2D**, immune sera from mice showed similar reactivity against CW-PSC and DPPC. In contrast, a serum sample from

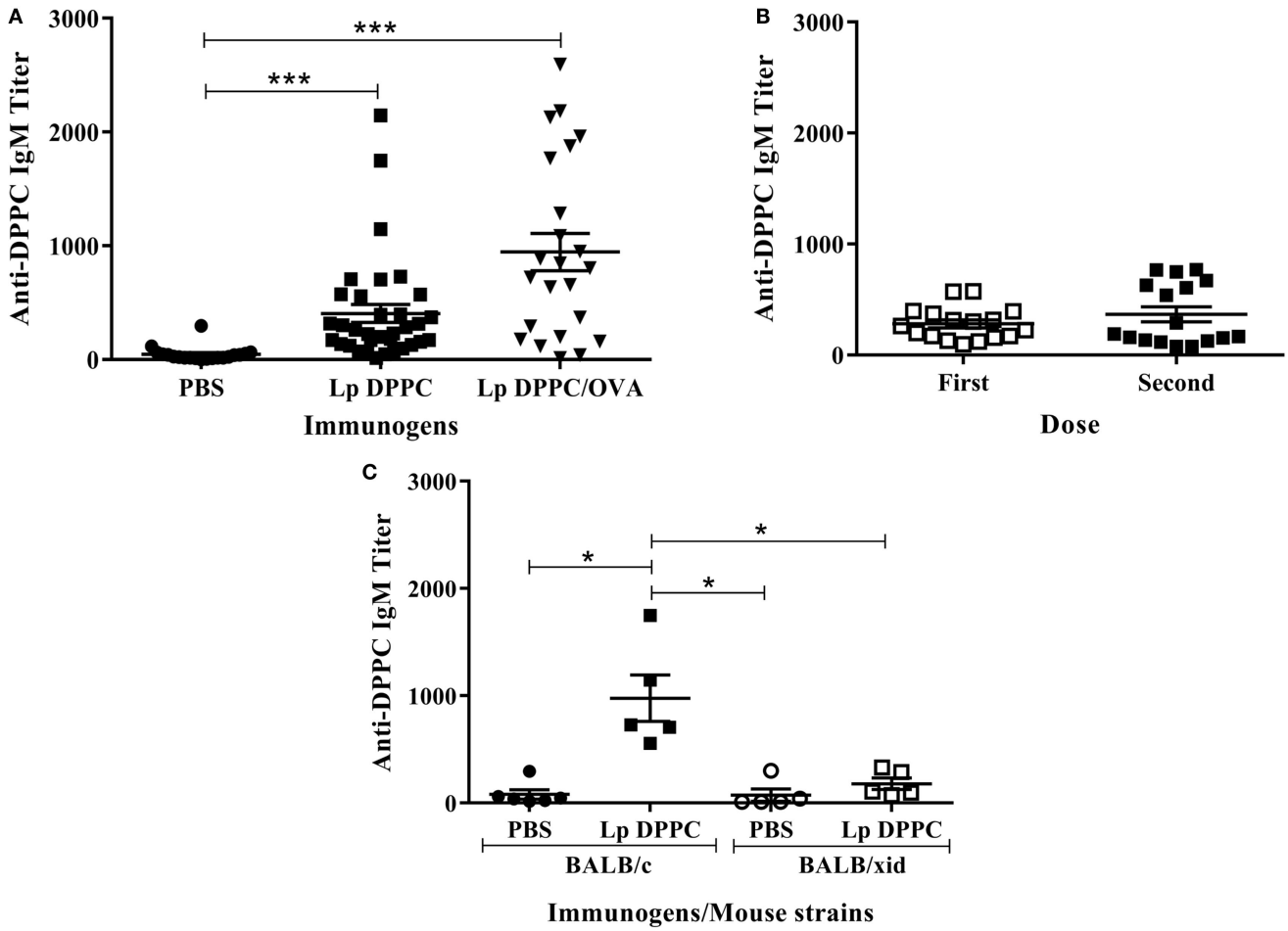


FIGURE 1 | Liposomal DPPC induces DPPC-specific IgM in BALB/c, but not in BALB/*xid* mice. DPPC-specific IgM titers were evaluated by ELISA in **(A)** sera from BALB/c mice immunized i.p. with two doses of PBS ($n = 20$), Lp DPPC ($n = 28$), or Lp DPPC/OVA ($n = 23$); **(B)** sera from BALB/c mice ($n = 17$) immunized i.p. with one or two doses of Lp DPPC; **(C)** sera from BALB/c and BALB/*xid* mice immunized i.p. with two doses of PBS or Lp DPPC ($n = 5$ in each group). Means ± SEM are shown. Statistical analysis was performed with Kruskal–Wallis test with Dunn posttest **(A,C)** and Wilcoxon signed-rank test **(B)**; $^{*}p < 0.05$ and $^{***}p < 0.001$.

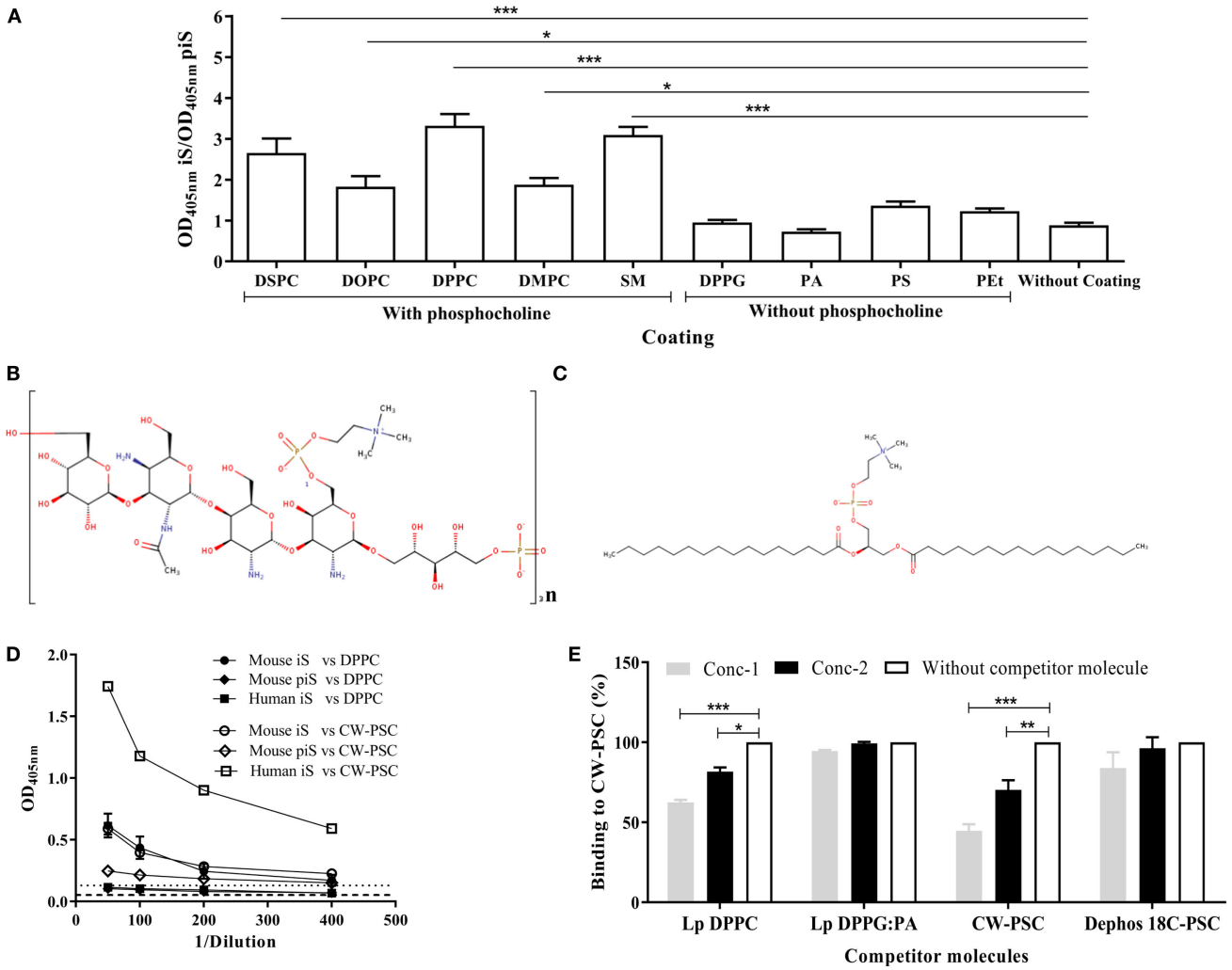


FIGURE 2 | Antibodies induced by liposomal DPPC are specific for the phosphocholine group. **(A)** The recognition of a panel of lipids containing or not phosphocholine in their structure by sera from BALB/c mice immunized i.p with empty Lp DPPC (n = 11) was evaluated by ELISA. Sera were diluted 1:100, and the ratio of optical density at 405 nm between immune sera ($OD_{405\,nm}$ iS) and preimmune sera ($OD_{405\,nm}$ piS) was calculated. The following lipids were assessed: distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), dipalmitoyl-phosphatidylcholine (DPPC), dimyristoyl-phosphatidylcholine (DMPC), sphingomyelin (SM), dipalmitoyl-phosphatidylglycerol (DPPG), phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylethanolamine (PEt). The 2D structures of **(B)** the cell wall polysaccharide (CW-PSC) from *S. pneumoniae* and **(C)** DPPC were drawn using the Marvin Sketch program version 15.2.23.0 (2015), ChemAxon (http://www.chemaxon.com). **(D)** Serial dilutions of iS and piS from BALB/c mice immunized with Lp DPPC (n = 7) were evaluated by ELISA against DPPC and CW-PSC. A serum from a human donor immunized with a pneumococcal vaccine was used as positive control of CW-PSC recognition. Dashed and dotted lines indicate the background signal (without well coating) with mice and human serum, respectively. **(E)** The ability of Lp DPPC to inhibit the binding of DPPC-specific antibodies to CW-PSC was evaluated by a competitive ELISA. Sera from mice immunized with Lp DPPC were diluted 1:100, mixed 1:1 (v/v) with suspensions of Lp DPPC at two different concentrations (conc-1: 80 μg mL^{-1} and conc-2: 20 μg mL^{-1}), and incubated for 2 h at 37°C. CW-PSC at conc-1: 50 μg mL^{-1} and conc-2: 12 μg mL^{-1} was used as positive control, while Lp DPPG:PA (a ratio 0.25:0.75) and dephos 18C PSC at the same concentrations that Lp DPPC and CW-PSC, respectively, were used as negative controls. The mixtures were added to an ELISA plate coated with CW-PSC and incubated overnight at 4°C. The percentage of binding to CW-PSC of sera in the presence of competitor molecule was calculated with respect to binding without competitor molecule. Means ± SEM are shown. Statistical analysis was performed with a Friedman test with Dunn posttest **(A)** and Kruskal–Wallis test with Dunn posttest **(E)**; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

a human donor immunized with a pneumococcal vaccine only recognized CW-PSC (**Figure 2D**). To confirm that the structure recognized by mouse sera was the phosphocholine group in both molecules, we carried out a competitive ELISA against CW-PSC. The previous incubation of sera with Lp DPPC inhibited binding to CW-PSC, in a concentration-dependent manner, similar to positive control CW-PSC. As expected, Lp DPPG:PA or dephos18C PSC did not affect the binding of sera to CW-PSC (**Figure 2E**) at any of the concentrations tested. Altogether, these results demonstrated the specificity for phosphocholine of the antibodies induced by empty Lp DPPC, which is in agreement with our previous data using OVA-encapsulating particles (20).

In the present work, this finding was reinforced by using a larger panel of phosphocholine-containing molecules.

Opsonization of Lp DPPC/OVA with Anti-Phosphocholine Antibodies-Containing Serum Total Ig Fraction Partially Mimics the B-1 Cells-Mediated Immunostimulatory Effect of This Formulation

As in our previous report (20), we confirmed that the ability of Lp DPPC to potentiate the antibody response against encapsulated OVA was impaired in BALB/*xid* mice (**Figure 3**). To evaluate whether anti-phosphocholine antibodies participate in the B-1 cells-mediated immunostimulatory effect of Lp DPPC, BALB/*xid* mice were immunized with Lp DPPC/OVA opsonized with the total Ig fraction from anti-DPPC antisera generated in wild-type animals (Lp DPPC/OVA + Ab). The Ig fraction ability to opsonize these particles was assessed by flow cytometry (**Figure 3A**). BALB/*xid* mice immunized with Lp DPPC/OVA + Ab exhibited significantly increased anti-OVA IgG titers in comparison with those induced by Lp DPPC/OVA alone, although without reaching the levels obtained in wild-type animals (**Figure 3B**). Whereas almost no IgG1 response was observed in any of the BALB/*xid* mouse groups (**Figure 3C**), the levels of IgG2a reproduced the results of the total IgG titers (**Figure 3D**). In summary, the opsonization with anti-phosphocholine antibodies-containing total serum Ig fraction partially rescued the immunostimulatory properties of Lp DPPC in B-1 cells-deficient mice.

Opsonization with Phosphocholine-Specific Antibodies Increases the Uptake of Lp DPPC/OVA by Peritoneal Macrophages

The more abundant large peritoneal macrophages (LPMs) express high levels of the canonical surface markers CD11b and F4/80 ($F4/80^{High}$ macrophages), while small peritoneal macrophages (SPMs) express lower levels of these molecules ($F4/80^{Low}$ macrophages) (26). We first assessed the uptake of Lp DPPC by both populations by immunizing BALB/*xid* mice with Lp DPPC/

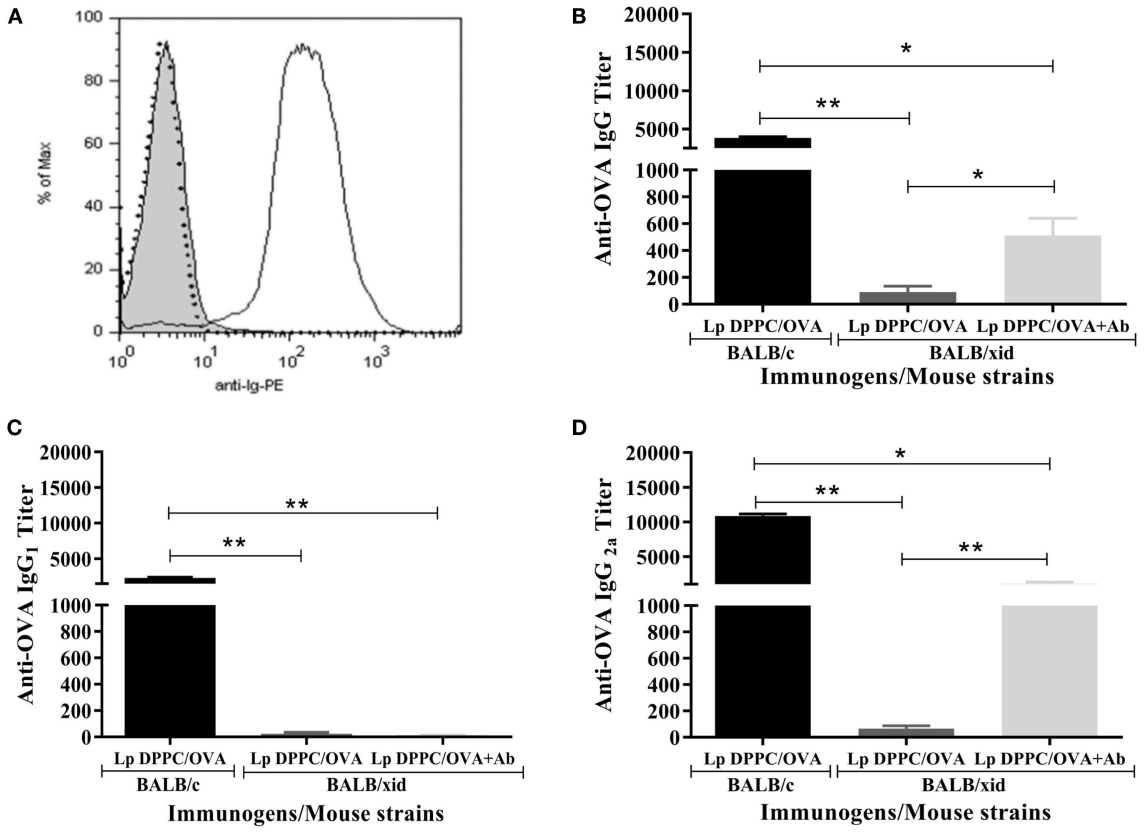


FIGURE 3 | Opsonization of Lp DPPC/OVA with anti-phosphocholine antibodies-containing serum total Ig fraction partially mimics in BALB/*xid* mice the OVA-specific antibody response induced by this liposomal formulation in wild-type animals. **(A)** Representative histograms of Lp DPPC/OVA opsonized with anti-phosphocholine antibodies-containing serum total Ig fraction (Lp DPPC/OVA + Ab) (black line), Lp DPPC/OVA alone (filled), and Lp DPPC/OVA opsonized with an isotype-matched control antibody (dotted line). OVA-specific **(B)** IgG, **(C)** IgG1, and **(D)** IgG2a were measured by ELISA in sera from BALB/*xid* mice ($n = 5$) immunized i.p. with two doses of Lp DPPC/OVA + Ab, and BALB/*xid* ($n = 5$) and BALB/c ($n = 6$) mice immunized i.p. with two doses of Lp DPPC/OVA. Means ± SEM are shown. Statistical analysis was performed with one-way ANOVA test with Tukey posttest **(C)** or Kruskal–Wallis test with Dunn posttest **(B,D)**; $^{*}p < 0.05$ and $^{**}p < 0.01$.

FITC-OVA previously opsonized or not with anti-phosphocholine antibodies-containing serum total Ig fraction. Peritoneal cells from each group were analyzed by flow cytometry following the gating strategy described in Figure S1 in Supplementary Material. BALB/*xid* mice immunized with opsonized Lp DPPC/FITC-OVA showed a higher frequency of both phenotypes of peritoneal macrophages internalizing the labeled antigen (19.6% F4/80^{Low} and 30% F4/80^{High}) than in the case of animals receiving non-opsonized particles (5.2% F4/80^{Low} and 17.7% F4/80^{High}) (**Figure 4A**). In addition, a significant increase in the total number of antigen-loaded F4/80^{Low} and F4/80^{High} peritoneal macrophages was detected in animals receiving the opsonized liposomes in comparison with those that received non-opsonized vesicles (**Figures 4B,C**, respectively).

In order to determine if this effect was due to IgM-specific DPPC, we purified the IgM fraction from the anti-phosphocholine antibodies-containing serum total Ig preparation (IgM_{DPPC}). Two affinity chromatography steps were necessary to obtain purified IgM. The eluted fraction from the IgM affinity chromatography step still contained IgG contaminants, as shown in lines one of **Figures 5A,C**. When this fraction was applied into the protein G chromatographic column, a purified IgM preparation

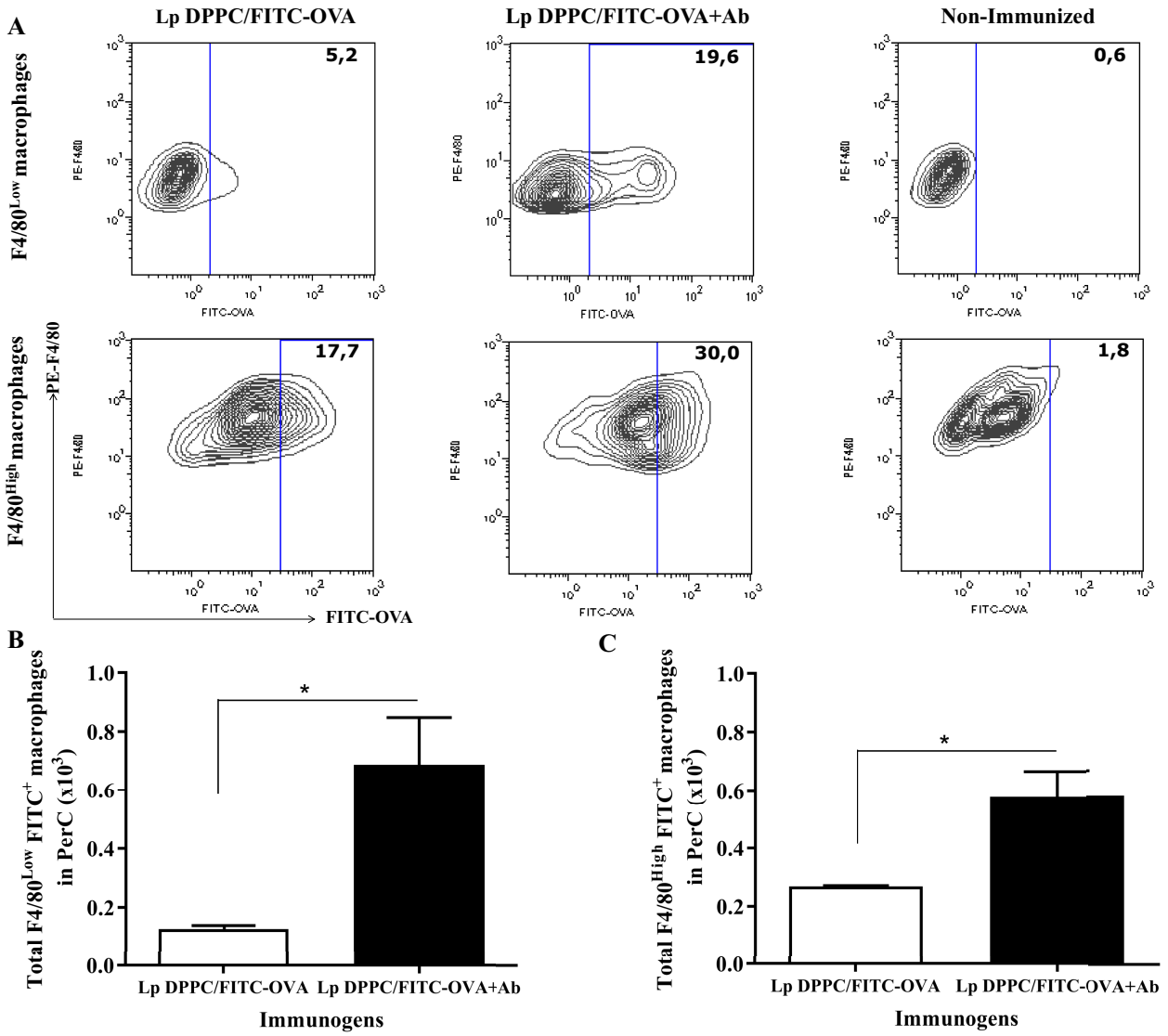


FIGURE 4 | Opsonization of Lp DPPC/OVA with anti-phosphocholine antibodies-containing serum total Ig fraction improves the *in vivo* uptake of encapsulated OVA by peritoneal macrophages. BALB/*xid* mice ($n = 3$) were immunized i.p. with FITC-OVA encapsulated into Lp DPPC opsonized or not with anti-phosphocholine antibodies-containing serum total Ig fraction (Lp DPPC/FITC-OVA + Ab and Lp DPPC/FITC-OVA, respectively). Non-immunized BALB/*xid* mice were used as control. **(A)** Representative contour graphs of FITC-OVA vs. PE-F4/80 for both macrophage populations (F4/80^{Low} and F4/80^{High}) from one representative animal per group. Means ± SEM of the total number of antigen-loaded **(B)** F4/80^{Low} and **(C)** F4/80^{High} macrophages are shown. Statistical analysis was performed with Mann–Whitney *U* test: $^*p < 0.05$.

was obtained, as determined by SDS-PAGE and Western blot (**Figures 5A–C**, lines 2). The absence of IgG in this preparation was also verified by ELISA (data not shown). The IgM fraction from sera of animals immunized with Lp DPPG was also purified using a similar strategy (IgM_{DPPG}, data not shown). Only the IgM fraction from sera of animals immunized with Lp DPPC recognized DPPC, while IgM_{DPPG} and $IgM_{irrelev}$ were not able to bind to it (**Figure 5D**). The opsonization of Lp DPPC/OVA with IgM_{DPPC}, IgM_{DPPG}, and $IgM_{irrelev}$ was assessed by flow cytometry (**Figure 5E**). As expected, the higher signal was obtained with the DPPC-specific IgM-enriched preparation.

Finally, the uptake of opsonized Lp DPPC/FITC-OVA by peritoneal macrophages was measured in BALB/*xid* mice. In agreement with the results showed in **Figure 4**, the frequency of both peritoneal macrophage populations ($F4/80^{Low}$ and $F4/80^{High}$) loaded with the labeled antigen was higher with liposomes opsonized with IgM_{DPPC} than with Lp DPPC/FITC-OVA opsonized with IgM_{DPPG}, $IgM_{irrelev}$, or non-opsonized (**Figure 6A**). Moreover, the total numbers of macrophages from both populations ($F4/80^{Low}$ and $F4/80^{High}$) internalizing the antigen were significantly higher in mice immunized with Lp DPPC/FITC-OVA opsonized with IgM_{DPPC} than in the other

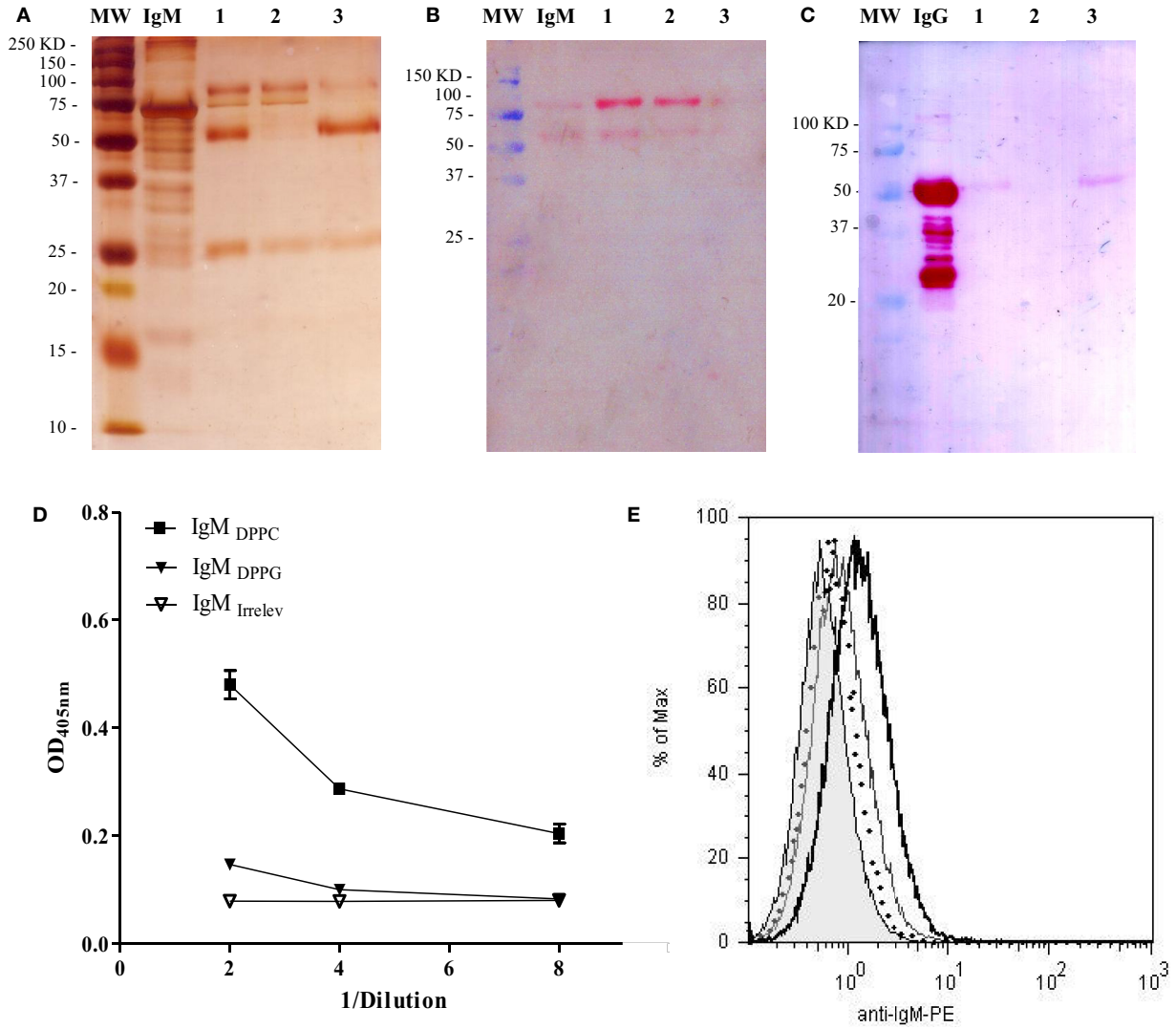


FIGURE 5 | Analysis of purity and DPPC-reactivity of IgM purified from anti-phosphocholine antibodies-containing sera. The IgM fraction purified from sera of BALB/c mice immunized with empty Lp DPPC was analyzed by SDS-PAGE and Western blot. **(A)** Silver-stained SDS-PAGE in 12% acrylamide under reducing conditions. The presence of **(B)** IgM and **(C)** IgG in each sample was determined by Western blot using alkaline phosphatase-conjugated goat anti-mouse IgM (μ-chain-specific) and anti-mouse IgG (whole molecule) antibodies, respectively. MW: molecular weight markers; IgM/IgG: standards. 1: eluted fraction from the IgM affinity chromatography applied into the protein G chromatography; 2: unbound fraction; and 3: eluted fraction. Recognition of DPPC by IgM from sera of animals immunized with Lp DPPC (IgM_{DPPC}) or Lp DPPG (IgM_{DPPG}), and by an irrelevant IgM ($IgM_{irrelev}$) was assessed by **(D)** ELISA and **(E)** flow cytometry. In **(E)**, representative histograms of Lp DPPC/OVA opsonized with IgM_{DPPC} (black line), IgM_{DPPG} (gray line), $IgM_{irrelev}$ (dotted line), and Lp DPPC/OVA alone (filled) are shown.

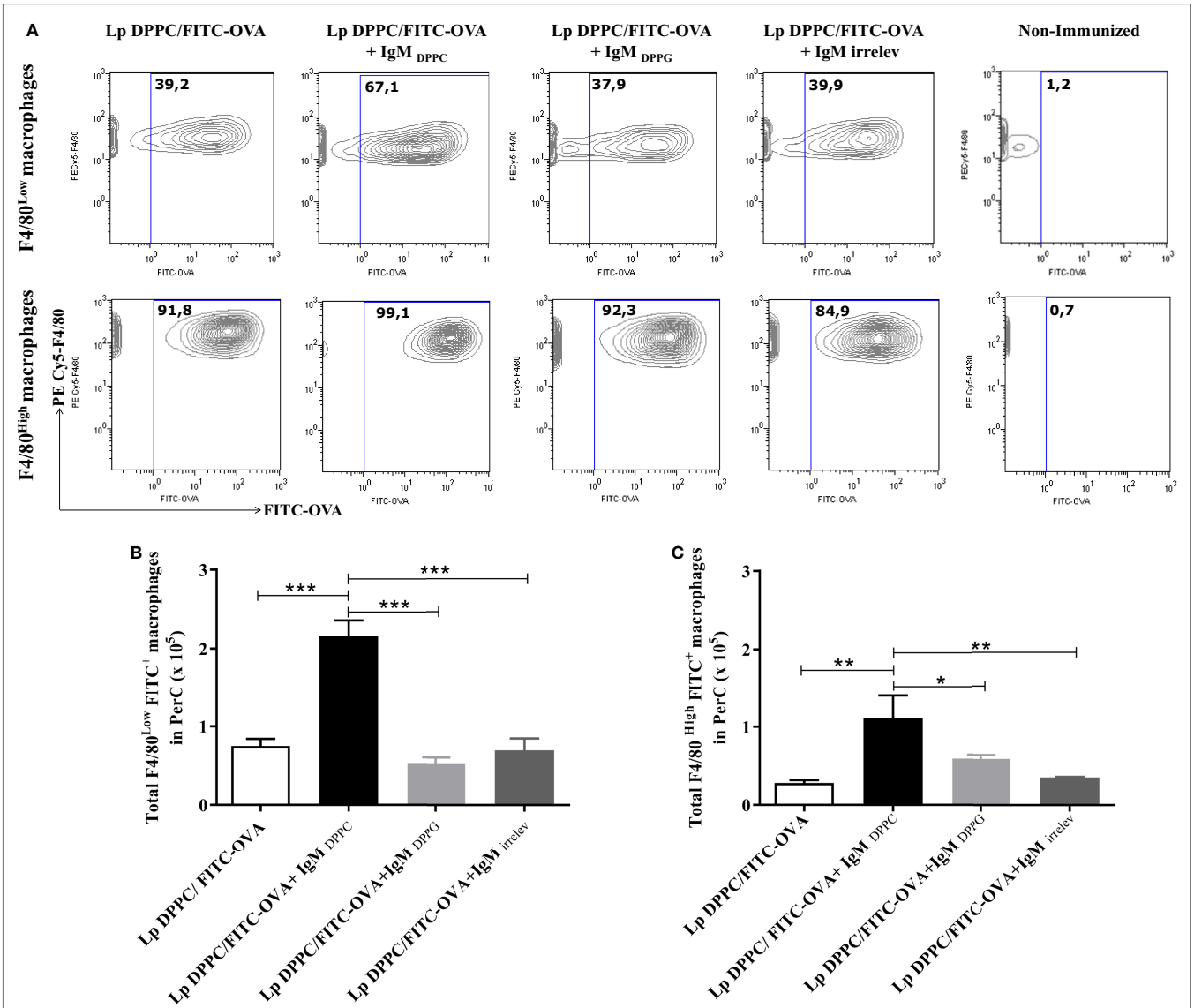


FIGURE 6 | Opsonization of Lp DPPC/OVA with anti-phosphocholine antibodies-containing serum IgM fraction improves the *in vivo* uptake of encapsulated OVA by peritoneal macrophages. BALB/*xid* mice ($n = 3$) were immunized i.p. with FITC-OVA encapsulated into Lp DPPC (Lp DPPC/FITC-OVA) opsonized or not with the IgM fraction from sera of mice immunized with empty Lp DPPC (Lp DPPC/FITC-OVA + IgM_{DPPC}) or Lp DPPG (Lp DPPC/FITC-OVA + IgM_{DPPG}). Non-immunized BALB/*xid* mice and a group of mice immunized with Lp DPPC/OVA opsonized with an irrelevant IgM (Lp DPPC/FITC-OVA + $IgM_{irrelev}$) were included as controls. **(A)** Representative contour graphs of FITC-OVA vs. PECy5-F4/80 for both macrophage populations ($F4/80^{Low}$ and $F4/80^{High}$) from one representative animal per group. Means ± SEM of the total number of antigen-loaded **(B)** $F4/80^{Low}$ and **(C)** $F4/80^{High}$ macrophages are shown. Statistical analysis was performed with one-way ANOVA test with Tukey posttest **(B)** or Kruskal–Wallis test with Dunn posttest **(C)**; $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$.

groups (**Figures 6B,C**, respectively). Neither IgM_{DPPG} nor $IgM_{irrelev}$ improved the uptake of liposomes by macrophages.

DISCUSSION

Secreted IgM is an important mediator in the optimal initiation of primary thymus-dependent humoral immune responses. It serves as a natural adjuvant by enhancing the immunogenicity of protein antigens, perhaps as a result of its ability to facilitate antigen deposition onto follicular dendritic cells and to promote rapid germinal center formation (1, 4, 27–29). Besides, the complex antigen–IgM is involved in affinity maturation (3, 28). It has also been reported that secreted IgM influences BCR signaling and promotes survival of splenic B cells (30).

We have previously shown that B-1 cells contribute to the ability of DPPC-containing liposomes to enhance the encapsulated antigen-specific antibody response. Besides, liposomal DPPC stimulates B-1 cells to produce IgM, specific for the phosphocholine polar head (20). We therefore addressed here whether these antibodies contributed to the immunostimulatory

properties of Lp DPPC. The results described in the present work demonstrate that these particles were able to induce similar levels of DPPC-specific antibodies irrespective of the presence of encapsulated OVA. This response depended on B-1 cells, since it was significantly reduced in B-1 cells-deficient BALB/*xid* mice, as we previously reported for liposomes encapsulating OVA (20). *Xid* mice have extensively been used as a model of B-1 cell deficiency (13), and although these animals exhibit defects also in the B-2 cell compartment, higher doses of soluble OVA than the one we used in our work (2 μg) induced similar IgG titers in BALB/c and BALB/*xid* mice (our unpublished data), suggesting that the B-2 cell response against this antigen is not affected in the latter animals. Moreover, no significant differences have been found in the marginal zone B cell population between these two mouse strains (31).

The recognition of different lipid species containing phosphocholine in their structure, as well as the CW-PSC from *S. pneumoniae* by the antibodies induced by liposomal DPPC corroborated their specificity for the phosphocholine group. On the other hand, the lack of reactivity with DPPC of the serum from a human donor immunized with a pneumococcal vaccine is in agreement with previous results in which rabbits immunized with this antigen conjugated to bovine serum albumin elicited antibodies that recognized the saccharide moiety but not the phosphocholine group (32).

The opsonization of Lp DPPC/OVA with phosphocholine-specific antibodies partially mimicked the immunostimulatory effect of DPPC-liposomes in the OVA-specific humoral response, as proven in B-1 cells-deficient mice. Our results extend previous observations demonstrating a role for B-1 cells-derived IgM in the enhancement of IgG production by B-2 cells (28, 33).

IgM antibodies can promote humoral immune responses through complement activation (34) and engagement of receptors, such as mannan-binding lectin (35), the polymeric Ig receptor (36), the Fc alpha/mu receptor (Fcα/μR) (37, 38), or the Fc receptor specific for IgM (FcμR) (38–41). The potential roles of different cellular receptors for IgM are a topic of active investigation. The Fcα/μR is constitutively expressed on macrophages, in addition of other cells, such as B cells and follicular dendritic cells, and recognizes IgM and IgA with high and intermediate affinity, respectively (37, 42). It mediates endocytosis of IgM-coated microbes (37, 43). On the other hand, the FcμR is expressed on macrophages and dendritic cells, although to a lesser extent in comparison with other immune cells like T and B lymphocytes (29, 39–42, 44). It plays an essential role in humoral immune responses to both thymus-dependent and -independent antigens (38, 44) and acts as an endocytic receptor, internalizing antigen–IgM complex (45). The engagement of this pathway could result in synergistic activation of B cells stimulated through the BCR (46).

Liposomes coated with the anti-phosphocholine IgM produced by B-1 cells could be taken up more efficiently by antigen-presenting cells through IgM-specific receptors, thus enhancing the presentation of encapsulated antigens. Particularly in this work, we demonstrated that administration of liposomes opsonized with anti-phosphocholine antibodies-containing serum total Ig or IgM fractions enhanced the uptake of the antigen by both the large and the small populations of peritoneal macrophages of BALB/*xid* mice, whose phagocytic activity *in vivo* has been demonstrated (26). This result is in agreement with other works showing that the incubation with polyclonal IgM enhances the phagocytosis of apoptotic cells in the lungs by alveolar macrophages (47) and promotes the clearance of apoptotic microparticles released from dying cells (48). The increase in the opsonized Lp DPPC/OVA uptake by macrophages *in vivo* could be related to the improvement of the OVA-specific antibody response observed in BALB/*xid* mice immunized with this preparation. Interestingly, this humoral response was not completely restored in comparison with wild-type animals. Notably, B-1 cells-deficient animals were persistently unable to produce specific IgG1, despite the restoration of the IgG2a levels. This could be due to the role of these cells as IL-10 producers, which inhibits IgG2a and favors IgG1 production (49). This observation suggests the direct participation of B-1 cells in the adjuvanticity of liposomes beyond the production of anti-DPPC IgM. In agreement with this, we previously described the ability of B-1 cells to uptake and transport the antigen from the PerC to the spleen after intraperitoneal immunization with Lp DPPC/OVA (20).

In conclusion, in the present work, it has been demonstrated for the first time that B-1 cells-derived phosphocholine-specific antibodies induced by liposomal DPPC contribute to the immunostimulatory properties of these particles.

AUTHOR CONTRIBUTIONS

YC-L: conception and design of the work; acquisition, analysis, and interpretation of the data and writing of the manuscript. AL-R: analysis and interpretation of the data and critical revision of the manuscript. IL-G and YH: acquisition, analysis, and interpretation of data. CA: analysis and interpretation of the data and revision of the manuscript. RP: conception of the work, critical scientific support, interpretation of the data, revision of the manuscript, and final approval of the manuscript. MEL: conception and design of the work and critical revision and final approval of the manuscript.

ACKNOWLEDGMENTS

We thank Gerardo Ramsés from CIGB (Havana, Cuba) for the lyophilization procedures, Dr. Janoi Chang from Finlay Institute (Havana, Cuba) for providing the serum from a human donor vaccinated with a pneumococcal vaccine and the dephosphorylated 18C polysaccharide from *S. penumoniae*. We also acknowledge the contribution of Dr. Kathya R. de la Luz and Dr. Adolfo Castillo from the Center of Molecular Immunology.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00374

REFERENCES

1. Ferguson AR, Youd ME, Corley RB. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *Int Immunol* (2004) 16(10):1411–22. doi:10.1093/intimm/dxh142
2. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* (2010) 10(11):778–86. doi:10.1038/nri2849
3. Corley RB, Morehouse EM, Ferguson AR. IgM accelerates affinity maturation. *Scand J Immunol* (2005) 62(Suppl 1):55–61. doi:10.1111/j.1365-3083.2005.01610.x
4. Youd ME, Ferguson AR, Corley RB. Synergistic roles of IgM and complement in antigen trapping and follicular localization. *Eur J Immunol* (2002) 32(8):2328–37. doi:10.1002/1521-4141(200208)32:8<2328::AID-IMMU2328>3.0.CO;2-T
5. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* (1999) 96(5):2250–5. doi:10.1073/pnas.96.5.2250
6. Hayakawa K, Hardy RR, Herzenberg LA. Peritoneal Ly-1 B cells: genetic control, autoantibody production, increased lambda light chain expression. *Eur J Immunol* (1986) 16(4):450–6. doi:10.1002/eji.1830160423
7. Hayakawa K, Hardy RR. Development and function of B-1 cells. *Curr Opin Immunol* (2000) 12(3):346–53. doi:10.1016/S0952-7915(00)00098-4
8. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* (2004) 21(3):379–90. doi:10.1016/j.immuni.2004.06.019
9. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med* (2008) 205(13):3053–64. doi:10.1084/jem.20080979
10. Mercolino TJ, Arnold LW, Haughton G. Phosphatidyl choline is recognized by a series of Ly-1+ murine B cell lymphomas specific for erythrocyte membranes. *J Exp Med* (1986) 163(1):155–65. doi:10.1084/jem.163.1.155
11. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G. Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J Exp Med* (1988) 168(2):687–98. doi:10.1084/jem.168.2.687
12. Masmoudi H, Mota-Santos T, Huetz F, Coutinho A, Cazenave PA. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. *Int Immunol* (1990) 2(6):515–20. doi:10.1093/intimm/2.6.515
13. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* (2002) 20:253–300. doi:10.1146/annurev.immunol.20.100301.064833
14. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* (2000) 105(12):1731–40. doi:10.1172/JCI8472
15. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* (2009) 119(5):1335–49. doi:10.1172/JCI36800
16. Friedman P, Horkko S, Steinberg D, Witztum JL, Dennis EA. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids. Importance of Schiff base formation and aldol condensation. *J Biol Chem* (2002) 277(9):7010–20. doi:10.1074/jbc.M108860200
17. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J Immunol* (1989) 142(10):3643–51.
18. Cox KO, Hardy SJ. Autoantibodies against mouse bromelain-modified RBC are specifically inhibited by a common membrane phospholipid, phosphatidylcholine. *Immunology* (1985) 55(2):263–9.
19. Hardy RR, Hayakawa K. Development of B cells producing natural autoantibodies to thymocytes and senescent erythrocytes. *Springer Semin Immunopathol* (2005) 26(4):363–75. doi:10.1007/s00281-004-0183-1
20. Cruz-Leal Y, Machado Y, Lopez-Requena A, Canet L, Laborde R, Alvares AM, et al. Role of B-1 cells in the immune response against an antigen encapsulated into phosphatidylcholine-containing liposomes. *Int Immunol* (2014) 26(8):427–37. doi:10.1093/intimm/dxu042
21. Rawlings DJ, Saffran DC, Tsukada S, Largaespada DA, Grimaldi JC, Cohen L, et al. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* (1993) 261(5119):358–61. doi:10.1126/science.8332901
22. Narendran A, Ramsden D, Cumano A, Tanaka T, Wu GE, Paige CJ. B cell developmental defects in X-linked immunodeficiency. *Int Immunol* (1993) 5(2):139–44. doi:10.1093/intimm/5.2.139
23. Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, et al. Defective B cell development and function in Btk-deficient mice. *Immunity* (1995) 3(3):283–99. doi:10.1016/1074-7613(95)90114-0
24. Kirby CJ, Gregoriadis G. Preparation of liposomes containing factor VIII for oral treatment of haemophilia. *J Microencapsul* (1984) 1(1):33–45. doi:10.3109/02652048409031535
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (1970) 227(5259):680–5. doi:10.1038/227680a0
26. Ghosn EE, Cassado AA, Govoni GR, Fukuhara T, Yang Y, Monack DM, et al. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci U S A* (2010) 107(6):2568–73. doi:10.1073/pnas.0915000107
27. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* (1999) 286(5447):2156–9. doi:10.1126/science.286.5447.2156
28. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* (1998) 160(10):4776–87.
29. Kubagawa H, Oka S, Kubagawa Y, Torii I, Takayama E, Kang DW, et al. Identity of the elusive IgM Fc receptor (FcmuR) in humans. *J Exp Med* (2009) 206(12):2779–93. doi:10.1084/jem.20091107
30. Notley CA, Baker N, Ehrenstein MR. Secreted IgM enhances B cell receptor signaling and promotes splenic but impairs peritoneal B cell survival. *J Immunol* (2010) 184(7):3386–93. doi:10.4049/jimmunol.0902640
31. Donahue AC, Hess KL, Ng KL, Fruman DA. Altered splenic B cell subset development in mice lacking phosphoinositide 3-kinase p85alpha. *Int Immunol* (2004) 16(12):1789–98. doi:10.1093/intimm/dxh180
32. Szu SC, Schneerson R, Robbins JB. Rabbit antibodies to the cell wall polysaccharide of *Streptococcus pneumoniae* fail to protect mice from lethal challenge with encapsulated pneumococci. *Infect Immun* (1986) 54(2):448–55.
33. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* (2000) 192(2):271–80. doi:10.1084/jem.192.2.271
34. Chen FH, Arya SK, Rinfret A, Isenman DE, Shulman MJ, Painter RH. Domain-switched mouse IgM/IgG2b hybrids indicate individual roles for C mu 2, C mu 3, and C mu 4 domains in the regulation of the interaction of IgM with complement C1q. *J Immunol* (1997) 159(7):3354–63.
35. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA, et al. Human serum IgM glycosylation: identification of glycoforms that can bind to mannan-binding lectin. *J Biol Chem* (2005) 280(32):29080–7. doi:10.1074/jbc.M504528200
36. Kaetzel CS. The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunol Rev* (2005) 206:83–99. doi:10.1111/j.0105-2896.2005.00278.x
37. Shibuya A, Sakamoto N, Shimizu Y, Shibuya K, Osawa M, Hiroyama T, et al. Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. *Nat Immunol* (2000) 1(5):441–6. doi:10.1038/80886
38. Ouchida R, Mori H, Hase K, Takatsu H, Kurosaki T, Tokuhisa T, et al. Critical role of the IgM Fc receptor in IgM homeostasis, B-cell survival, and humoral immune responses. *Proc Natl Acad Sci U S A* (2012) 109(40):E2699–706. doi:10.1073/pnas.1210706109
39. Basten A, Warner NL, Mandel T. A receptor for antibody on B lymphocytes. II. Immunochemical and electron microscopy characteristics. *J Exp Med* (1972) 135(3):627–42. doi:10.1084/jem.135.3.627
40. Lamon EW, Andersson B, Whitten HD, Hurst MM, Ghanta V. IgM complex receptors on subpopulations of murine lymphocytes. *J Immunol* (1976) 116(5):1199–203.
41. Shima H, Takatsu H, Fukuda S, Ohmae M, Hase K, Kubagawa H, et al. Identification of TOSO/FAIM3 as an Fc receptor for IgM. *Int Immunol* (2010) 22(3):149–56. doi:10.1093/intimm/dxp121
42. Klimovich VB. IgM and its receptors: structural and functional aspects. *Biochemistry (Mosc)* (2011) 76(5):534–49. doi:10.1134/S0006297911050038

43. Honda S, Kurita N, Miyamoto A, Cho Y, Usui K, Takeshita K, et al. Enhanced humoral immune responses against T-independent antigens in Fc alpha/muR-deficient mice. *Proc Natl Acad Sci U S A* (2009) 106(27):11230–5. doi:10.1073/pnas.0809917106
44. Choi SC, Wang H, Tian L, Murakami Y, Shin DM, Borrego F, et al. Mouse IgM Fc receptor, FCMR, promotes B cell development and modulates antigen-driven immune responses. *J Immunol* (2013) 190(3):987–96. doi:10.4049/jimmunol.1202227
45. Vire B, David A, Wiestner A. TOSO, the Fcmicro receptor, is highly expressed on chronic lymphocytic leukemia B cells, internalizes upon IgM binding, shuttles to the lysosome, and is downregulated in response to TLR activation. *J Immunol* (2011) 187(8):4040–50. doi:10.4049/jimmunol.1100532
46. Vanden Bush TJ, Bishop GA. TLR7 and CD40 cooperate in IL-6 production via enhanced JNK and AP-1 activation. *Eur J Immunol* (2008) 38(2):400–9. doi:10.1002/eji.200737602
47. Litvack ML, Djiadeu P, Renganathan SD, Sy S, Post M, Palaniyar N. Natural IgM and innate immune collectin SP-D bind to late apoptotic cells and enhance their clearance by alveolar macrophages in vivo. *Mol Immunol* (2010) 48(1–3):37–47. doi:10.1016/j.molimm.2010.09.014
48. Litvack ML, Post M, Palaniyar N. IgM promotes the clearance of small particles and apoptotic microparticles by macrophages. *PLoS One* (2011) 6(3):e17223. doi:10.1371/journal.pone.0017223
49. Houston KM, Wilson EH, Eyres L, Brombacher F, Harnett MM, Alexander J, et al. Presence of phosphorylcholine on a filarial nematode protein influences immunoglobulin G subclass response to the molecule by an interleukin-10-dependent mechanism. *Infect Immun* (2000) 68(9):5466–8. doi:10.1128/IAI.68.9.5466-5468.2000

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 August 2016
doi: 10.3389/fimmu.2016.00296

The Global Self-Reactivity Profile of the Natural Antibody Repertoire Is Largely Independent of Germline D_H Sequence

Andre M. Vale[1,2,3,4], Cecília B. Cavazzoni[4], Alberto Nobrega[5†] and Harry W. Schroeder Jr.[1,2,3*†]*

[1] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA, [2] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA, [3] Department of Genetics, University of Alabama at Birmingham, Birmingham, AL, USA, [4] Program in Immunobiology, Laboratory of Immunereceptors and Signaling, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [5] Department of Immunology, Paulo de Goes Institute of Microbiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

OPEN ACCESS

Edited by:
Ana María Hernández, Center of Molecular Inmunology, Cuba

Reviewed by:
Patrick C. Wilson, University of Chicago, USA
Laurence Morel, University of Florida, USA

***Correspondence:**
Alberto Nobrega
afnobrega@gmail.com;
Harry W. Schroeder Jr.
hwsj@uab.edu

†Alberto Nobrega and Harry W. Schroeder Jr. contributed equally.

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *25 May 2016*
Accepted: *22 July 2016*
Published: *10 August 2016*

Citation:
Vale AM, Cavazzoni CB, Nobrega A and Schroeder HW Jr (2016) The Global Self-Reactivity Profile of the Natural Antibody Repertoire Is Largely Independent of Germline D_H Sequence. Front. Immunol. 7:296. doi: 10.3389/fimmu.2016.00296

Natural antibodies (NAbs) are produced in the absence of exogenous antigenic stimulation and circulate in the blood of normal, healthy individuals. These antibodies have been shown to provide one of the first lines of defense against both bacterial and viral pathogens. Conservation of the NAb repertoire reactivity profile is observed both within and across species. One view holds that this conservation of NAb self-reactivities reflects the use of germline antibody sequence, whereas the opposing view holds that the self-reactivities reflect selection driven by key conserved self-antigens. In mice, B-1a B cells are a major source of NAbs. A significant fraction of the B-1a antibody repertoire is devoid of N nucleotides in H chain complementarity determining region 3 (CDR-H3) and, thus, completely germline encoded. To test the role of germline D_H sequence on the self-reactivity profile of the NAb repertoire, we examined the composition and self-antigen specificity of NAbs produced by a panel of D_H gene-targeted BALB/c mice, each strain of which expresses a polyclonal, altered CDR-H3 repertoire that differs from the wild-type norm. We found that in most cases the same key self-antigens were recognized by the NAbs created by each D_H-altered strain. The differences in reactivity appeared to represent the genetic signature of the NAb repertoire of each mouse strain. These findings suggest that although germline CDR-H3 sequence may facilitate the production of certain NAbs, a core set of self-antigens are likely the main force driving the selection of Nab self-specificities.

Keywords: natural antibodies, self-antigen recognition, CDR-H3 repertoire, peritoneal B cell subsets

Abbreviations: ΔD-DFL, depleted D_H locus with a single DFL16.1 gene segment; ΔD-iD, depleted D_H locus with a single mutated DFL16.1 gene segment containing inverted DSP2.2 sequence; CDR-H3, complementarity determining region 3 of the immunoglobulin heavy chain; LDA, limiting dilution assay; NAb, natural antibody; PCA, principal components analysis; PerC, peritoneal cavity; TdT, terminal deoxynucleotidyl transferase; WT, wild type.

INTRODUCTION

"Natural antibodies" (NAbs) are immunoglobulins present in the healthy organism in the absence of intentional immunization (1). The stimuli behind the production of NAbs by B cells have been a topic of considerable interest since NAbs were first recognized. Several studies have shown that germ-free (GF) and even antigen-free (AgF) mice produce serum IgM in amounts equivalent to mice raised under normal vivarium conditions, suggesting that NAbs are largely independent of stimulation by external antigens, including antigens derived from the microbiota (2–4). Instead there is considerable support for NAb production being driven by endogenous antigen stimulation (5). Self-antigen recognition appears to play a physiological role in the distribution of B cells within subpopulations, often into distinct anatomical niches (6–8).

B-1a lymphocytes are generated preferentially early in ontogeny and have been implicated as a main source of NAbs. The B-1a cells demonstrate decreased or absent N-region addition at the V-D and D-J junctions and are thus, enriched for entirely germline-encoded Ig variable domains (9–17). Given that B-1a cells are among the first B cells to develop in the fetus, their participation in the secretion of NAbs might provide a reasonable explanation for the conservation of the Nab repertoire and its establishment early in ontogeny (18, 19). However, studies with irradiated chimeras reconstituted with fetal liver and adult bone marrow from wild-type (WT) mice have shown that most NAb reactivities are produced even when B-1a are profoundly reduced in numbers, thus suggesting a role for somatic selection in regenerating the NAb repertoire of specificities (20).

Although previous studies have shown that the reactivity profile of NAbs remains conserved regardless of gut colonization or depletion of B-1 cell compartment (2, 3, 20); the influence of inherited CDR-H3 antigen-binding sites on the NAb repertoire remains unclear. BALB/c mice IgH loci contain 13 functional D_H gene segments, each belonging to one of four families (DFL, DSP, DST, and DQ52). Theoretically, each D_H gene segment gives the developing B cell access to six reading frames (RFs) of differing peptide sequence. In practice, however, the use of RF1 is preferred, RF2 and RF3 are used less frequently, and the three inverted RF are rarely used (21). We had previously used techniques of cre–loxP-based gene targeting to delete 12 of the 13 D_H gene segments in the BALB/c D_H locus, retaining only the single DFL16.1 segment (ΔD-DFL mice) (22). We then generated the ΔD-iD strain by replacing the center of the single DFL16.1 segment with an inverted DSP2.2 gene segment (23). D_H gene-targeted BALB/c mice express polyclonal, altered CDR-H3 repertoires that differ from the WT norm [reviewed in Ref. (24)].

We have previously observed that the ΔD-iD strain lacks NAbs that protect against infection with *Streptococcus pneumoniae* while continuing to exhibit protective reactivity against a key altered self-antigen, oxidized LDL (25). In the present work, we sought to investigate, in depth, the nature and extent of the conservation of NAb reactivities with self-antigens from brain tissue. To test the extent to which germline antibody gene content controls the composition of NAbs and the innate antibody response to self-antigens, we examined the NAb repertoire in a panel of WT and D_H gene-targeted BALB/c mice raised under specific pathogen-free conditions. We found that most, but not all, of the reactivities against self-antigens were found in the serum of mice irrespective of changes in germline D_H content and, thus, CDR-H3 sequence and structure. Our findings suggest that for most self-reactivities the primary force driving the generation of NAbs is exposure to a key set of self-antigens.

RESULTS

Conservation of Serum IgM Reactivity Profile (Actual Repertoire) in D_H-Altered Mice

Serum IgM repertoire of antigen specificities have been screened using a semi-quantitative immunoblot assay that enables accurate *en bloc* analysis of the self-reactivity of IgM present in the serum (*actual repertoire*) of unmanipulated mice (26–28). We used the same assay to analyze the serum IgM reactivities of D_H-altered mice with self-antigens from brain tissue. Shown in **Figure 1A** is a comparison of the pattern of self-reactivity expressed by the WT BALB/c, ΔD-DFL, and ΔD-iD strains. To test whether the reactivity levels correlate with immunoglobulin specificity or with the relative abundance of the proteins, we compared protein staining with immunoreactivity densities in the same membrane. The comparison shown in **Figure 1B** reveals highly abundant proteins that are not recognized by natural serum IgM, as well as highly IgM-recognized proteins that are not abundant in the tissue extract. With a few notable exceptions, the mean sera IgM reactivity profiles were remarkably conserved among mouse lineages that generate very different primary CDR-H3 repertoires (22, 23) (**Figure 1C**).

It is noteworthy that these data revealing restriction of self-antigen recognition comply with the idea of the *immunological homunculus,* as proposed by Cohen (29). Furthermore, these data demonstrate that the reactivities found in the immunoblot assay cannot be attributed to simple non-specific interactions.

Impact of D_H Alterations on the Available Repertoire

The results shown above suggest that strong mechanisms of antigen-driven somatic selection may operate at the cellular level to recreate a normal WT NAb repertoire of specificities in genetically modified mice. In mice, the peritoneal cavity (PerC) is the major site for both B-1 and B-2 cells. We, thus, sought to investigate whether these somatic selection pressures would generate similar repertoires among the PerC B-1 and B-2 B cells (available repertoire) from the different mouse strains. The reactivity profiles of these B cell populations provided a privileged opportunity for comparison between different B cell populations that share the same anatomical niche. After sorting each PerC B cell population (B-1a, B-1b, and B-2), we cultured the cells under LPS stimulation to induce polyclonal IgM secretion and tested the supernatants using the immunoblot against brain extract assay.

All three subsets from D_H-altered PerC B cells demonstrated frequencies of response to LPS that were similar to their corresponding WT B cell subsets (**Figure 2A**). Production of IgM in culture was also similar (**Figure 2B**). Thus, D_H alteration and

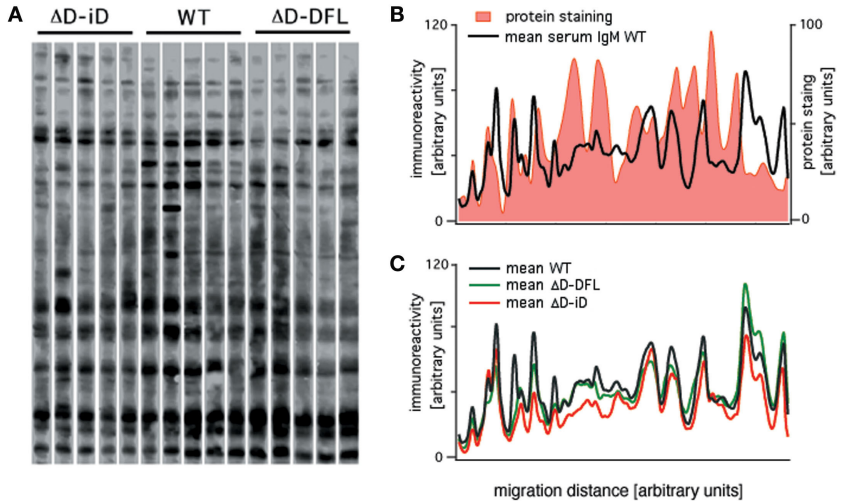


FIGURE 1 | Serum natural IgM pattern of self-reactivity expressed by ΔD-DFL, ΔD-iD, and WT BALB/c mice. **(A)** Serum IgM reactivities against syngeneic brain extract from five animals of each strain. **(B)** Total protein staining by colloidal gold densitometric profile was compared to natural IgM recognition profile in the same membrane. **(C)** Mean serum IgM reactivity profiles of each mouse strain. Raw data adapted from Ref. (25).

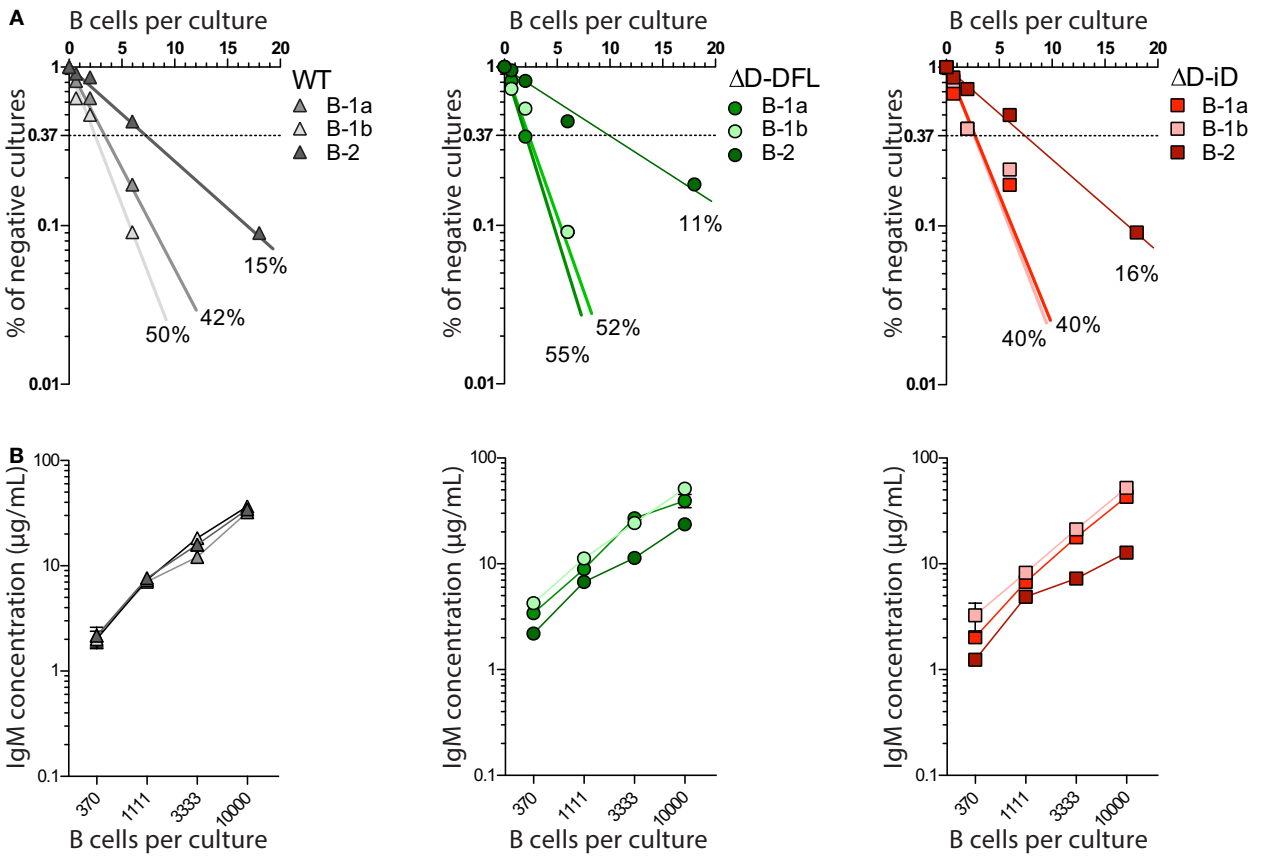


FIGURE 2 | D_H-altered B cells response to polyclonal stimuli. **(A)** Frequencies of response to LPS of PerC B cell subsets of each mouse strain were calculated by limiting dilution analysis. Presence of IgM in culture supernatants of 18, 6, 2, and 0.66 cells/culture on day 7 was assessed by ELISA. **(B)** IgM production by each PerC B cell subset in culture was measured by ELISA on culture supernatants of increasing number of cells/culture (370, 1111, 3333, and 10,000 cells/culture). PerC B-1a cells were sorted as $B220^{lo}CD5^{+}$, B-1b as $B220^{lo}CD5^{-}$ Mac-$1^{lo/+}$, and PerC B-2 cells were sorted as $B220^{hi}CD5^{-}$Mac-1^{-}. All cultures received 30 μg/mL of LPS and 5×10^3 S17 feeder cells. Supernatants were collected on day 7.

restriction of the CDR-H3 repertoire did not affect the B cell response to polyclonal stimulus from LPS.

We then performed the same analysis of self-reactivities shown for serum IgM with the supernatants from the B-1 and B-2 cultures (**Figure 3**). As there is a difference in the frequency of response to LPS among B cell subsets, we diluted each supernatant to normalize all to the same IgM concentration per growing clone (30). We performed an immunoblot analysis of the reactivities against syngeneic brain extract from seven separate culture supernatants from the three strains of mice, for a total of 21 supernatants for each B cell subset.

The mean intensity of reactivities present in B-1a cells culture supernatants remained almost completely invariant despite the genetic alteration of the D_H gene sequences (**Figure 3A**, right). The mean reactivity profiles for B-1b cells supernatants (**Figure 3B**, right) also demonstrated similarities in self-antigen reactivities irrespective of genotype, although the replicates in the membrane suggested a greater degree of variation around the mean values (**Figure 3B**, left). Conversely, the mean reactivity profiles of the B-2 cells were divergent both within and across the different mouse strains. This corresponded well with the different sets of reactivities of the B-2

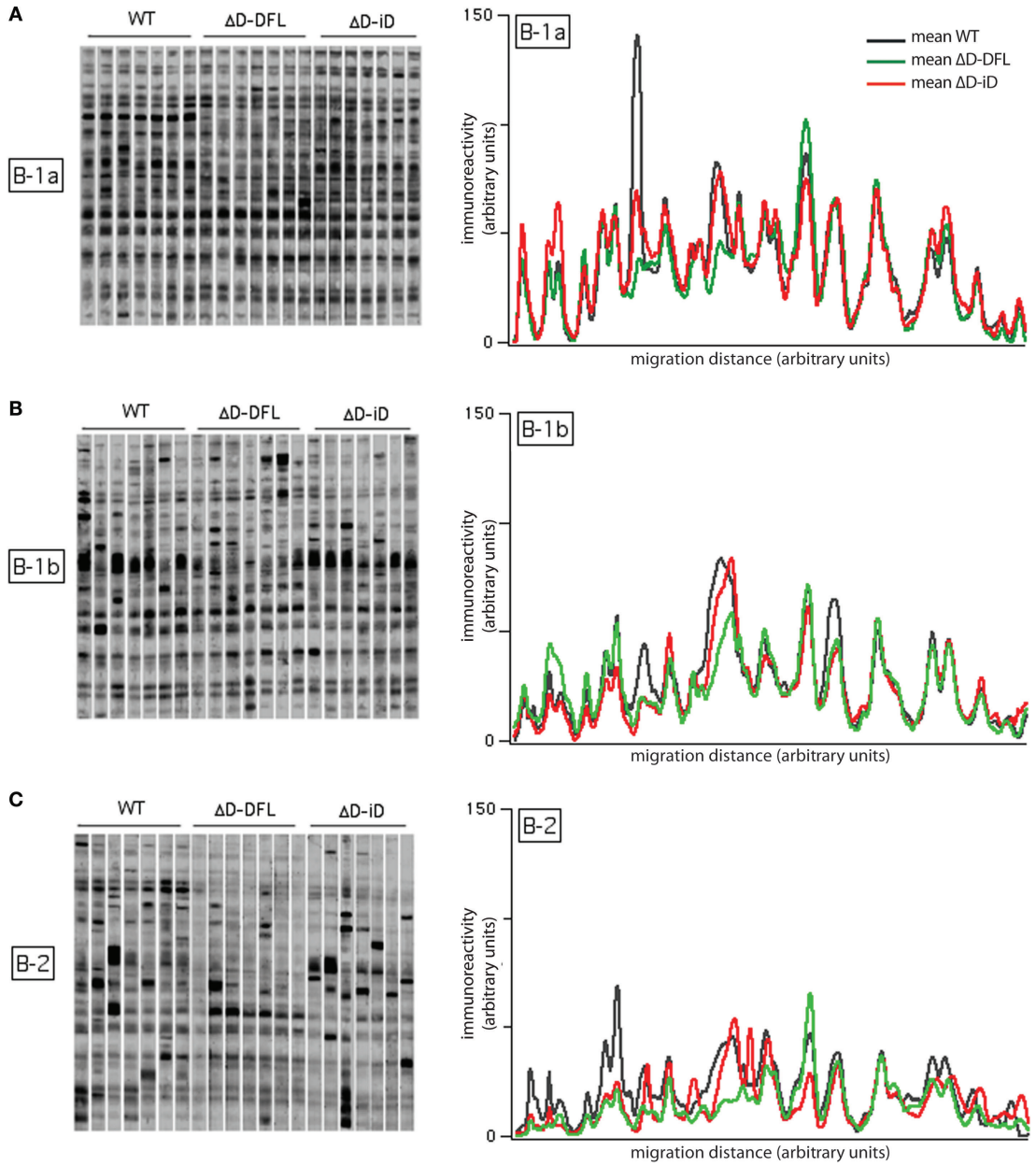


FIGURE 3 | Self-reactivities of the available repertoire in PerC B cell subsets. Left panels **(A–C)** show 21 B-1a, B-1b, and B-2 cells culture supernatants (~1,500 responding B cell per culture), respectively, reacting with syngeneic brain extract. Right panels show the mean intensity of reactivities displayed by B-1a **(A)**, B-1b **(B)**, and B-2 **(C)** cells from each mouse genotype. Raw data adapted from Ref. (25).

cells supernatants (**Figure 3C**, right). The individual variability of immunoreactivities of the 21 B-2 cell culture supernatants were also highly increased when compared to the B-1 reactivity profiles (**Figure 3C**, left). These findings suggested that differences in the variance of the reactivities were intrinsic to the B cell subset, and not dependent on the germline sequence of the immunoglobulin repertoire.

To address the issue of variance in depth, we divided the immunoreactivity profiles into 36 sections, which corresponded to the major bands of reactivity. We plotted the magnitudes of reactivity against these sections for each supernatant from the B-1a, B-1b, and B-2 subsets (**Figures 4A–C**, respectively). The solid lines indicate the mean profile of all supernatants for each B cell subset. In this format, the variance of the distribution around the mean profile can be properly visualized. Despite few exceptions, we observed that the variance around the mean was much lower for the B-1a supernatants when compared to B-1b and B-2 (**Figure 4**).

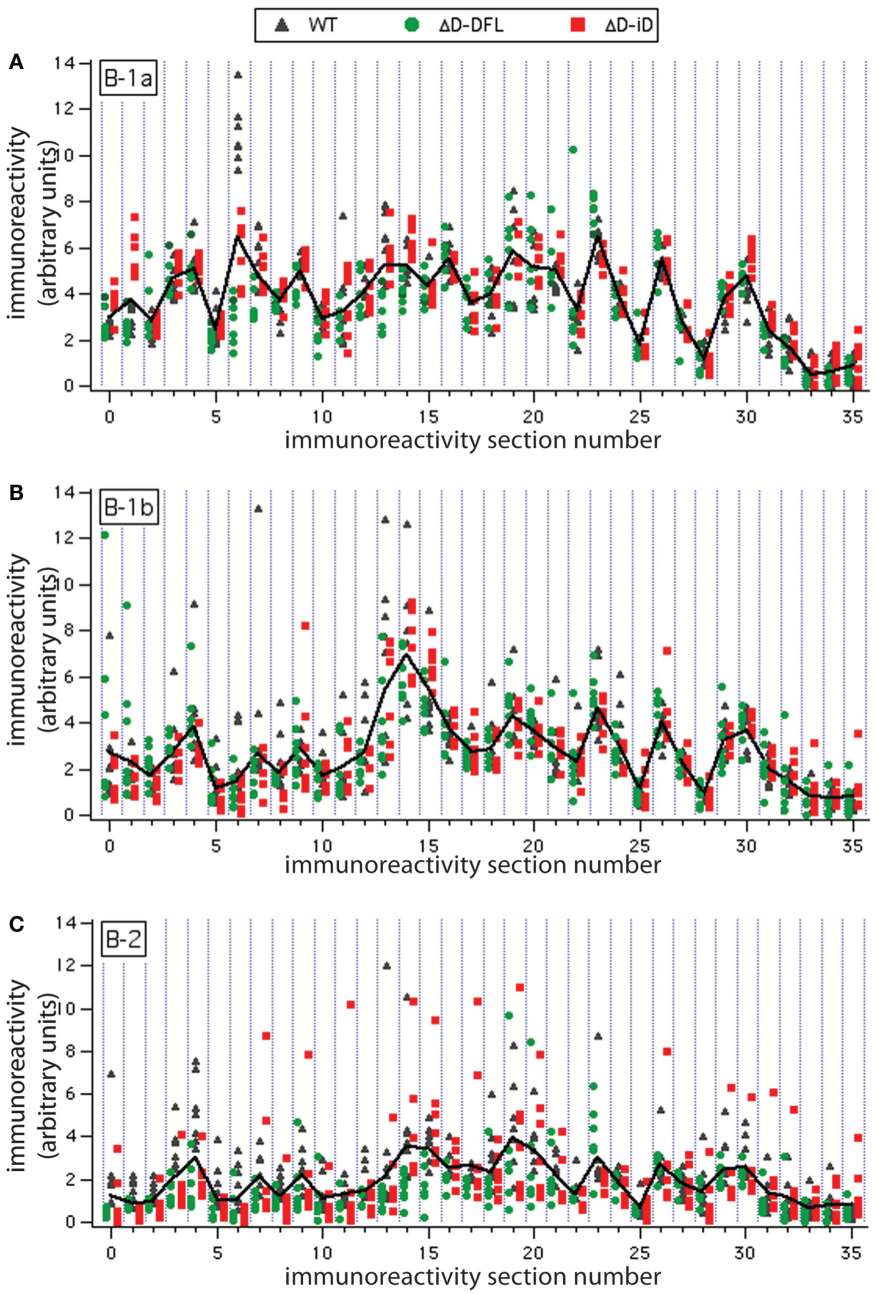


FIGURE 4 | Variance distribution of reactivities around the mean densitometric profile for each PerC B cell subset. Supernatant magnitudes of reactivity against the 36 sections in which reactivity profiles divided are plotted in **(A–C)**. These corresponded to B-1a, B-1b, and B-2 subsets, respectively. Solid lines indicate the mean profile of all supernatants for each B cell subset.

Each PerC B Cell Subset Displays a Characteristic Reactivity Repertoire Signature

To further confirm that each B cell subset displays a unique repertoire, we analyzed the reactivity profiles within each mouse strain. Irrespective of D_H content, the B-1a individual culture supernatants profiles proved very homogeneous, with little variance around their mean (**Figure 5A**, top; and Figure S1 in Supplementary Material). The reactivity profiles from B-1b subset were observed to differ from the B-1a for all mouse

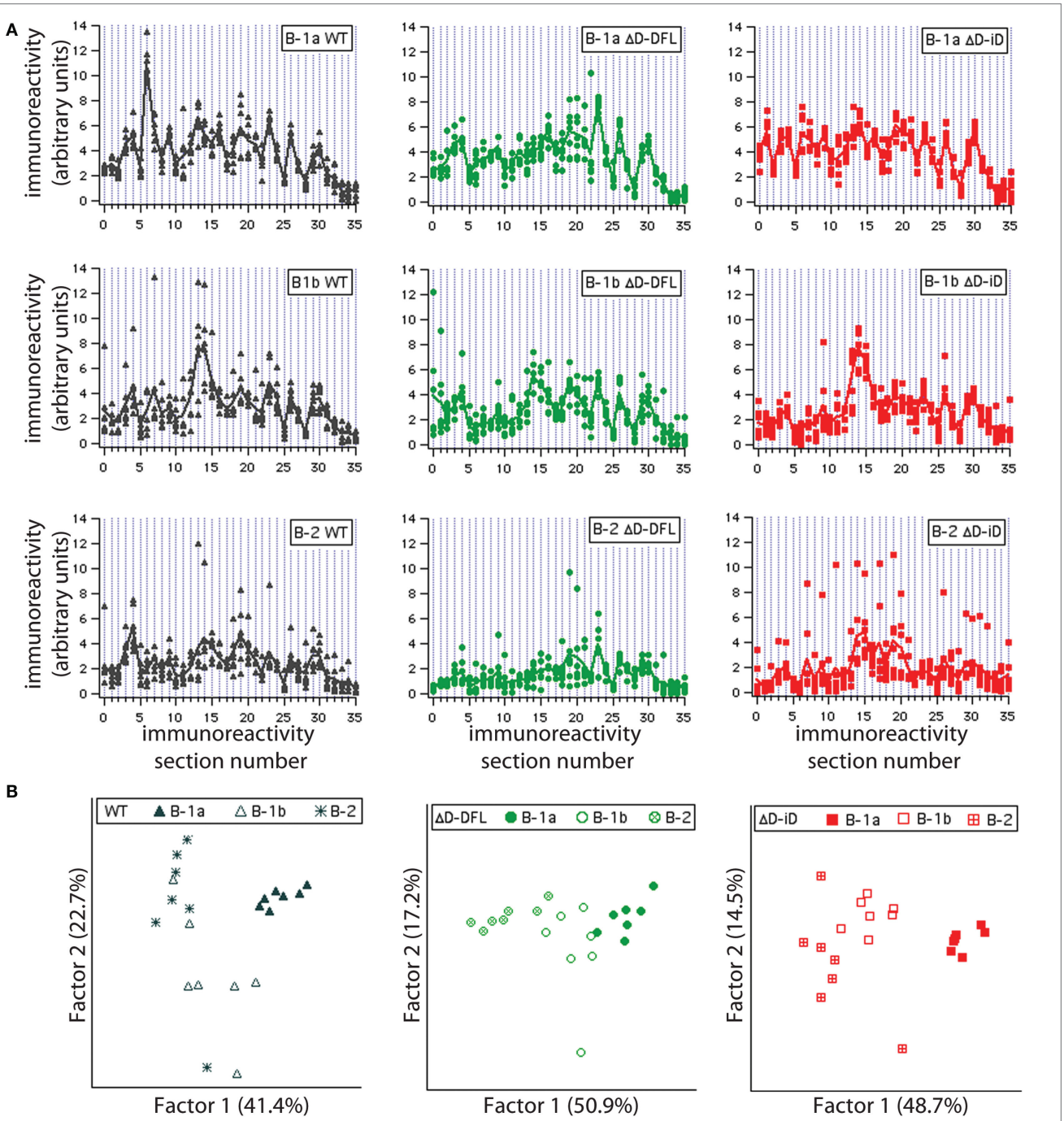


FIGURE 5 | Graphic representation of variance distribution of each B cell subset reactivity profile within each mouse strain. (A) Profiles of reactivities and distribution around the mean profile of each PerC B cell subset from WT, ΔD-DFL, and ΔD-iD are represented graphically. **(B)** Principal component analysis (PCA) of reactivity profiles from each mouse strain discriminates B cell subsets.

strains (**Figure 5A**, middle). The B-2 reactivity profiles displayed increased variance of the distribution in WT mice and especially for the ΔD-iD strain (Figure S1 in Supplementary Material), whereas the single normal D_H control (ΔD-DFL) displayed less variability and intensity of reactivity (**Figure 5A**, bottom).

To substantiate these observations, we performed principal component analysis (PCA) for each mouse strain. This multivariate approach takes in consideration all the reactivities together, providing an unbiased evaluation of the data (26, 31). All PerC B cell subset repertoires were discriminated within each mouse strain, suggesting distinct repertoire signatures for each population.

The Conservation of the Self-Reactivity Profile Correlates with B Cell Subset in Preference to Germline D_H Sequence Content

Although the mean reactivity profiles were generally conserved among the three mouse strains (**Figure 3A**), individual differences between the repertoires were also apparent (**Figures 2** and **3**). We, thus, performed a PCA of the data presented in **Figure 4**, which are grouped by B cell subset. This multivariate analysis revealed the impact of the genetic alteration of D_H sequences on the B-1a available repertoire of specificities (**Figure 6A**, left),

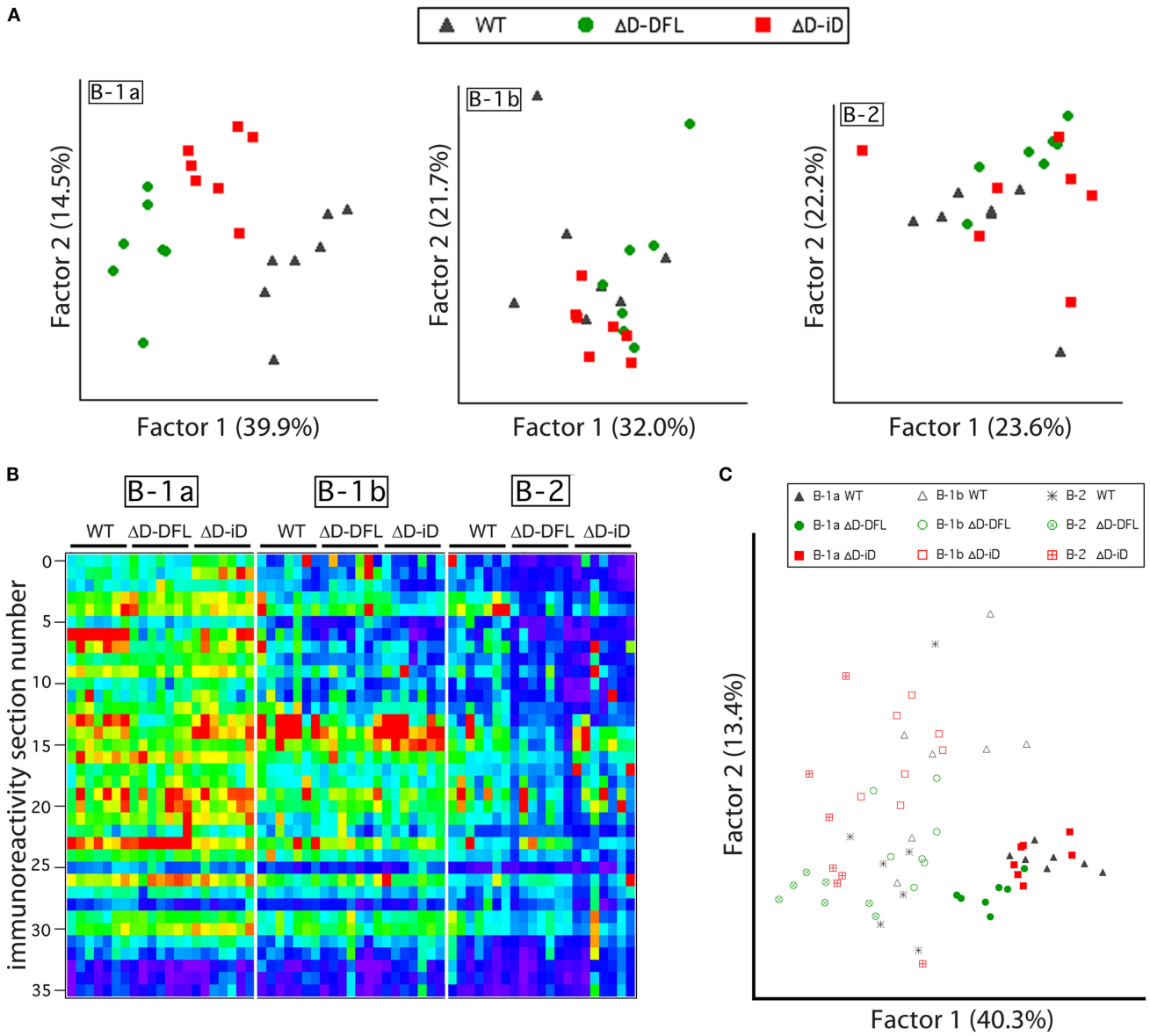


FIGURE 6 | B cell subset-intrinsic and genotype-dependent features of the repertoire. **(A)** Principal component analysis (PCA) of reactivities for each PerC B cell subset discriminates the B-1a genetic alterations of D_H sequences in the available repertoire of specificities but not B-1b or B-2 subsets available repertoire. **(B)** All data from reactivity profiles (B-1a, B-1b, and B-2 subsets from the three mouse strains) are shown on a single heat map indicating similarities between reactivity profiles. **(C)** Subjecting all data matrix to PCA results in subset characteristics prevailing over genetic distinctions imposed by D_H alterations.

demonstrating distinct differences between the strains. To better evaluate the contribution of each factor to the resulting B cell subset repertoires, we collected all the data from the reactivity profiles (B-1a, B-1b, and B-2 subsets from the three mouse strains) on a single heat map (**Figure 6B**). This large data matrix was similarly subjected to PCA.

Both the immunoblot assay (**Figures 2** and **3**) and the heat map of the reactivities (**Figure 6B**) disclosed a set of major bands that were unique to each strain and were shared among individual mice from each strain. These shared similarities and differences were much less apparent in the B-1b and B-2 subsets (**Figure 6A**). When analyzed by PCA together, the characteristics common to the subsets appeared to exert a greater effect than the characteristics provided by the differences in D_H content (**Figure 6C**).

DISCUSSION

Natural antibodies are formed early in ontogeny and are, thus, mostly germline encoded (18, 19). We sought to test whether the specificity of the NAb repertoire is the product of natural selection of germline immunoglobulin sequence or whether self-antigen is the driving force. These two hypotheses are not mutually exclusive, because even if self-antigen were the stimuli, the use of conserved germline sequence could facilitate the development of antibodies with certain specific epitope recognition properties that could provide protection in an anticipatory fashion (25).

In this work, we focused on the role of the sequence encoded by CDR-H3, which is created *de novo* by VDJ rearrangement and N addition and lies at the center of the antigen-binding site. This central position allows CDR-H3 to often play a major role in defining the epitope specificity of the antibody (32). The diversity gene segment (D_H) contributes significantly to the amino acid composition of CDR-H3. The ΔD-DFL strain expresses that portion of the CDR-H3 repertoire that is typically generated through use of the DFL16.1 gene segment, and is thus enriched for tyrosine, serine, and glycine. The ΔD-iD strain forces use of amino acids contributed by DSP gene segment inverted sequence. This repertoire is enriched for arginine, histidine, and asparagine.

Even though the CDR-H3 repertoire differs between the three strains of mice, the global self-antigen specificity of serum IgM in all three strains was largely the same (**Figure 1**), suggesting that antigen reactivity, and not germline immunoglobulin sequence content, was the driving force. The immunoreactivity profiles were similar in mice missing that portion of the immunoglobulin repertoire that is normally created by the 12 other deleted D_H gene segments in the ΔD-DFL mice. The same finding was obtained in the ΔD-iD mice that are not only missing the contribution of 12 D_H gene segments but also preferentially use an inverted RF sequence that completely alters the normal contribution of D_H encoded amino acids to the repertoire.

In previous studies in WT BALB/c mice, we found evidence of categorical selection of CDR-H3 sequence during B cell development, with B cell subsets, including those from the PerC, often exhibiting characteristic global sequence signatures (15, 33–35). These signatures were in addition to the bias for germline sequence exhibited by B-1a. To test whether the IgM repertoire expressed by these subsets in our panel of mice varied in its self-antigen specificities, we sorted B-1a, B-1b, and B-2 cells from the PerC, cultured them in the presence of LPS, and examined the IgM that was produced (**Figure 3**). If the self-specificity of the IgM were to reflect the influence of germline sequence, then the prediction was that B-1a IgM expressed by the D-altered B cells would demonstrate the greatest deviation from the WT norm. However, the opposite was observed. The reactivity of the IgM produced was most conserved in B-1a with the least variance, and least conserved in B-2 with the most variance (**Figures 3** and **4**). The B-1a repertoire, thus, demonstrated major convergent self-antigen-binding selection specificities in the presence of divergent germline sequence, whereas the B-1b and B-2 repertoires did not. PCA confirmed the distinct nature of the repertoire produced by each of the three subsets (**Figure 5**). The mechanisms behind these differences in variability are unclear, but potentially could reflect B cell subset-intrinsic characteristics, such as self-renewal capacity, clonal size, and repertoire polyreactivity.

The dominance of the biology of the B-1a component of the repertoire over the genetic alteration of CDR-H3 content among the activated PerC B cells, which is revealed by the heat-map PCA (**Figure 6C**), is in agreement with the substantial homology of the self-reactivities of the IgM in the sera of the WT, ΔD-DFL, and ΔD-iD mice (**Figure 1**), which has been argued to derive primarily from the B-1a subset (16). Although the biology of each individual subset exerted the greater effect on the reactivity of the IgM produced, a multivariate analysis revealed individual differences between the repertoires as a function of D_H sequence (**Figure 6**). By inspection, it is clear that some of these individual differences reflect the presence or absence of specific bands.

ΔD-DFL mice bear a D_H segment that is expressed in 20–30% of B-1a WT; this may result in a large overlap of B-1a repertoires and explain the similarity of their NAb repertoires. However, this explanation does not hold for ΔD-iD mice. One possible mechanism that could explain the convergence of the reactivities would be an antigen-influenced shift in the immunoglobulin repertoire expressed by the D-altered PerC B cells in the use of V_H or J_H, the extent of N nucleotide insertion or exonucleotide nibbling, or use of alternative D_H RFs. Studies are underway in our laboratory to test these hypotheses.

Our findings of similar immunoreactivity profiles between D-altered mice indicate that NAb reactivity is likely driven by a key subset of self-antigens. The nature of these antigens and the epitopes that define them remain largely unknown. In WT animals, they are preferentially recognized by IgM formed by germline-conserved CDR-H3 sequences. In mice forced to use alternative sequences, the majority, but not all, of these self-antigens seems to be able to induce a NAb repertoire with a similar reactivity profile. The exceptions may represent epitopes that require unique, germline-encoded D_H sequence in the antigen-binding site (25). These findings strongly suggest that it is the self-antigens, rather than conserved CDR-H3 germline sequence, that play the greater role in driving NAb production and reactivity.

MATERIALS AND METHODS

Mice

The panel of D_H-altered BALB/c mouse strains was bred in our mouse colony at the University of Alabama at Birmingham (UAB). Mice bearing ΔD-DFL (22) or ΔD-iD (23) D_H alleles were created by cre–loxP targeting of a BALB/c ES cell line. For each allele, 12 of the 13 BALB/c D_H gene segments were deleted and then the single, remaining DFL16.1 gene segment was either retained or altered. All WT and D_H-altered mice were studied at from 8 to 10 weeks of age. The mice were maintained in a SPF barrier facility and in a climate-controlled environment with a 12 h light/12 h dark cycle, with diet and water supplied *ad libitum*. Animal care was conducted in accordance with established guidelines and protocols approved by the UAB Animal Care and Use Committee.

Flow Cytometry and Cell Sorting

Flow cytometric analysis and cell sorting were performed as previously described (36). Briefly, peritoneal washout cells were obtained from five different mice of each D_H-altered strain. The following mAbs were used to isolate PerC B cells into the B-1a, B-1b, and B-2 subpopulations: anti-B220 (RA 3.6B2) (BD Pharmingen) (Southern Biotechnology, Birmingham, AL, USA), anti-Mac-1 (BD Pharmingen, San Diego, CA, USA) and anti-CD5 (BD Pharmingen, San Diego, CA, USA). PerC B-1a cells were sorted as $B220^{lo}CD5^{+}$, B-1b as $B220^{lo}CD5^{-}Mac\text{-}1^{lo/+}$, and PerC B-2 cells were sorted as $B220^{hi}CD5^{-}Mac\text{-}1^{-}$. A MoFlo instrument (Dako) was used for cell sorting.

Electrophoresis and Immunoblot

The preparation of brain extract, as well as determination of protein concentration, electrophoretic separation, blotting onto nitrocellulose membranes, and the test of immunoreactivities in the Mini-Cassette System, was performed as previously described (27) with secondary anti-IgM antibody coupled to alkaline phosphatase from Southern Biotechnology (Birmingham, AL, USA). The four gels used for limiting dilution assay (LDA) of reactivities were polymerized and run in parallel, such that profiles of total proteins blotted (revealed with colloidal gold) and the immunoreactivity profiles of lanes containing the standard pooled supernatant matched accurately between the gels, both qualitatively and quantitatively.

Rescaling of the Immunoblots and Data Analysis

The densitometric profiles of immunoreactivities were analyzed as previously described (30). Briefly, profiles were acquired first by scanning (Silverscanner II). This was followed by colloidal gold staining in order to reveal the migration position of the proteins. A comparison between any two immunoreactivity profiles could subsequently be performed by referring to the corresponding protein profiles. Data analysis was performed using the software IGOR Pro (Wavemetrics, Lake Oswego, OR, USA). Special software packages were written by the authors for the analysis and statistical treatment of the data and can be obtained from the authors.

B Cell Culture and Limiting Dilution Assay

B cell cultures were performed as described previously (36). Sorted B-1a, B-1b, and B-2 cells were cultured in 250 µl of complete RPMI medium in 96 well flat-bottom plates in the presence of 30 µg/ml of LPS (*Salmonella typhimurium*, Sigma-Aldrich). All cultures contained 5×10^3 S17 feeder cells/well for growth support as described by Ref. (37) with some modifications. Briefly, 1 day before the start of the LDA cultures, 5×10^3 S17 cells were added per well and incubated overnight at 37°C with 5% CO_2. The next day, the S17 culture plates were irradiated with 3000 rad and various numbers of the sorted B cells were added to the cultures. Culture supernatants were used to determine the frequency of IgM secreting clones by ELISA according to the Poisson distribution (38, 39) and contained sorted B cells with 22 replicates for each cell number (18, 6, 2, and 0.66 B cells per well). Another culture set was performed to analyze reactivity profiles by immunoblot, with 22 replicates of 10,000; 3333; 1111; and 370 B cells added per well. Culture supernatants were typically harvested on the fifth day of culture, unless indicated otherwise in the text.

IgM ELISA

To determine IgM concentration in the supernatants, ELISA was performed as previously described (36), using anti mouse IgM-specific reagents (Southern Biotechnology). Standard curves, obtained by using polyclonal, serially threefold diluted, mouse IgM (Southern Biotechnology), were used to quantify IgM.

Statistical Analysis

Differences between populations were assessed by Student's *t*-test, two tailed; Fisher's exact test, two tailed; $\chi 2$-test; or Levene's tests for the homogeneity of variance, as appropriate. Analysis was performed with JMP version 7.0 (SAS Institute, Inc., Cary, NC, USA). Means are reported with the SE of the mean.

AUTHOR CONTRIBUTIONS

AV, AN, and HS designed research; AV performed the experiments; AV, CC, and AN performed the statistical analysis; AV, CC, AN, and HS analyzed data and wrote the manuscript.

FUNDING

The present study was supported, in part, by the US National Institutes of Health (NIH) AI48115, AI090902, AI090742, and AI117703. Flow cytometry was supported by NIH AR48311 and AI027767. AN was supported by CNPq, FAPERJ, and FINEP. AV was supported in part by CNPq, FAPERJ, and a fellowship from CAPES (CsF-BJT; 024/2012). CC was supported by a fellowship from CNPq.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00296

Figure S1 | The immunoblot reactivity profiles of culture supernatants from LPS-stimulated B cells were subdivided into 36 reactivity sections,

as shown in Figure 4. For each mouse lineage and each B cell subpopulation, seven different supernatants were tested in the immunoblot assay for immunoreactivity against self-antigens derived from brain tissue extract (**Figure 4**). The variance of the magnitude of immunoreactivity for all 36 sections of reactivity was calculated for each mouse lineage and each B cell population. Histograms are shown in the figure. In all cases, most of the reactivity sections presented variance values below 10^6 units. For comparison between histograms, the number of sections with variance values inferior, or superior, to 10^6 units was indicated for each case. The differences between histograms were tested for statistical significance with Fischer exact test applied to 2 × 2 table containing the numbers of reactivity sections with variance values inferior, or superior, to 10^6 units.

REFERENCES

1. Avrameas S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol Today* (1991) 12(5):154–9. doi:10.1016/S0167-5699(05)80045-3
2. Hooijkaas H, Benner R, Pleasants JR, Wostmann BS. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. *Eur J Immunol* (1984) 14(12):1127–30. doi:10.1002/eji.1830141212
3. Bos NA, Kimura H, Meeuwsen CG, De Visser H, Hazenberg MP, Wostmann BS, et al. Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. *Eur J Immunol* (1989) 19(12):2335–9. doi:10.1002/eji.1830191223
4. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *Eur J Immunol* (1997) 27(6):1557–63. doi:10.1002/eji.1830270635
5. Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, et al. Positive selection of natural autoreactive B cells. *Science* (1999) 285(5424):113–6. doi:10.1126/science.285.5424.113
6. Wen L, Brill-Dashoff J, Shinton SA, Asano M, Hardy RR, Hayakawa K. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity* (2005) 23(3):297–308. doi:10.1016/j.immuni.2005.08.007
7. Gaudin E, Hao Y, Rosado MM, Chaby R, Girard R, Freitas AA. Positive selection of B cells expressing low densities of self-reactive BCRs. *J Exp Med* (2004) 199(6):843–53. doi:10.1084/jem.20030955
8. Cancro MP, Kearney JF. B cell positive selection: road map to the primary repertoire? *J Immunol* (2004) 173(1):15–9. doi:10.4049/jimmunol.173.1.15
9. Hayakawa K, Hardy RR, Honda M, Herzenberg LA, Steinberg AD, Herzenberg LA. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc Natl Acad Sci U S A* (1984) 81(8):2494–8. doi:10.1073/pnas.81.8.2494
10. Sidman CL, Shultz LD, Hardy RR, Hayakawa K, Herzenberg LA. Production of immunoglobulin isotypes by Ly-1+ B cells in viable motheaten and normal mice. *Science* (1986) 232(4756):1423–5. doi:10.1126/science.3487115
11. Feeney AJ. Junctional sequences of fetal T cell receptor beta chains have few N regions. *J Exp Med* (1991) 174(1):115–24. doi:10.1084/jem.174.1.115
12. Tornberg UC, Holmberg D. B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. *EMBO J* (1995) 14(8):1680–9.
13. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* (1997) 158(3):1175–86.
14. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity* (2005) 23(1):7–18. doi:10.1016/j.immuni.2005.04.011
15. Vale AM, Tanner JM, Schelonka RL, Zhuang Y, Zemlin M, Gartland GL, et al. The peritoneal cavity B-2 antibody repertoire appears to reflect many of the same selective pressures that shape the B-1a and B-1b repertoires. *J Immunol* (2010) 185(10):6085–95. doi:10.4049/jimmunol.1001423
16. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) 11(1):34–46. doi:10.1038/nri2901
17. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol* (2012) 42(1):120–9. doi:10.1002/eji.201141890
18. Mouthon L, Nobrega A, Nicolas N, Kaveri SV, Barreau C, Coutinho A, et al. Invariance and restriction toward a limited set of self-antigens characterize neonatal IgM antibody repertoires and prevail in autoreactive repertoires of healthy adults. *Proc Natl Acad Sci U S A* (1995) 92(9):3839–43. doi:10.1073/pnas.92.9.3839
19. Merbl Y, Zucker-Toledano M, Quintana FJ, Cohen IR. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. *J Clin Invest* (2007) 117(3):712–8. doi:10.1172/JCI29943
20. Nobrega A, Stransky B, Nicolas N, Coutinho A. Regeneration of natural antibody repertoire after massive ablation of lymphoid system: robust selection mechanisms preserve antigen binding specificities. *J Immunol* (2002) 169(6):2971–8. doi:10.4049/jimmunol.169.6.2971
21. Ivanov II, Link JM, Ippolito GC, Schroeder HW Jr. Constraints on hydropathicity and sequence composition of HCDR3 are conserved across evolution. In: Zanetti M, Capra JD, editors. *The Antibodies*. London: Taylor and Francis Group (2002). p. 43–67.
22. Schelonka RL, Ivanov II, Jung DH, Ippolito GC, Nitschke L, Zhuang Y, et al. A single DH gene segment creates its own unique CDR-H3 repertoire and is sufficient for B cell development and immune function. *J Immunol* (2005) 175(10):6624–32. doi:10.4049/jimmunol.175.10.6624
23. Ippolito GC, Schelonka RL, Zemlin M, Ivanov II, Kobayashi R, Zemlin C, et al. Forced usage of positively charged amino acids in immunoglobulin CDR-H3 impairs B cell development and antibody production. *J Exp Med* (2006) 203(6):1567–78. doi:10.1084/jem.20052217
24. Schroeder HW Jr, Zemlin M, Khass M, Nguyen HH, Schelonka RL. Genetic control of DH reading frame and its effect on B-cell development and antigen-specifc antibody production. *Crit Rev Immunol* (2010) 30(4):327–44. doi:10.1615/CritRevImmunol.v30.i4.20
25. Vale AM, Kapoor P, Skibinski GA, Elgavish A, Mahmoud TI, Zemlin C, et al. The link between antibodies to OxLDL and natural protection against pneumococci depends on DH gene conservation. *J Exp Med* (2013) 210(5):875–90. doi:10.1084/jem.20121861
26. Nobrega A, Haury M, Grandien A, Malanchere E, Sundblad A, Coutinho A. Global analysis of antibody repertoires. II. Evidence for specificity, self-selection and the immunological "homunculus" of antibodies in normal serum. *Eur J Immunol* (1993) 23(11):2851–9. doi:10.1002/eji.1830231119
27. Haury M, Grandien A, Sundblad A, Coutinho A, Nobrega A. Global analysis of antibody repertoires. 1. An immunoblot method for the quantitative screening of a large number of reactivities. *Scand J Immunol* (1994) 39(1):79–87. doi:10.1111/j.1365-3083.1994.tb03343.x
28. Mouthon L, Haury M, Lacroix-Desmazes S, Barreau C, Coutinho A, Kazatchkine MD. Analysis of the normal human IgG antibody repertoire. Evidence that IgG autoantibodies of healthy adults recognize a limited and conserved set of protein antigens in homologous tissues. *J Immunol* (1995) 154(11):5769–78.
29. Cohen IR. The cognitive paradigm and the immunological homunculus. *Immunol Today* (1992) 13(12):490–4. doi:10.1016/0167-5699(92)90024-2
30. Nobrega A, Grandien A, Haury M, Hecker L, Malanchere E, Coutinho A. Functional diversity and clonal frequencies of reactivity in the available antibody repertoire. *Eur J Immunol* (1998) 28(4):1204–15. doi:10.1002/(SICI)1521-4141(199804)28:04<1204::AID-IMMU1204>3.0.CO;2-G
31. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* (1998) 95(25):14863–8. doi:10.1073/pnas.95.25.14863
32. Kabat EA, Wu TT. Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. Relative contributions of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites. *J Immunol* (1991) 147(5):1709–19.
33. Ivanov II, Schelonka RL, Zhuang Y, Gartland GL, Zemlin M, Schroeder HW Jr. Development of the expressed Ig CDR-H3 repertoire is marked by focusing of constraints in length, amino acid use, and charge that are first established

in early B cell progenitors. *J Immunol* (2005) 174(12):7773–80. doi:10.4049/jimmunol.174.12.7773
34. Schelonka RL, Tanner J, Zhuang Y, Gartland GL, Zemlin M, Schroeder HW Jr. Categorical selection of the antibody repertoire in splenic B cells. *Eur J Immunol* (2007) 37(4):1010–21. doi:10.1002/eji.200636569
35. Schelonka RL, Szymanska E, Vale AM, Zhuang Y, Gartland GL, Schroeder HW Jr. DH and JH usage in murine fetal liver mirrors that of human fetal liver. *Immunogenetics* (2010) 62(10):653–66. doi:10.1007/s00251-010-0469-5
36. Vale AM, Hayashi E, Granato A, Schroeder HW Jr, Bellio M, Nobrega A. Genetic control of the B cell response to LPS: opposing effects in peritoneal versus splenic B cell populations. *Immunogenetics* (2010) 62(1):41–8. doi:10.1007/s00251-009-0404-9
37. Collins LS, Dorshkind K. A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. *J Immunol* (1987) 138(4):1082–7.
38. Andersson J, Coutinho A, Lernhardt W, Melchers F. Clonal growth and maturation to immunoglobulin secretion in vitro of every growth-inducible B lymphocyte. *Cell* (1977) 10(1):27–34. doi:10.1016/0092-8674(77)90136-2
39. Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J Immunol* (1981) 126(4):1614–9.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

METHODS
published: 04 November 2016
doi: 10.3389/fimmu.2016.00457

IMPre: An Accurate and Efficient Software for Prediction of T- and B-Cell Receptor Germline Genes and Alleles from Rearranged Repertoire Data

*Wei Zhang[1], I-Ming Wang[2], Changxi Wang[1], Liya Lin[1], Xianghua Chai[1], Jinghua Wu[1], Andrew J. Bett[2], Govindarajan Dhanasekaran[2], Danilo R. Casimiro[2] and Xiao Liu[1]**

[1] Beijing Genomics Institute (BGI-Shenzhen), Shenzhen, China, [2] Merck Research Laboratories, West Point, PA, USA

OPEN ACCESS

Edited by:
Nichol E. Holodick,
The Feinstein Institute for Medical Research, USA

Reviewed by:
Hedda Wardemann,
German Cancer Research Center (HZ), Germany
Christof Von Kalle,
Nationales Centrum für Tumorerkrankungen, Germany
Christian Busse contributed to the review of Hedda Wardemann

***Correspondence:**
Xiao Liu
liuxiao@genomics.cn

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: 26 April 2016
Accepted: 12 October 2016
Published: 04 November 2016

Citation:
Zhang W, Wang I-M, Wang C, Lin L, Chai X, Wu J, Bett AJ, Dhanasekaran G, Casimiro DR and Liu X (2016) IMPre: An Accurate and Efficient Software for Prediction of T- and B-Cell Receptor Germline Genes and Alleles from Rearranged Repertoire Data. Front. Immunol. 7:457. doi: 10.3389/fimmu.2016.00457

Large-scale study of the properties of T-cell receptor (TCR) and B-cell receptor (BCR) repertoires through next-generation sequencing is providing excellent insights into the understanding of adaptive immune responses. Variable(Diversity)Joining [V(D)J] germline genes and alleles must be characterized in detail to facilitate repertoire analyses. However, most species do not have well-characterized TCR/BCR germline genes because of their high homology. Also, more germline alleles are required for humans and other species, which limits the capacity for studying immune repertoires. Herein, we developed "Immune Germline Prediction" (IMPre), a tool for predicting germline V/J genes and alleles using deep-sequencing data derived from TCR/BCR repertoires. We developed a new algorithm, "Seed_Clust," for clustering, produced a multiway tree for assembly and optimized the sequence according to the characteristics of rearrangement. We trained IMPre on human samples of T-cell receptor beta (TRB) and immunoglobulin heavy chain and then tested it on additional human samples. Accuracy of 97.7, 100, 92.9, and 100% was obtained for TRBV, TRBJ, IGHV, and IGHJ, respectively. Analyses of subsampling performance for these samples showed IMPre to be robust using different data quantities. Subsequently, IMPre was tested on samples from rhesus monkeys and human long sequences: the highly accurate results demonstrated IMPre to be stable with animal and multiple data types. With rapid accumulation of high-throughput sequence data for TCR and BCR repertoires, IMPre can be applied broadly for obtaining novel genes and a large number of novel alleles. IMPre is available at https://github.com/zhangwei2015/IMPre.

Keywords: immune repertoire, novel germline gene, novel germline allele, TCR, BCR

INTRODUCTION

The "immune repertoire" is defined as the collection of diverse T-cell receptors (TCRs) and B-cell receptors (BCRs) created by somatic recombination of many germline V (variable), D (diversity), J (joining), and C (constant) gene segments. The immune repertoire (hereafter termed "repertoire") comprises the adaptive wing of the immune system. In recent years, advances in next-generation sequencing technology have enabled assessment of millions of B- or TCRs from a single sequencing

assay. This strategy allows researchers to study the repertoire in a more comprehensive way. Sequencing of the repertoire (Rep-seq) is being applied in several research areas: (i) monitoring of residual disease and immune reconstitution in cancers; (ii) understanding the diversity of T- and B-cell repertoires generated upon vaccination or infection; (iii) investigation of the mechanisms of immune surveillance in specific diseases (especially in infectious and autoimmune diseases); and (iv) production of monoclonal antibodies targeting specific antigens (1–5).

Well-characterized TCR/BCR germline genes are critical for analyses and interpretation of Rep-seq data. The publically available ImMunoGeneTics (IMGT) database[1] collects the genes of certain species. However, such information is not available for most species, which makes studying of repertoires highly challenging (if not unattainable). Deciphering of TCR and BCR germline loci requires additional resource-intensive efforts beyond conventional sequencing of the whole genome because these loci comprise multiple highly homologous and polymorphic gene family members. For instance, according to the IMGT database, the human immunoglobulin heavy-chain (IGH) locus, located at chromosome 14 (6), is composed of 123–129 V genes, 27 D segments, 9 J segments, and 9 C genes. The other two immunoglobulin light chain and four TCR loci are organized in a similar way, so exact identification of the many homologous gene sequences is difficult. In addition, like gene loci from human leukocyte antigens, germline genes also exhibit high polymorphism of alleles. There are >470 IGH V alleles in the IMGT database, but recently reported novel alleles (7, 8) from a few individuals suggest that numerous V alleles are absent. Many alleles for humans and other species have not been found. Absence of alleles may influence analyses of repertoires greatly. If the segment allele is assigned in error, the somatic hypermutation (SHM) cannot be identified exactly and may result in misleading clinically relevant decision-making processes (9). Well-characterized TCR/BCR germline alleles (polymorphisms) are critical for Rep-seq analyses.

Investigation of BCR/TCR germline genes and alleles is problematic, a validated method is lacking, and few studies in this area have been published. The conventional method employs a polymerase chain reaction (PCR)-based cloning strategy. That is, primers are designed based on human germline genes and are used to extract the species' counterparts through PCR amplification using genomic DNA (10–12). This is the most direct approach for obtaining sequences with high accuracy but is suitable only for species that are significantly homologous with humans. With this approach, iterative PCR optimization might be needed, which requires designing of primers on multiple occasions. Greenaway and colleagues showed germline genes to be inferred from the genomes of assembled species (13). However, precise and accurate assembly of genes from the V/J region is difficult due to their high homology; the inferred germline genes extracted from the genome could, therefore, contain errors. In addition, Gadala-Maria and coworkers discovered 11 unreported alleles (polymorphisms) from Rep-seq data on human IGH (8). However, that research focused on human IGH alleles with five or fewer mutations, required known germline sequences, and could not be used to predict novel genes. To resolve these issues, a tool for inferring novel germline genes/alleles without a genome and known germline genes/alleles is needed.

We designed primers at the conservative C region to capture the TCR/BCR rearranged repertoires using high-throughput sequencing. We developed a *de novo* tool, Immune Germline Prediction (IMPre), to infer novel TCR/BCR germline genes and alleles using Rep-seq data without known germline sequences or assembled genome data. Without knowing V/J gene segments, primers designed at the C region are available for all species, which is a perfect perspective to infer the highly homologous germline genes/alleles, and IMPre is the first tool to do this. IMPre is implemented using C and Perl programs and comprises four main steps: data processing, clustering, assembly, and optimization. As part of this effort, we developed a clustering algorithm, Seed_Clust, based on the same seed k-mer (i.e., all the possible substrings of length k contained in a string) to classify sequences. Subsequently, a multiway tree structure was used in the assembly step to extend seeds in both directions. T-cell receptor beta (TRB) and IGH samples from humans were sequenced to train IMPre, and additional human samples were used for testing. Accuracies were 97.7, 100, 92.9, and 100% for TRBV, TRBJ, IGHV, and IGHJ, respectively. Subsampling performance was estimated, and results showed IMPre to be robust even with different data quantities, and that 1 million sequences were sufficient for germline prediction. IMPre performs with good efficiency and speed while using less memory. TRB samples from rhesus monkeys and additional long-sequence human samples were used to retest IMPre. The highly accurate results obtained suggest that IMPre is stable with animal and long-sequence data.

MATERIALS AND METHODS

Design and Overview of the IMPre System

Two main recombination characteristics support the notion that rearranged repertoire data can be used to infer germline genes. First, the probability of V(D)J insertion/deletion at a gene terminus in an individual tends to decrease with increasing length of the deletion (14, 15). We analyzed our data (Table S1 in Supplementary Material) on the distribution of length of the V/J deletion (Figures S1A,B in Supplementary Material). We found that a length of the V deletion >50% for TRB and 70% for IGH were within 1 bp, whereas the length of the J deletion was much more diverse; however, the value tended to decrease if the deletion length increased. Then, we downloaded the rearranged sequences from the IMGT database and observed the same trend from 3907 fully annotated sequences (Figure S1C in Supplementary Material). Second, researchers have reported that immunoglobulin (Ig) genes introduce SHMs at 10^{-3} per bp per cell division (16, 17). SHMs are, in general, random with a low frequency ($\leq$5%), except for known G/C "hotspots" ($\leq$10%) among large-scale repertoire data (7). Germline genes can be predicted after elimination of SHM effects. These phenomena provide a strong theoretical basis for accurate identification of germline genes and alleles.

The IMPre pipeline mimics the reverse process of VDJ rearrangement in TCRs and BCRs (**Figure 1A**) and comprises

[1]http://www.imgt.org/.

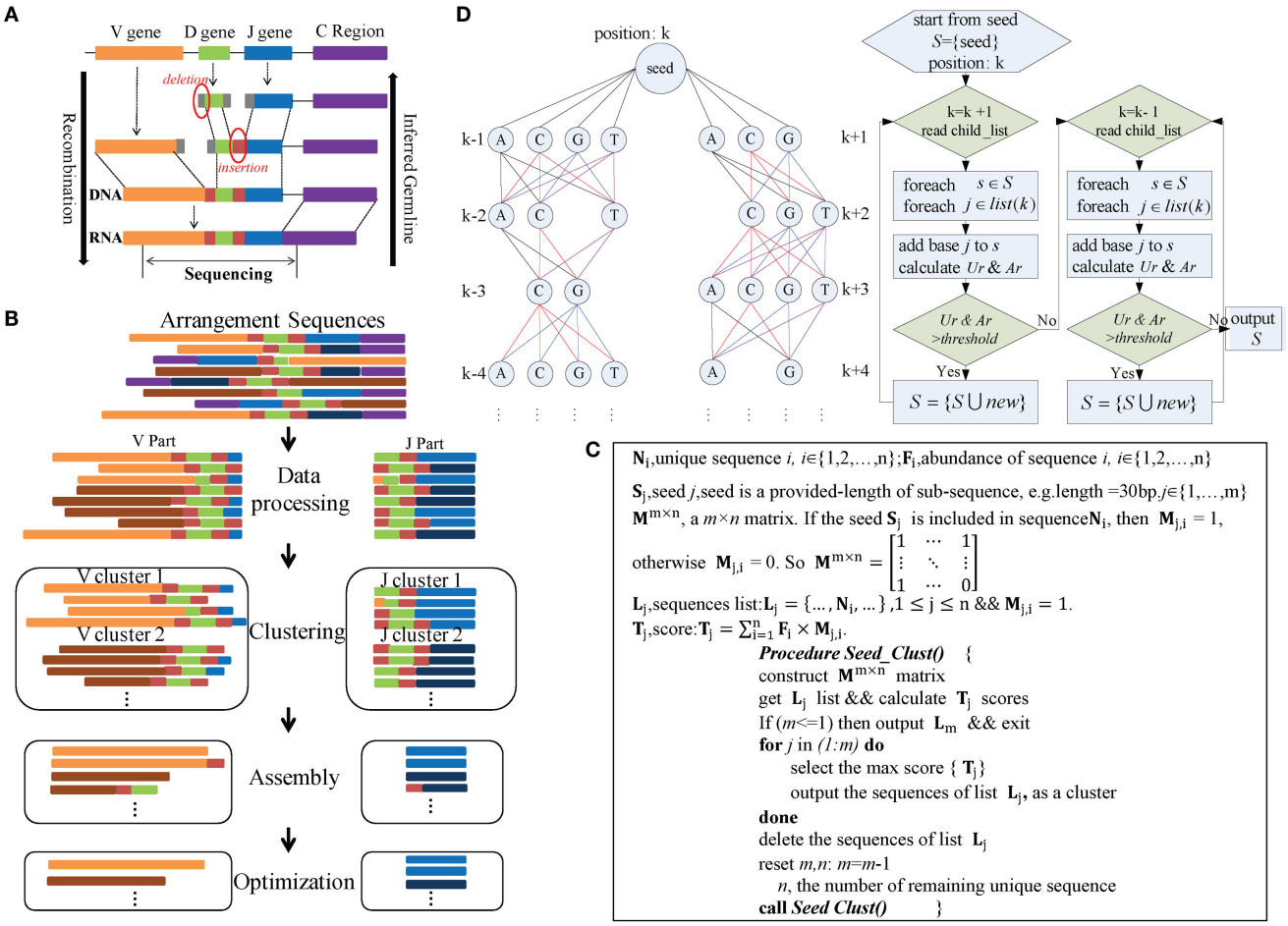


FIGURE 1 | Overview of IMPre workflow. **(A)** Rearrangement. **(B)** IMPre flowchart shows four steps and uses rearranged sequences as the input. Sequences are divided into V and J parts during the data-processing step. The Seed_Clust algorithm is used to cluster sequences for each part. For each cluster, sequences are used for assembly and further optimization. **(C)** Clustering. The pseudocode outlines the Seed_Clust clustering algorithm. **(D)** Assembly. A multiway tree structure stores nucleotides and assembles using a one-nucleotide extension strategy. The V gene begins to extend from the right subtree and then from the left subtree. Two parameters, *Ar* and *Ur*, determine whether a nucleotide should be added or not.

four steps: data processing, clustering, assembly, and optimization (**Figure 1B**). During data processing, rearranged repertoire sequences are converted into forward sequences and partitioned into V and J parts. During clustering, the Seed_Clust algorithm is based on seed k-mers and used for sequence clustering that prioritizes large clusters with the same seed k-mers. A pseudocode that describes this step in detail is shown in **Figure 1C**. Each sequence is assigned to one cluster. During assembly, for each cluster, a multiway tree structure is constructed to store the seed and upstream/downstream nucleotides. It begins at the seed and is extended using a one-nucleotide extension strategy for *de novo* assembly. The *Ar* and *Ur* (detailed definitions are shown below) are used to ascertain if a nucleotide should be added or not added (**Figure 1D**). During optimization, three steps are used. The *Trim5 Ratio*, *Trim5 Rate*, and *More5 Rate* (definitions are shown below) are calculated to filter out false-positive (FP) readings. Sequences with the same overlapping region are merged to eliminate redundancy. Similar low-frequency sequences are removed to filter out sequences with SHM and PCR/sequencing errors. Finally, inferred sequences are annotated by known germline sequences.

Data Processing

Immune Germline Prediction can process the rearranged sequences with or without the C region. If the C region is embodied, it is identified using previously reported publically available C sequences with a limit of two mismatches for the first 18 bp. The identified C region is used to convert all the sequences to forward sequences and then is trimmed for further analyses. To predict V and J genes independently, rearranged sequences are partitioned into two parts. IMPre extracts the last 60 bp (parameter: $-jm$) from the 3' end of sequences to define the "J part." Sequences that are trimmed in the last 40 bp (parameter: $-vm$) of the 3' end are defined as the "V part." Parameters can be adjusted, but they must cover the entire potential V and J regions. To avoid missing regions, the V portion of the sequence is tolerated to contain a D gene (for IGH and TRB) and a partial

J gene. Also, the J portion may contain a D gene (for IGH and TRB) and a partial V gene (**Figure 1B**).

Clustering

Immune Germline Prediction clusters V and J sequences separately (**Figure 1B**), and both use the same clustering strategy detailed in **Figure 1C**. Heyer et al. reported a quality cluster algorithm (QT_Clust) for expression data (18). Similarly, to discover large clusters, we developed a clustering algorithm for V and J sequences. The algorithm prioritizes the largest cluster that included the same seed k-mer, defined as Seed_Clust.

Seed_Clust operates in five main steps (**Figure 1C**). In step (1), length-provided subsequences (k-mer) are created as seeds (S) from all sequences. In step (2), a matrix $\boldsymbol{M}^{m \times n}$ is constructed using the seed k-mer, where m is the number of seeds and n is the number of unique sequences. If the seed $\boldsymbol{S}_j$ is included in the sequence $\boldsymbol{N}_i$, then $\boldsymbol{M}_{j,i=1}$; otherwise, $\boldsymbol{M}_{j,i=0.2}$. The score $\boldsymbol{T}_j$ (defined in **Figure 1C**) is calculated for each seed $\boldsymbol{S}_j$. In step (3), the maximum score $\boldsymbol{T}_j$ is selected, and the sequences that embody the seed $\boldsymbol{S}_j$ are regarded as one cluster and are outputted. In step (4), the output sequences are removed from the matrix $\boldsymbol{M}^{m \times n}$. In step (5), steps (1)–(4) are repeated under iteration until all sequences belong to one cluster.

The seed k-mer is dependent upon sequence length and other characteristics. However, the last 10 bp of the 3' end at the "V part" sequence are excluded for seed creation because this region might not truly belong to the V gene; similarly, the first 5 bp at the 5' end of the J portion are not considered.

Assembly

Each cluster is assembled independently (**Figure 1B**). We developed an assembly strategy based on seeds and one-nucleotide-by-one-nucleotide extension (**Figure 1D**). In this step, a multiway tree is generated to store data and to aid assembly. First, the seed of each sequence is set as the center position k, and the upstream nucleotides are set at positions $k-1, k-2, \ldots$, in order, whereas the downstream nucleotides are set at positions $k+1, k+2, \ldots$, in order. Each position may consist of four nucleotides, except position k, and the number of supporting sequences for each nucleotide is calculated.

The assembly begins from the seed and extends in the direction of the right subtree [usually in the direction toward CDR3 (complementarity-determining regions) sequences]. After the right subtree is complete, it extends further in the direction toward the left subtree. Criteria and rules are identical for left and right subtrees (**Figure 1D**). For each time, one nucleotide ("note") is considered to be added for extension, and then all "brother-notes" are considered successively. After finishing all brother-notes, the algorithm extends to all "child-notes." For example, three nucleotides ("A," "C," and "G") at position $k+1$ are considered for addition to the seed, and three extended sequences are created if the nucleotides meet requirements. Then, three nucleotides ("C," "G," and "T") at position $k+2$ are considered for addition to the extended sequences separately. Nucleotides at the remaining positions comply with the same rule iteratively. Finally, multiple qualified extended sequences can be retained.

For each time, to ascertain if the nucleotide (note) should or should not be added for extension, we defined two parameters, *Ar* and *Ur*:

$$\mathrm{Ar}(i,j) = \frac{\text{Number of supporting reads for an extended sequence}}{\text{Number of total reads at position } i}$$

$$\mathrm{Ur}(i,j) = \frac{\text{Number of unique supporting reads for an extended sequence}}{\text{Total number of unique reads at position } i},$$
$$i \in \{.., k-2, k-1, k, k+1, k+2, \ldots\}, j \in \{A, C, T, G\}$$

Here, an "extended sequence" is defined as the sequence after one nucleotide j is added in position i. We provide an example to illustrate these two parameters (**Figure 2A**). Here, there are 10 reads in the cluster. When it extends to position i, three reads contain the nucleotide "A" in a total of seven reads, so *Ar(i,A)* is equal to 42.9%. Likewise, two unique reads contain the "A" in the total of six unique reads, so *Ur(i,A)* is equal to 33.3%. The two values can be calculated for other nucleotides in the same way. Defaults for the two parameters are V: $Ar > 0.15$, $Ur > 0.12$; J: $Ar > 0.12$, and $Ur > 0.10$. Actually, most SHMs can be filtered in this step.

Optimization

Numerous potential germline sequences are obtained from the assembly step, but they must be optimized further to eliminate incorrect sequences. The assembly step yields four types of sequences (**Figure 2B**), among which three are not accurate or precise, and are defined as FPs. Among them, one is derived from a non-specific PCR amplification and the other two types exhibit missing portions of the germline sequence (too short) or contain extra V–D/D–J additions (too long).

Three steps are used to filter incorrect sequences (**Figure 3A**). First, FP sequences are filtered. Genuine rearranged V/J gene segments feature diverse V/J deletion/insertion lengths and recombine with multiple adjacent D genes. Therefore, the diversity of the 5 bp-trimmed or extended sequences is significantly higher for the correct type of sequence (**Figure 2B**). Three factors (*Trim5 Ratio*, *Trim5 Rate*, and *More5 Rate*) derived from these characteristics are introduced to filter out the incorrect sequences, and the definitions are listed as shown below: (The *Trim5 Ratio* includes *Trim5 Ratio1* and *Trim5 Ratio2*, whereas the *Trim5 Rate* includes *Trim5 Rate1* and *Trim5 Rate2*.)

$$\text{Trim5 Ratio1} = \frac{\text{Number of supporting reads for } S_{\text{trim5}}}{\text{Number of supporting reads for } S} \times 100$$

$$\text{Trim5 Ratio2} = \frac{\text{Number of unique supporting reads for } S_{\text{trim5}}}{\text{Number of unique supporting reads for } S} \times 100$$

$$\text{Trim5 Rate1} = \frac{\text{Number of supporting reads for } S_{\text{trim5}}}{\frac{1}{n}\sum_{i=1}^{n}\{\text{Number of supporting reads for } S_{\text{trim5}}(i)\}} \times 100$$

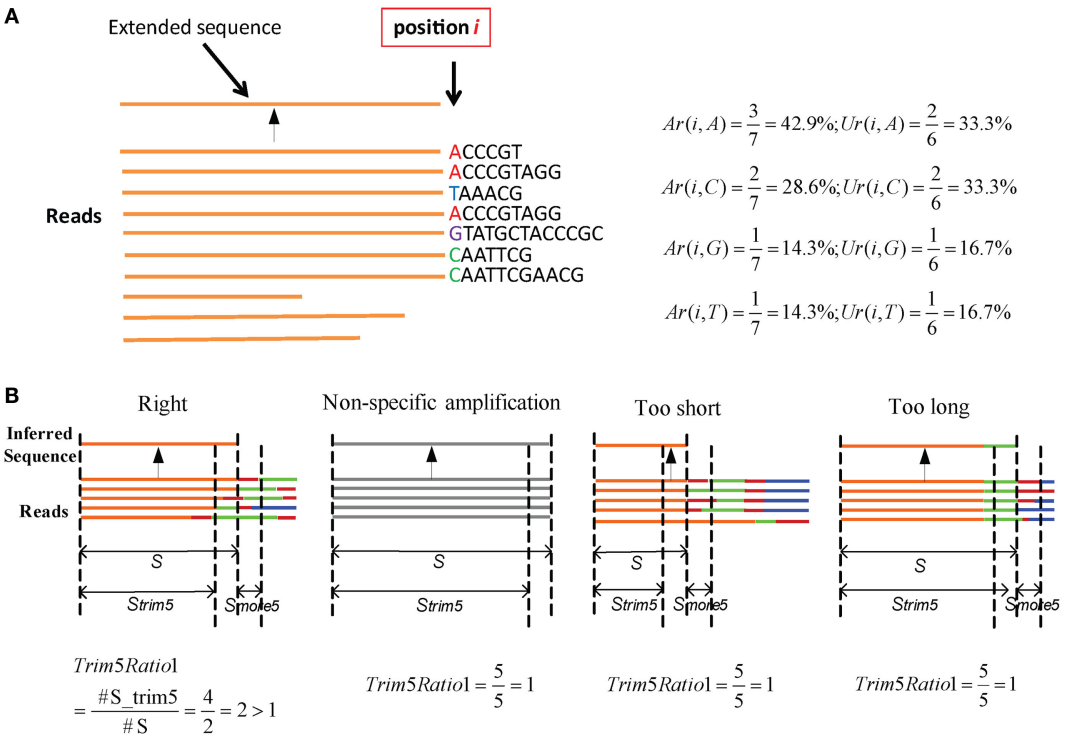


FIGURE 2 | Parameters in assembly and optimization steps (schematic). **(A)** An example for *Ar* and *Ur* calculation in the assembly step. There are 10 reads in a cluster. Each time, we determine whether the nucleotides in position *i* should be added for extension or terminated using the values of *Ar* and *Ur*. **(B)** Four types of inferred sequences obtained from the assembly step. For the reads, orange denotes the V region, red is inserted nucleotides, green is the D region, blue is the J region, and gray is the reads from non-specific amplification. S_{trim5} are 5 bp shorter than *S*, and S_{more5} is a 5 bp fragment.

$$\text{Trim5 Rate2} = \frac{\text{Number of unique supporting reads for } S_{\text{trim5}}}{\frac{1}{n}\sum_{i=1}^{n}\{\text{Number of unique supporting reads for } S_{\text{trim5}}(i)\}} \times 100$$

$$\text{More5 Rate} = \frac{\text{Number of unique supporting reads for } S_{\text{more5}}}{\frac{1}{n}\sum_{i=1}^{n}\{\text{Number of unique supporting reads for } S_{\text{more5}}(i)\}} \times 100$$

where n is total number of inferred sequences, S is the inferred sequence from the assembly step, S_{trim5} is the 5 bp-trimmed S at the end (the V gene is trimmed at the 3′ end and the J gene is trimmed at the 5′ end), S_{more5} is a 5-mer subsequence which is extended from S (extended from the 3′ end of S for V and the 5′ end of S for the J gene). S, S_{trim5}, and S_{more5} are illustrated in **Figure 2B**. For example, taking *Trim5 Ratio*1 in **Figure 2B**, the value of *Trim5 Ratio*1 for the "Right" type sequence is 2, whereas the values for other three types of sequences are equal to 1.

Defaults of three factors are set as: *Trim5 Ratio* >1.5, *Trim5 Rate* >2 (*for TRB-J* >0.5), and *More5 Rate* >5.

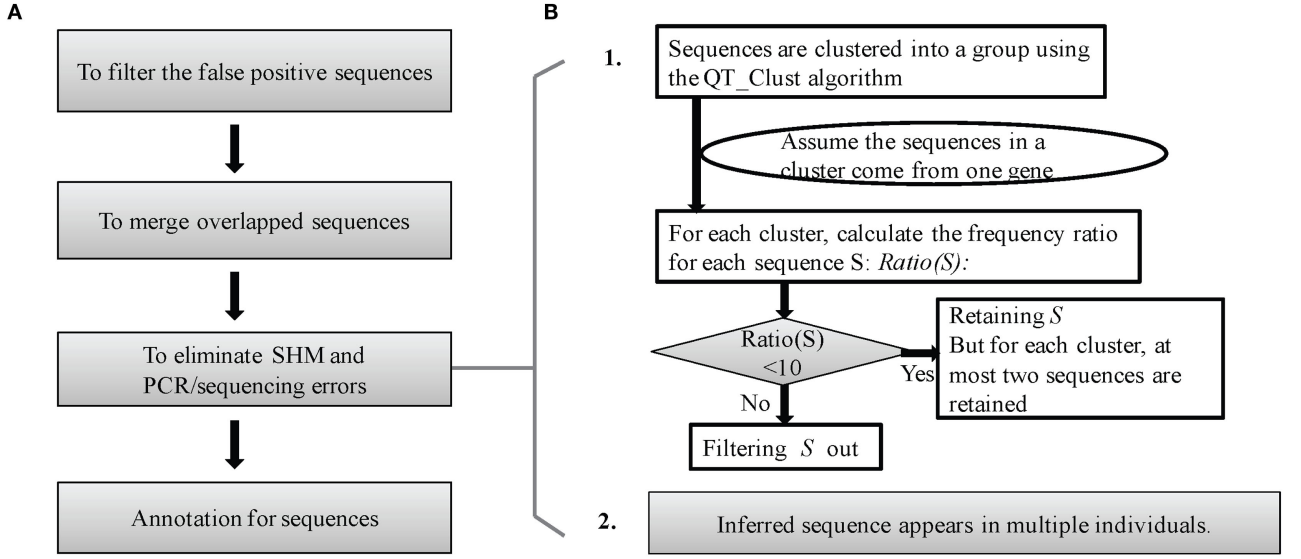
Second, to eliminate redundancy in inferred germline sequences, sequences are merged if they have identical overlapping regions (two mismatches are allowed for the V gene at the 3′ end and two mismatches are allowed for the J gene at the 5′ end).

Third, elimination of SHM and PCR/sequencing errors is attempted (**Figure 3B**). The predicted germline sequences are clustered into multiple groups using the QT_Clust algorithm (18). Sequences with the most reads are defined as the "center sequence," and other sequences with more than three mismatches (two mismatches for the J gene) are clustered into the same group with the center sequences (the last 3 bp at the 3′ end for the V gene, and the first 3 bp at the 5′ end for the J gene are masked). According to analyses of human germline sequences from the IMGT database (Figure S2 in Supplementary Material), we assumed that the sequences within one group arose from the same germline genes. Theoretically, observation of more than two alleles for each gene in one sample is not possible, so some sequences must be filtered if there are more than two sequences in one cluster. Thus, Ratio(*S*) for each sequence *S* is introduced as

$$\text{Ratio}(S) = \frac{\text{number of supporting reads for the center sequence}}{\text{number of supporting reads of } S}.$$

The predicted sequence *S* is filtered out if the Ratio(*S*) >10 because Boyd and colleagues reported that almost all ratios of the two alleles in an individual were <10 (7). Furthermore, at most, two sequences in a cluster are retained [retention of the center sequence and another sequence with the lowest Ratio(*S*)].

Another effective way to eliminate SHMs is using multiple individuals to infer germline genes/alleles (**Figure 3B**). If the inferred sequence is observed in multiple individuals, it is likely to be the "true" germline sequence. SHMs would occur at random, and the chances of multiple individuals using identical alleles with the same SHMs are rare.



C

#ID	Score	Total_reads_support	Unique_reads_support	Recommend_Gene/allele	Flag	Mapped_to_known_germline	Mapped_identity	Mapped_mismatch	Mapped_Deviated_bases
V-infer-3	87	6525	5552	*V7-9*08*	novel_allele	*TRBV7 -9*01*_F_Human#*TRBV7 -9*02*_(F)_Human	99.11	1	1
V-infer-16	73	2412	2360	*V20-1*01*	known	*TRBV20 -1*01*_F_Human	100	0	0
V-infer-25	72	2342	2276	*V7-2*01*	known	*TRBV7 -2*01*_F_Human	100	0	2
V-infer-30	69	1533	1435	*V-A*01*	novel_gene	*TRBV7 -2*01*_F_Human	90	9	0

Partial sequences:

>V-infer-3

TGCTCAGTGATCGGTTCTCTGCAGAGAGGCCTAAGGGATCTTTCTCCACCTTGGAGATCCAGCGCACAGAGCAGGGGGACTCGGCCA

>V-infer-16

AAGGACAAGTTTCTCATCAACCATGCAAGCCTGACCTTGTCCACTCTGACAGTGACCAGTGCCCATCCTGAAGACAGCAGCTTCTACA

>V-infer-25

GCCCAGTGATCGCTTCTCTGCAGAGAGGACTGGGGAATCCGTCTCCACTCTGACGATCCAGCGCACACAGCAGGAGGACTCGGCCGT

FIGURE 3 | Detailed optimization strategy and output format. **(A)** Pipeline for the optimization step. **(B)** Details for elimination of SHM and PCR/sequencing errors. **(C)** Output format of IMPre. IMPre outputs two files: one is the annotation file and the other is the inferred sequence.

Annotation and Output

To measure the certainty of an inferred germline sequence, we provide a score for each inferred germline sequence according to seven important factors with different weight W (the total weight is 100 and the range of scores is between 0 and 100, **Figure 3C**). The score for each sequence S is defined as

$$\begin{aligned}\mathrm{Score}(S) = &\frac{\text{Trim5 Ratio1}\,(S)}{\max\{\text{Trim5 Ratio1}\}} \times \mathrm{W}_{\text{Trim5 ratio1}} \\ &+ \frac{\text{Trim5 Ratio2}\,(S)}{\max\{\text{Trim5 Ratio2}\}} \times \mathrm{W}_{\text{Trim5 ratio2}} \\ &+ \frac{\text{More5 Rate}\,(S)}{\max\{\text{More5 Rate}\}} \times \mathrm{W}_{\text{More5 rate}} \\ &+ \frac{\text{Trim5 Rate1}\,(S)}{\max\{\text{Trim5 Rate1}\}} \times \mathrm{W}_{\text{Trim5 rate1}} \\ &+ \frac{\text{Trim5 Rate2}\,(S)}{\max\{\text{Trim5 Rate2}\}} \times \mathrm{W}_{\text{Trim5 rate2}} \\ &+ \frac{\#\text{merged sequences}}{\max\{\#\text{merged sequences}\}} \times \mathrm{W}_{\text{merged num}} \\ &+ \{\mathrm{W}_{\text{major}} | \mathrm{W}_{\text{minor}}\}\end{aligned}$$

$$(\mathrm{W}_{\text{Trim5 ratio1}}, \mathrm{W}_{\text{Trim5 ratio2}}, \mathrm{W}_{\text{More5 rate}}, \mathrm{W}_{\text{Trim5 rate1}}, \mathrm{W}_{\text{Trim5 rate2}}, \mathrm{W}_{\text{merged num}}, \mathrm{W}_{\text{major}}, \mathrm{W}_{\text{minor}}) = (20, 20, 15, 10, 10, 15, 10, 5)$$

where "#merged sequences" is the number of sequences merged in the optimization step.

Similarity of human alleles for α and β chains, as well as for heavy and light chains, in IMGT, was analyzed. Hamming distances between any alleles and the *01 allele in a gene were calculated (Figure S5 in Supplementary Material, Inner_gene). As

a control, Hamming distances between the *01 allele and one of the alleles in other genes (Figure S5 in Supplementary Material, Outer_gene) were also calculated and had the highest similarity with the *01 allele. Thus, the most probable dividing lines between the Inner_gene and Outer_gene were a Hamming distance for the V and J gene of 7 and 5, respectively (Figure S5 in Supplementary Material). To distinguish between inferred germline genes and alleles, and to determine which alleles belonged to the same genes, two steps were used to provide a recommended name for each inferred sequence. First, sequences were aligned to the known germline sequences of the human and mouse from the IMGT database, where other known germline sequences could also be added for annotation using the parameter "*−known*." If the mismatch number between the sequence and nearest known germline sequence was <7 bp (for J: ≤ 5 bp), the sequence was regarded as a novel allele belonging to the gene of the nearest known germline. Second, for sequences with a mismatch number >7 bp (for J: >5 bp), we clustered sequences into a group if their Hamming distance was <7 bp (for J: ≤ 5 bp) using the QT_Clust algorithm (18) and assumed that the sequences in a group belonged to one gene. We named the group using a character as an assumed gene name followed by an asterisk and allele number (**Figure 3C**) according to the nomenclature rules set by the IMGT collaboration. Notably, accurate nomenclature requires complete assembly of the genome for TCR and BCR germline loci, which is not available for most species. Besides, a few independent V segments are high homologous between each other, with the mismatch number less than 7 bp. Therefore, the gene and allele names provided here are just as reference, and much more sufficient evidence are required by further work.

To annotate the inferred sequences, information of the mapped nearest known germline sequence was provided in the output file, which included the nearest allele, number of mismatches, and number of deviated bases (**Figure 3C**). Each sequence was marked as "known," "novel_allele," or "novel_gene," and a name recommended in terms of the standards and rules stated above. Overall, IMPre outputs two files: one is the annotation file and the other is the inferred sequence with the FASTA format (**Figure 3C**).

Simulation of *In Silico* Sequences

The method to generate simulated BCR repertoire sequences were described in the previous paper (14). Here, we create 12 datasets and each one includes 10^5 rearranged sequences. First, we generate sequencing error with the rate of 0.5% (per base) for all sequences. Second, we generate the SHM for 80% of sequences of dataset, with different SHM level rates: 0, 1, 5, 10, 15, and 20% (per base) for six dataset separately, and with the SHM rate 1% for the other six datasets, where the mutations occur at random. Third, for the latter six datasets, we generate the hotspot mutation in them, with the hotspot rate of 5, 10, 15, 20, 25, and 30%, respectively. We generate the hotspot mutations like that: for the sequences derived from the same germline allele, partial of them (hotspot rate 5% means 5% of them) create a same specific mutation. For example, there are 100 rearranged sequences derived from the germline *IGHV1-1*01*, 20 of them have the same mutation at position 120, such as A->C, so the hotspot rate is 20%. Therefore, all germline alleles made up the dataset generate the same hotspot mutation rate.

Collection and Preprocessing of Samples

Research was reviewed prospectively and approved by a duly constituted ethics committee (Institutional Review Board on Bioethics and Biosafety of BGI-Shenzhen).

All human samples were recruited after obtaining written informed consent. Mononuclear cells were isolated from the peripheral blood of five healthy individuals using Ficoll-Paque (GE Healthcare, Little Chalfont, UK) gradient centrifugation. RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA). Three samples were used to capture TRB, and two samples were used for IGH. We used a Rapid Amplification of cDNA Ends (5′ RACE) kit (v2.0, Invitrogen) to amplify the target region with primers at a C region. TRB primers were used based on the study by Warren and colleagues (19). PCR products were sheared using a Covaris S2 system (Applied Biosystems, Foster City, CA, USA). Biotinylated fragments were purified and excised in 100–200 fragments to prepare a library. Samples were sequenced using a HiSeq 2000 system (Illumina, San Diego, CA, USA) with a paired-end (PE) 100 bp (Table S1 in Supplementary Material). An additional human sample was collected. Multiplex PCR was used to amplify IGH with BIOMED-2 primers (20) at the FR1 region and C region. We undertook sequencing with MiSeq and PE 300 bp kits (Table S1 in Supplementary Material). Data of two healthy Indian rhesus monkeys were collected [we adhered to the *Guidelines for the Care and use of Animals for Scientific Purposes* (November 2004) established by the Singapore National Advisory Committee for Laboratory Animal Research]. TRB samples were sequenced using HiSeq 2000 with PE 150 bp kits (Table S1 in Supplementary Material). All sequence preprocessing was done and PE reads merged using IMonitor (14).

Raw deep-sequencing data of human samples are available at the NCBI Short Read Archive[2] under the accession numbers SRA339484 and RJNA309577.

RESULTS

Training Parameters of Software Using Human Samples

Currently, there are relatively complete germline sequences for humans in the IMGT database. If we align the TRB or IGH repertoires to the human known germline sequences, and we can identify which gene and allele exists in the TRB and IGH samples. Therefore, rearranged repertoire data for humans were adopted to train IMPre. Two TRB (S01-R and S03-R) samples and another IGH (H09, of which the mutation rate is 5.36% per base and 72.25% sequences contain error bases) sample from healthy humans (Table S1 in Supplementary Material), amplified by 5′ RACE and sequenced using the Illumina platform, were used as the training dataset. The assembly step used *Ar* and *Ur* to judge whether a sequence extension should continue or terminate. To train these two parameters, we calculated the *Ar* and *Ur* for the true germline sequence (TGS) and error germline sequence (EGS) in each cluster outputted by the clustering step of IMPre. After clustering, if the sequence contained the V or J germline allele

[2]http://www.ncbi.nlm.nih.gov/Traces/sra/.

without mismatch and <3 bp deletion at the junction region, we defined it as the TGS, otherwise it was defined as an EGS. Human germline sequences from the IMGT database were trimmed 3 bp at the 3′ end for the V gene (or trimmed 3 bp at the 5′ end for the J gene) for use as the reference database. Sequences after clustering were aligned to the reference database using a global-alignment strategy. TGSs and EGSs were determined using the criteria stated above, and the *Ar* and *Ur* for them calculated (**Figure 4A**). *Ar* and *Ur* values for TGSs are shown in red, and EGSs are shown in black, in **Figure 4A**. Remarkably, TGSs exhibited much higher values than EGSs for *Ur* and *Ar*. Hence, we could use *Ar* and *Ur* (V: $Ar > 0.15$, $Ur > 0.12$; J: $Ar > 0.12$, $Ur > 0.10$) to distinguish most TGSs from EGSs.

For the optimization step, we used five parameters (*Trim5 Ratio1*, *Trim5 Ratio2*, *Trim5 Rate1*, *Trim5 Ratio1*, and *More5 Rate*) to filter FP sequences. We observed four types of sequences from the assembly step (**Figure 2B**). Herein, we define the extended sequences of the "right" type as "true positive" (TP) and the other three types as FP. We aligned the extended sequences to known germline sequences in humans (as reference, from the IMGT database). If the identity was >90%, missed nucleotides at the terminus (compared with the reference) were <20 bp and extra nucleotides at the terminus were <5 bp, the extended sequence was regarded to be a TP, otherwise it was defined as a FP. Then, we calculated the values of five parameters for TPs and FPs (**Figures 4B,C**). As expected, TPs exhibited higher values for all parameters than FPs, which demonstrated their discriminatory power on TPs and FPs for TRB and IGH samples [*Trim5 Ratio* >1.5, *Trim5 Rate* >2 (for *TRB-J* >0.5), and *More5 Rate* >5].

Evaluation of IMPre Accuracy Using Human Samples

A TRB sample (S02-R) and IGH sample (H08) from healthy humans were used to evaluate the accuracy of IMPre. Predicted germline sequences were aligned to known human germline genes and alleles, and the nearest allele was determined for each sequence to calculate the mismatch number, identity, and deviated bases (**Figure 5**). Deviating bases were the number of missed nucleotides (−) or extra nucleotides (+) at the 3′ end of V (and 5′ end of J) compared with the nearest allele. Currently, 48 V and 13 J TCR-β functional human genes as well as 53 V and 6 J IGH functional human genes have been reported in the IMGT database. For these samples, most VJ genes could be predicted with at least one allele using this method. All 13 J genes, 42 of 48 V genes for TRB, all 6 J genes and 36 of 53 V genes for IGH were observed in addition to 2 pseudogenes per sample. Compared with the nearest allele, the mismatch number, deviated bases, and identity were

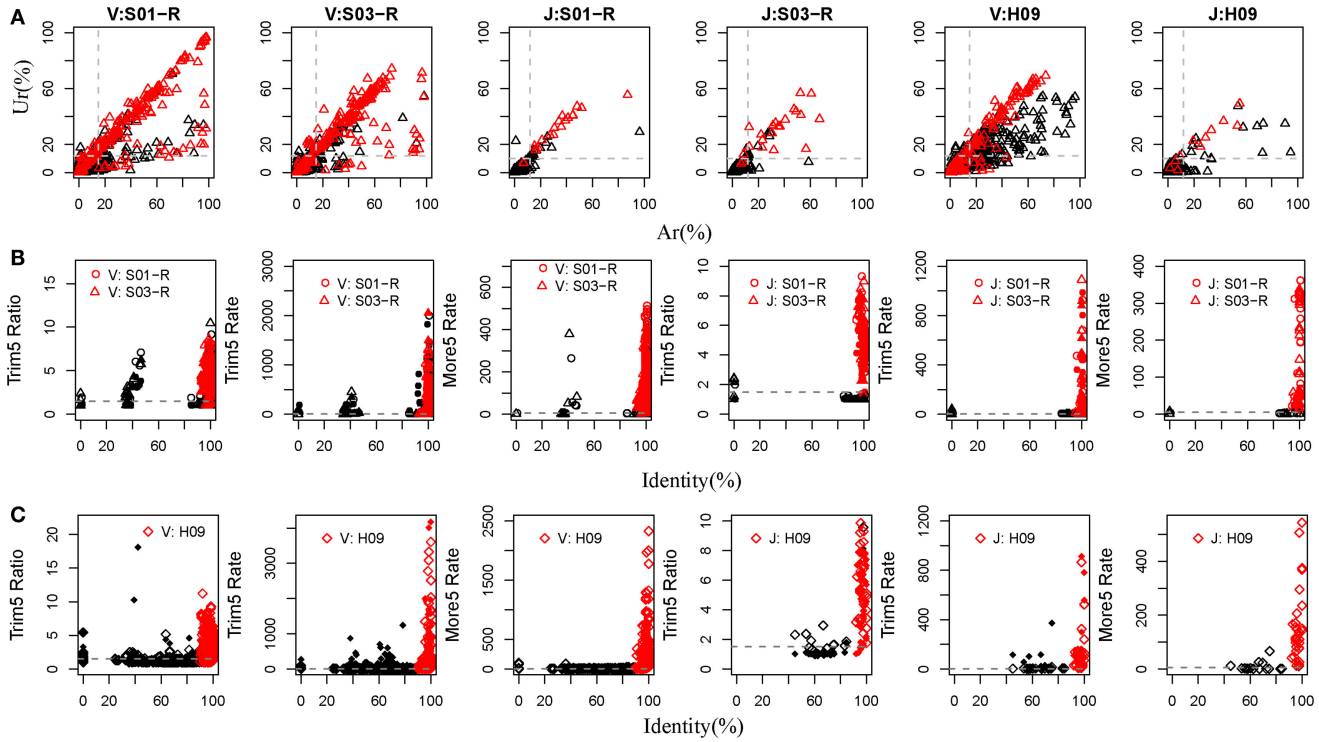


FIGURE 4 | Training IMPre parameters using human samples. **(A)** Two human TRB samples and an IGH sample are used for training parameters, *Ar* and *Ur*, used in the assembly step. Red triangle: TGSs; black triangle: EGSs. The dashed line for TRB indicates the following: $Ar(V) = 0.15$, $Ur(V) = 0.12$, $Ar(J) = 0.12$, and $Ur(J) = 0.10$. The dashed line for IGH indicates the following: $Ar(V) = 0.15$, $Ur(V) = 0.12$, *Ar* (J) = 0.12, and $Ur(J) = 0.10$. **(B)** Two human TRB samples are used for training five parameters (*Trim5 Ratio1*, *Trim5 Ratio2*, *Trim5 Rate1*, *Trim5 Rate2*, and *More5 Rate*) used in the optimization step. The *Trim5 Ratio* includes *Trim5 Ratio1* and *Trim5 Ratio2* (solid), and the *Trim5* Rate includes *Trim5 Rate1* (hollow) and *Trim5 Rate2* (solid). Red: TP; black: FP. The dashed line denotes the following: *Trim5 Ratio* = 1.5, *Trim5 Rate* = 2, *Trim5 Rate*(J) = 0.5, and *More5 Rate* = 5. **(C)** A human IGH sample is used for training five parameters (*Trim5 Ratio1*, *Trim5 Ratio2*, *Trim5 Rate1*, *Trim5 Rate2*, and *More5 Rate*) used in the optimization step. Red: TP; black: FP. The dashed line denotes the following: *Trim5 Ratio* = 1.5, *Trim5 Rate* = 2, and *More5 Rate* = 5.

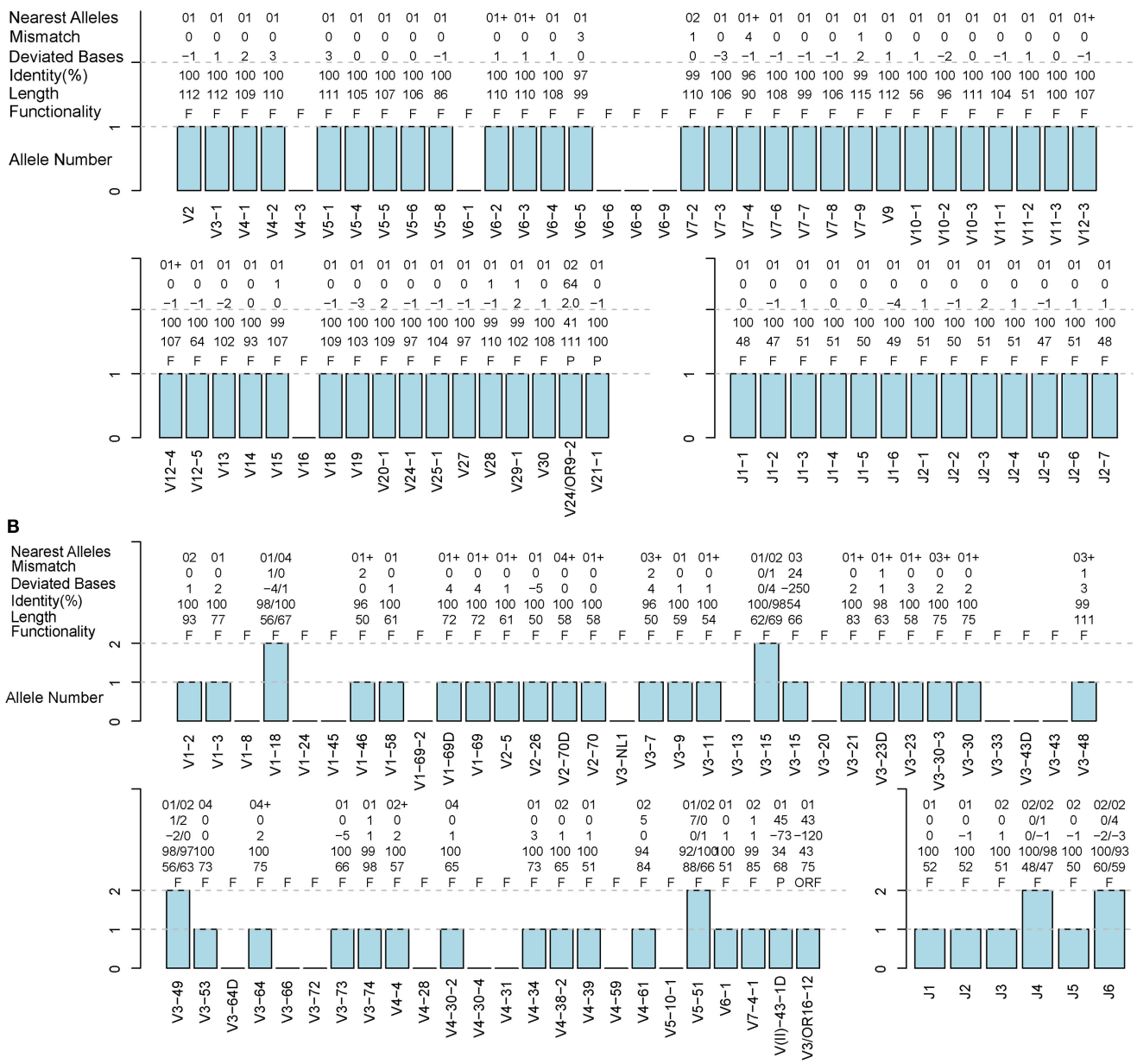


FIGURE 5 | Detailed evaluation of the predicted sequences for human samples. Predicted sequences were aligned to known human germline genes to determine the nearest alleles and calculate the mismatch number, deviated base, and identity. A "+" for the nearest alleles indicates that the sequence has multiple nearest genes. A "+/−" in deviated bases indicates that it includes extra nucleotides/missed nucleotides at the terminus. F, functional; P, pseudogene; ORF, open reading frame (definition from the IMGT). **(A)** TRB S02-R sample. **(B)** IGH H08 sample.

used to assess the quality of the predicted sequence. The sequence was considered to be correct if the identity was >90%, and the absolute deviated base was <5. Accuracies for TRBV, TRBJ, IGHV and IGHJ were 97.7, 100, 92.9, and 100%, respectively (**Table 1**), which showed that IMPre is highly accurate for TRB and IGH. Average identity, average deviated base, and average mismatch were, respectively, 99.7%, 1.1, and 0.3 for TRBV; 100%, 1.1, and 0 for TRBJ; 99.1%, 1.7, and 0.6 for IGHV; and 98.9%, 1.1, and 0.6 for IGHJ. High identity, few deviated bases, and fewer mismatches suggest that IMPre could predict human germline genes/alleles accurately and precisely. Furthermore, most nearest alleles for samples were *01 for TRB and IGH samples, suggesting that *01 is the major allele in the population. The few heterozygous alleles observed in the present study are comparable to those noted by Boyd and coworkers (7).

Evaluation of Efficiency and Robustness

Immune Germline Prediction was implemented using Perl and C programs. We optimized the programs multiple times to reduce memory use and increase the efficiency of clustering and

TABLE 1 | Evaluation of the predicted V/J germline sequences of humans and rhesus monkey.

Sample	Species-chain	Predicted V germline genes/alleles[c]					Predicted J germline genes/alleles[c]				
		Number	Accuracy (%)[a]	AVE identity (%)	AVE |deviated bases (bp)|[b]	AVE mismatch (bp)	Number	Accuracy (%)[a]	AVE identity (%)	AVE |deviated bases (bp)|[b]	AVE mismatch (bp)
S02-R	Human-TRB	44	43 (97.7)	99.7	1.1	0.3	13	13 (100.0)	100.0	1.1	0
H08	Human-IGH	42	39 (92.9)	99.1	1.7	0.6	8	8 (100.0)	98.9	1.1	0.6
05D328	Monkey-TRB	46	44 (95.7)	99.0	1.3	1.6	15	15 (100.0)	99.0	1.1	0.5
A8L087	Monkey-TRB	46	43 (93.5)	98.9	1.3	1.8	14	14 (100.0)	99.2	0.9	0.4
AVE1[d]	Monkey-TRB	46	44 (94.6)	99.0	1.3	1.7	15	15 (100.0)	99.1	1.0	0.5
H88-LS	Human-IGH	35	34 (97.1)	100.0	1.1	0	6	6 (100.0)	100.0	1.2	0

[a]*Identity ≥90% and |deviated bases| ≤5 were defined as correct and used to calculate accuracy.*
[b]*AVE, average; absolute values of deviated bases were used to calculate average values.*
[c]*Identity ≥90% and |deviated bases| ≤5 were defined as correct and used to calculate the average identity, deviated bases, and mismatches.*
[d]*Average of 05D328 and A8L087 samples.*

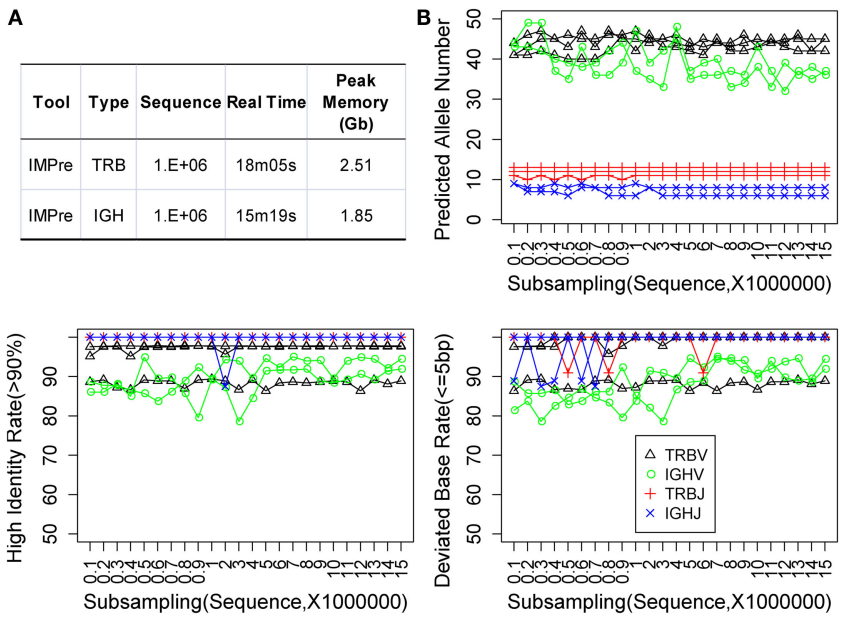


FIGURE 6 | Performance assessment. (A) Time and memory test. The following parameters were used: "—v_seed 40, —v_min_e 1, —j_min_e 1" and default for the remaining parameters. **(B)** Subsampling from five human samples. Predicted allele number, high identity, and deviated bases were used to assess the accuracy of predicted sequences.

assembly. One million subsequences were extracted at random from TRB and IGH samples, which were used to test IMPre performance. IMPre used 38 min and 41 s and 1.63 Gb peak memory for the entire TRB analysis, whereas 25 min and 17 s and 1.11 Gb peak memory were used for the IGH analysis (**Figure 6A**).

Immune Germline Prediction exhibited good accuracy for human deep-sequencing data. However, we did not know if the method was stable for lower throughput data or for optimal and minimal data requirements. Random sampling was done using five samples at a sequence interval of 1 million from 1 million to 15 million sequences. For each subsample, germline sequences were predicted using IMPre and aligned to human known germline genes for assessment. The predicted allele number, high identity (>90%) rate, and deviated base (≤5) rate were calculated (**Figure 6B**). Predicted numbers were fairly stable with a change in data size for TRB and IGHJ. Certain fluctuations were observed for IGHV, and the number tended to decrease with increase in data size. Similarly, the high identity and deviated base rate for TRB and IGHJ presented an almost flat line for most samples except for one TRBV sample (which produced lower values for both measures). IGHV exhibited a slight fluctuation for both measures, but they tended to have slightly high rates with increasing data size. In summary, the predicted accuracy was relatively stable for various subsamplings and demonstrated that IMPre is robust and reliable. Furthermore, accuracies between 1 million and 15 million sequences were similar, so 1 million sequences were sufficient for this analysis. The data size for IGH could be larger for greater accuracy.

Evaluation of TRB Samples from Monkeys

Immune Germline Prediction performed well using human samples, so the next step was to test its performance with non-human species. TCR-β germline alleles are incomplete, but the germline genes for rhesus monkeys are relatively complete in the IMGT database, thereby providing positive controls for our evaluation. Two rhesus monkeys (Table S1 in Supplementary Material) were sequenced for TCR-β using a 5′ RACE approach, and these data were used to predict TRB germline genes. IMPre parameters were the same as for human samples, and the predicted sequences were aligned to the known germline genes for rhesus monkeys (see text footnote 1). Assessment details are provided in **Figure 7**, Figure S3 in Supplementary Material, and **Table 1**. Accuracy was 94.6% for V genes and 100% for J genes, values which were similar to the human results. Most V/J genes were predicted for at least one allele. All J genes and, on average, 39 of 59 V genes, were observed per sample. The inferred V sequences exhibited, on average, 99% identity, 1.3 deviated bases, and 1.7 mismatches per sample, whereas the J sequences exhibited 99.1% identity, 1.0% deviated bases, and 0.5% mismatches per sample (**Table 1**). We also observed two V pseudogenes for each sample; only two predicted identities for sequences were <90% for each sample; four deviated bases of sequences (absolute value) were >5 bp (**Figure 7B**). However, compared with human samples, slightly more mismatches were observed (**Figure 7B**), which was probably due to incomplete alleles in the database. These data showed that IMPre and the parameters it employs can infer high-quality germline sequences for non-human samples precisely.

Evaluation Using Long-Sequence Human Samples

The analyses carried out above were based on prevalent short-sequence data (Illumina Hiseq). To ascertain if all IMPre parameters were suitable for long sequences (the entire V region was sequenced using MiSeq), a human IGH sample (Table S1 in Supplementary Material) with primers designed at FR1 and C regions were used to test IMPre. The parameters applied were the same as those mentioned above except for the changed seed length

FIGURE 7 | Detailed evaluation of predicted sequences for TRB samples from rhesus monkeys. Predicted sequences were aligned to known germline genes for rhesus monkeys for the nearest alleles and to calculate the mismatch number, deviated bases, and identity. **(A)** 05D328 sample. A "+" in the nearest alleles indicates that the sequence exhibits multiple nearest genes. A "+/−" in the deviated bases indicates that it exhibits extra nucleotides/missed nucleotides at the terminus. F, functional; P, pseudogene; ORF, open reading frame. **(B)** Statistics of the predicted sequences for two TRB samples. The left panel is the predicted V/J gene and allele number, including functional gene (F) and pseudogene (P). The identity, deviated base, and mismatch number for predicted sequences are calculated and displayed. V and J sequences are combined together.

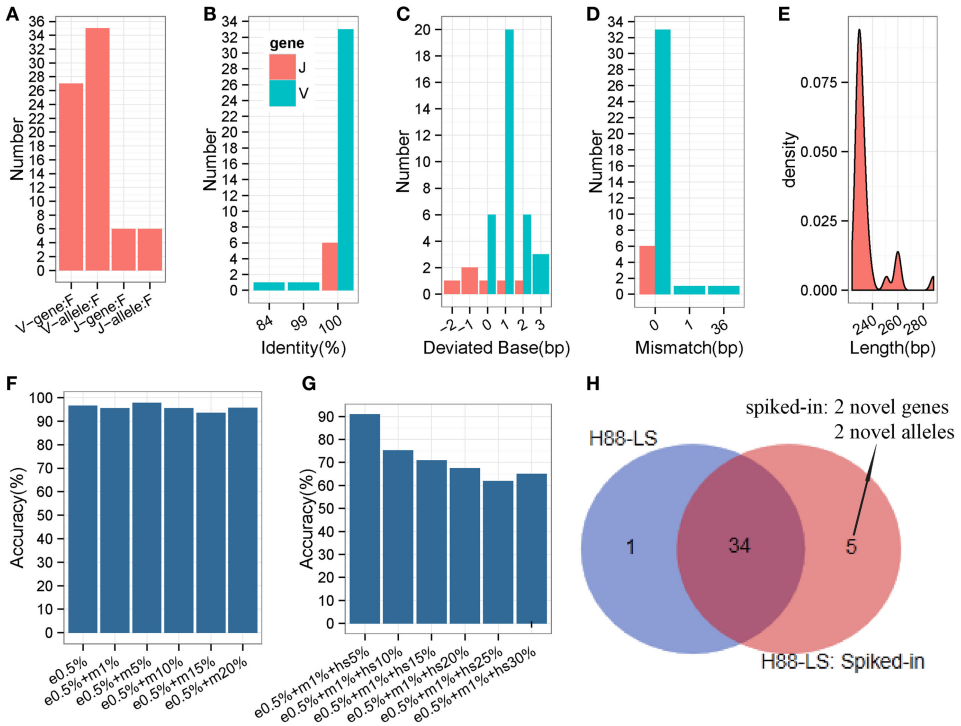


FIGURE 8 | Detailed evaluation of predicted long sequences for an IGH sample (H88-LS). Predicted sequences were aligned to known human IGH germline alleles, and accuracy evaluated. **(A)** Predicted V/J gene and allele number. **(B)** Identity distribution for predicted V/J sequences. **(C)** Distribution of deviated bases for predicted V/J sequences. **(D)** Distribution of mismatch number for predicted V/J sequences. **(E)** Length distribution of predicted V sequence. The V primer is trimmed for these predicted V sequences. **(F)** The accuracy of inferred germline sequences for simulated datasets. e0.5%: 5% of sequencing error rate; m1%: 1% of mutation (occur at random) rate. Each dataset includes 10^5 sequences. **(G)** The accuracy of inferred germline sequences for simulated datasets (containing hotspot). hs5%: 5% of hotspot rate, that means 5% of rearranged sequences derived from the same germline allele occur a same specific mutation. Each dataset includes 10^5 sequences. **(H)** Comparison of inferred germline sequences from H88-LS sample and spiked-in sample. Venn diagram shows the number of inferred germline sequences.

(200 bp). Assessment results showed that IMPre performed well using a long sequence (**Table 1**; **Figure 8**). The assessment detail for each sequence is displayed in Figure S4 in Supplementary Material. We predicted 27 V genes and most V3 genes were lost (Figure S4 in Supplementary Material) due to V3 accounting for only 3.7% of the raw sample sequences; all 6 J genes were identified (**Figure 8A**). Except for two sequences containing a mismatch, predicted V sequences were completely consistent with known germline gene/alleles in the IMGT database (**Figure 8D**), and the identities were 100% (**Figure 8B**); all predicted J germline sequences were consistent with known germline gene/alleles (**Figures 8B,D**). All deviated bases of predicted V/J sequences (absolute values) were between −2 and 3 bp (**Figure 8C**). All predicted V sequences were >224 bp after trimming of the V primer (**Figure 8E**). Overall, this surprising accuracy showed that IMPre is suitable for long-sequence data. It was much better than the results obtained from short sequences, suggesting that long sequences are favorable for improved accuracy of prediction.

Somatic Hypermutation Analysis and Novel Gene/Allele Effect

To evaluate the different SHM levels effects on IMPre, we generated 12 datasets by computer with 0.05% sequencing error rate and different mutation rates, and 6 of datasets included different hotspot levels. Surprisingly, the accuracy is not declined along with the mutation (occur at random) rate increased (**Figure 8F**), which proves the good stability and performance of IMPre. Furthermore, for the dataset that 5% of simulated sequences of each germline allele contain a specific hotspot mutation, 90.91% of the germline sequences are inferred completely correct (**Figure 8G**). However, 65% of them can be identified when the hotspot rate reaches to 30% (**Figure 8G**). The decreasingly accuracy is expectable because it is difficult to discriminate between the mutation and real novel allele when 30% of sequences appear a same mutation.

In our method, theoretically, we assemble the potential germline sequence using the reads in an independent cluster, where most of reads derive from the same V/J segments, so the presence of one segment do not influence the processing of other V/J segments inference. To test this speculation, the rearranged sequences were simulated (with 0.5% sequencing error rate and 1% mutation rate) from two novel V genes and two novel V alleles and then were spiked in the raw sequencing data of H-88 sample. As a result, the spiked-in 4 novel genes or alleles were inferred successfully by IMPre, and 34 (97.14%) germline alleles were repeated from the spiked-in data; however, 1 gene inferred from H-88 sample was not detected anymore in the spiked-in data (**Figure 8H**). The result demonstrates that the presence of one segment has little effect on other segments' inference.

DISCUSSION

In the present study, we introduced a novel tool, IMPre, based on rearranged repertoire data to predict novel genes and alleles. This method involves data processing, clustering, assembly, and optimization (**Figure 1**). We developed a clustering algorithm, Seed_Clust, to cluster sequences using the same seed k-mer. Then, a multiway tree was put in place to store nucleotides from a cluster, and a one-nucleotide extension strategy used for sequence assembly beginning with the seed. *Ur* and *Ar* values were applied further to determine where the extension should stop, which could be used to discriminate between a real gene segment and a FP sequence (**Figure 4A**). The three-step optimization process was designed to filter out FPs, merge redundant sequences, and remove SHM and PCR/sequencing errors (**Figures 4B,C**). We first trained this method using human samples and then assessed accuracy using additional samples (**Figure 5**; **Table 1**).

This method is based on certain probabilities of occurrence, including assembly and optimization parameters, so its stability had to be validated. We tested stability using three approaches. First, we selected data randomly from human samples at an interval of 1 million sequences with sizes from 1 million to 15 million sequences to evaluate the accuracy of the predicted sequences. Second, two non-human samples (TRB of rhesus monkeys) were used to test this method under the same key parameters. Third, three human IGH samples with the entire V region were used to ascertain if long-sequence data were suitable for this method using the same key parameters. The accuracy of all three tests was stable and similar to the results from the original human samples. Most parameters were derived from the characteristics of the V(D)J combinatorial mechanism, and we used human samples to train these parameters. Thus, if the V(D)J combinatorial mechanism of other species was similar to those of humans, IMPre could predict germline genes and alleles precisely. However, IMPre could miss a gene if its frequency in a sample was very low. To improve accuracy and infer all genes, more individuals are required. Also, the predicted sequence observed in multiple individuals is more credible and regarded to be the authentic germline gene or allele.

Somatic hypermutation is one of the obstacles for inferring BCR germline sequence. We utilized some strategies to process this problem and proved it good (**Figure 8**). It was reported that the SHM creates at random and at low rate (7, 16, 17). Therefore, most of SHMs can be filtered in the assembly step, because the two parameters *Ar* and *Ur* for extension used in assembly step filter the low-frequency sequence in the cluster group. We simulated six datasets with different SHM levels and found our method can eliminate the SHM effect (**Figure 8F**). However, some cells experiencing continuous antigen exposure (such as HIV) or lymphocytes proliferating (such as leukemia) result in high rate of SHM (hotspot). We also simulated six datasets with different hotspot rates and found the accuracy declined when the hotspot rate more than 10%. In this case, we recommend using multiple individuals to infer the germline genes/alleles and select the inferred sequence appeared in different individuals.

For some species, the rearranged repertoire cannot be amplified using multiplex PCR if the V/J germline genes are not known. However, the repertoire can be amplified using the 5′ RACE method for almost all species because the C region is conservative and it is easier to design the primer in this region. The arranged repertoire data generated by 5′ RACE provide an opportunity to infer the germline gene using IMPre. Unlike conventional PCR-based cloning strategies, we do not: (i) need to consider if the species is homologous with humans; (ii) use the known germline genes in a publically available database; or (iii) need the genome sequence. Hence, this is a simpler and more direct method to find germline genes. Unlike a method that infers the germline gene from a species genome, we predicted genes more accurately because exact and correct assembly for the highly homologous and polymorphic region is difficult.

In the near future, there will be a rapid accumulation of high-throughput sequence data for TCR and BCR repertoires, including various large-scale disease studies and application projects. These data can be used to infer novel alleles for humans and other species using IMPre. Many novel V/J alleles could be identified from these data. The rearranged repertoire data generated by multiplex PCR are also available to infer novel alleles because the latter will be included among them.

For future novel studies on prediction of germline genes, D genes can also be added using deep sequencing of repertoire data using the same strategy. Distributions of the length of 5′ and 3′ D deletions are similar to those in V genes (14). After the V and J germline genes have been identified for a rearranged sequence, the subsequence between V and J segments can be extracted and used to infer D germline segments. The IMPre tool that we developed provides a comprehensive approach for identification of novel BCR/TCR genes and alleles in certain species with greatly improved speed, cost, and accuracy.

AUTHOR CONTRIBUTIONS

XL, WZ, and I-MW designed the project; WZ developed the methodology and wrote the manuscript; LL and JW carried out the experiments; WZ designed the bioinformatic workflow; WZ and XC developed the code for bioinformatic processing; WZ and CW analyzed the data; I-MW, AB, GD, and DC contributed reagents/materials.

ACKNOWLEDGMENTS

We thank the donors who denoted samples.

FUNDING

This project was funded by the Beijing Genomics Institute. The funder provided support in the form of salaries for WZ, CW, LL, XC, JW, and XL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00457.

REFERENCES

1. Jackson KJ, Liu Y, Roskin KM, Glanville J, Hoh RA, Seo K, et al. Human responses to influenza vaccination show seroconversion signatures and convergent antibody rearrangements. *Cell Host Microbe* (2014) 16(1):105–14. doi:10.1016/j.chom.2014.05.013
2. Parameswaran P, Liu Y, Roskin KM, Jackson KK, Dixit VP, Lee JY, et al. Convergent antibody signatures in human dengue. *Cell Host Microbe* (2013) 13(6):691–700. doi:10.1016/j.chom.2013.05.008
3. Morris H, DeWolf S, Robins H, Sprangers B, LoCascio SA, Shonts BA, et al. Tracking donor-reactive T cells: evidence for clonal deletion in tolerant kidney transplant patients. *Sci Transl Med* (2015) 7(272):272ra10. doi:10.1126/scitranslmed.3010760
4. Jiang N, He J, Weinstein JA, Penland L, Sasaki S, He XS, et al. Lineage structure of the human antibody repertoire in response to influenza vaccination. *Sci Transl Med* (2013) 5(171):171ra19. doi:10.1126/scitranslmed.3004794
5. Cheung WC, Beausoleil SA, Zhang X, Sato S, Schieferl SM, Wieler JS, et al. A proteomics approach for the identification and cloning of monoclonal antibodies from serum. *Nat Biotechnol* (2012) 30(5):447–52. doi:10.1038/nbt.2167
6. Croce CM, Shander M, Martinis J, Cicurel L, D'Ancona GG, Dolby TW, et al. Chromosomal location of the genes for human immunoglobulin heavy chains. *Proc Natl Acad Sci U S A* (1979) 76(7):3416–9. doi:10.1073/pnas.76.7.3416
7. Boyd SD, Gaeta BA, Jackson KJ, Fire AZ, Marshall EL, Merker JD, et al. Individual variation in the germline Ig gene repertoire inferred from variable region gene rearrangements. *J Immunol* (2010) 184(12):6986–92. doi:10.4049/jimmunol.1000445
8. Gadala-Maria D, Yaari G, Uduman M, Kleinstein SH. Automated analysis of high-throughput B-cell sequencing data reveals a high frequency of novel immunoglobulin V gene segment alleles. *Proc Natl Acad Sci U S A* (2015) 112(8):E862–70. doi:10.1073/pnas.1417683112
9. Xochelli A, Agathangelidis A, Kavakiotis I, Minga E, Sutton LA, Baliakas P, et al. Immunoglobulin heavy variable (IGHV) genes and alleles: new entities, new names and implications for research and prognostication in chronic lymphocytic leukaemia. *Immunogenetics* (2015) 67(1):61–6. doi:10.1007/s00251-014-0812-3
10. Griffin LM, Snowden JR, Lawson AD, Wernery U, Kinne J, Baker TS. Analysis of heavy and light chain sequences of conventional camelid antibodies from *Camelus dromedarius* and *Camelus bactrianus* species. *J Immunol Methods* (2014) 405:35–46. doi:10.1016/j.jim.2014.01.003
11. Bible JM, Howard W, Robbins H, Dunn-Walters DK. IGHV1, IGHV5 and IGHV7 subgroup genes in the rhesus macaque. *Immunogenetics* (2003) 54(12):867–73. doi:10.1007/s00251-003-0536-2
12. Howard WA, Bible JM, Finlay-Dijsselbloem E, Openshaw S, Dunn-Walters DK. Immunoglobulin light-chain genes in the rhesus macaque I: kappa light-chain germline sequences for subgroups IGKV1, IGKV and IGKV3. *Immunogenetics* (2005) 57(3–4):210–8. doi:10.1007/s00251-005-0794-2
13. Greenaway HY, Kurniawan M, Price DA, Douek DC, Davenport MP, Venturi V. Extraction and characterization of the rhesus macaque T-cell receptor beta-chain genes. *Immunol Cell Biol* (2009) 87(7):546–53. doi:10.1038/icb.2009.38
14. Zhang W, Du Y, Su Z, Wang C, Zeng X, Zhang R, et al. IMonitor: a robust pipeline for TCR and BCR repertoire analysis. *Genetics* (2015) 201(2):459–72. doi:10.1534/genetics.115.176735
15. Freeman JD, Warren RL, Webb JR, Nelson BH, Holt RA. Profiling the T-cell receptor beta-chain repertoire by massively parallel sequencing. *Genome Res* (2009) 19(10):1817–24. doi:10.1101/gr.092924.109
16. Papavasiliou FN, Schatz DG. Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity. *Cell* (2002) 109(Suppl):S35–44. doi:10.1016/S0092-8674(02)00706-7
17. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* (2000) 102(5):553–63. doi:10.1016/S0092-8674(00)00078-7
18. Heyer LJ, Kruglyak S, Yooseph S. Exploring expression data: identification and analysis of coexpressed genes. *Genome Res* (1999) 9(11):1106–15. doi:10.1101/gr.9.11.1106
19. Warren RL, Freeman JD, Zeng T, Choe G, Munro S, Moore R, et al. Exhaustive T-cell repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. *Genome Res* (2011) 21(5):790–7. doi:10.1101/gr.115428.110
20. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* (2003) 17(12):2257–317. doi:10.1038/sj.leu.2403202

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 01 March 2016
doi: 10.3389/fimmu.2016.00065

Heterozygous Mutation in IκBNS Leads to Reduced Levels of Natural IgM Antibodies and Impaired Responses to T-Independent Type 2 Antigens

Gabriel K. Pedersen[1†], Monika Ádori[1†], Julian M. Stark[1], Sharesta Khoenkhoen[1], Carrie Arnold[2], Bruce Beutler[3] and Gunilla B. Karlsson Hedestam[1*]*

[1] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, [2] Department of Genetics, The Scripps Research Institute, La Jolla, CA, USA, [3] Center for the Genetics of Host Defense, University of Texas Southwestern Medical Center, Dallas, TX, USA

OPEN ACCESS

Edited by:
Nichol E. Holodick,
The Feinstein Institute for Medical Research, USA

Reviewed by:
Ronald B. Corley,
Boston University School of Medicine, USA
David Nemazee,
The Scripps Research Institute, USA

***Correspondence:**
Gabriel K. Pedersen
gabriel.pedersen@ki.se;
Gunilla B. Karlsson Hedestam
gunilla.karlsson.hedestam@ki.se

[†]Gabriel K. Pedersen and Monika Ádori contributed equally.

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: 23 November 2015
Accepted: 12 February 2016
Published: 01 March 2016

Citation:
Pedersen GK, Ádori M, Stark JM, Khoenkhoen S, Arnold C, Beutler B and Karlsson Hedestam GB (2016) Heterozygous Mutation in IκBNS Leads to Reduced Levels of Natural IgM Antibodies and Impaired Responses to T-Independent Type 2 Antigens. Front. Immunol. 7:65. doi: 10.3389/fimmu.2016.00065

Mice deficient in central components of classical NF-κB signaling have low levels of circulating natural IgM antibodies and fail to respond to immunization with T-independent type 2 (TI-2) antigens. A plausible explanation for these defects is the severely reduced numbers of B-1 and marginal zone B (MZB) cells in such mice. By using an ethyl-*N*-nitrosourea mutagenesis screen, we identified a role for the atypical IκB protein IκBNS in humoral immunity. IκBNS-deficient mice lack B-1 cells and have severely reduced numbers of MZB cells, and thus resemble several other strains with defects in classical NF-κB signaling. We analyzed mice heterozygous for the identified IκBNS mutation and demonstrate that these mice have an intermediary phenotype in terms of levels of circulating IgM antibodies and responses to TI-2 antigens. However, in contrast to mice that are homozygous for the IκBNS mutation, the heterozygous mice had normal frequencies of B-1 and MZB cells. These results suggest that there is a requirement for IκBNS expression from two functional alleles for maintaining normal levels of circulating natural IgM antibodies and responses to TI-2 antigens.

Keywords: B-1 cells, transitional B cells, *nfkbid*, IκBNS, NF-κB

INTRODUCTION

Innate-like B cells play significant roles in the early defense against pathogens and, at steady state, anti-inflammatory mediators, such as IL-10, and polyreactive IgM antibodies are secreted by these cell subsets to help maintain homeostasis. There is a clear division of labor between the different innate-like B cells. B-1a cells are thought to secrete most of the natural polyreactive antibodies found in the serum (1). Innate response activator B cells exert protective effects against sepsis by secreting GM-CSF (2). Marginal zone B (MZB) cells, through their location at the interface between the blood and the immune system, help initiate responses against blood-borne antigens [reviewed in Ref. (3)]. B cells regulate inflammation through various mechanisms, including production of anti-inflammatory cytokines, such as IL-10 and IL-35 (4, 5). The main IL-10 producing innate-like B cells, collectively named B10, are found within the MZB (5) and B-1a cell subsets (4, 6). Innate-like B cells are also

pivotal for the response against viral and bacterial infections (1). Particularly, the innate-like B cell subsets B-1b and MZB cells are the predominant responding B cells to T-independent type 2 (TI-2) antigens found on the surface of a number of pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitides* (3, 7, 8).

In addition to their distinct roles in homeostasis and response to different stimuli, the innate-like B cell subsets also have distinct developmental pathways. B-1 cells are generated readily from the yolk salk, paraaortic splanchnopleura, and liver during early fetal development (9, 10), while these organs are less effective at generating follicular B cells. In contrast, hematopoietic stem cells from adult bone marrow predominantly generate follicular B and MZB cells (9), collectively referred to as B-2 cells. Immature B cells mature in the spleen and undergo selection at various transitional stages before becoming naive B cells (11). B-2 cells are continuously replenished from the adult bone marrow and diverge into follicular B cells and MZB cells at the transitional B cell stage (12, 13). B-1 cells may develop from a separate progenitor population (14) and mature *via* a phenotypically distinct B-1 transitional B cell intermediate, which is found at high frequencies in the spleen of neonatal mice (15). The different B cell subsets require distinct stimuli for development and maintenance. For example, MZB cells are dependent on Notch signaling, and therefore mice with impaired Notch2 completely lack MZB cells. However, Notch signaling is not required for B-1 or follicular B cell development (12). The distinct B cell subsets also show different requirements for NF-κB signaling (16).

The NF-κB transcription factors, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel (Rel), and RelB, regulate transcription by binding to promoters of target genes. p50 and p52 induce gene transcription by forming heterodimers with p65, c-Rel, or RelB, all of which contain a transactivation domain. In contrast, homodimers of p50 or p52 lack a transactivation domain and thus generally function as repressors of transcription. In classical NF-κB signaling, the NF-κB transcription factors are sequestered in the cytoplasm as dimers of p50:p65 by a protein family known as inhibitors of κB (IκB), including IκB-α, IκB-β, IκB-ϵ, and the p50 precursor p105. The IκB proteins are characterized by their ankyrin repeat structure, which functions to mask nuclear localization signals (17). IκB kinases (IKK), IKK-α (IKK1), IKK-β (IKK2), and IKK-γ (NF-kappa-B essential modulator, NEMO), target IκBs for polyubiquitination and proteasomal degradation, thereby releasing the sequestered NF-κB1 p50 to nuclear localization (18, 19). In lymphocytes, this requires the CARD11, BCL-10, MALT1 (CBM) complex. Through an alternative NF-κB signaling pathway, NF-κB-inducing kinase (NIK) can activate IKK-α, facilitating proteasomal processing of NF-κB2 p100. This ultimately leads to nuclear localization of NF-κB p52/RelB (20). A number of atypical IκB proteins have recently been identified, defined by their ankyrin repeat structure and comprise BCL-3, IκBζ, IκBNS, and IκBη. Atypical IκB proteins may either augment or repress transcription depending on cell type, context, and timing. Recent studies have revealed important roles of atypical IκB proteins in lymphopoiesis and immunological responses [reviewed in Ref. (21)].

Classical NF-κB signaling is required for the generation of B-1 cells, particularly the B-1a subset, which is absent in a number of mouse strains where this pathway has been ablated [reviewed in Ref. (22)]. Reduction in MZB cell numbers is also seen in the absence of classical NF-κB signaling, while follicular B cells are less affected (23, 24). Although relatively little is known about the function of atypical IκB proteins in B cell development, roles for BCL-3 and IκBNS have recently been demonstrated. BCL-3 deficiency leads to increased numbers of MZB cells (25), while decreased B-1 and MZB cellularity was observed upon overexpression of BCL-3 (26). Absence of functional IκBNS leads to reductions in B-1b and MZB cell frequencies (27, 28) and complete absence of B-1a cells, while follicular B cell frequencies are intact (15, 28). In terms of B cell lymphopoiesis, IκBNS-deficient mice thus resemble other mouse strains with impaired classical NF-κB signaling. In addition to the role of classical NF-κB signaling in B cell development, it is also required for normal function of mature B cells. B cells from p50, BCL10, and CARMA1-deficient mice display reduced proliferation and antibody production to anti-IgM, anti-CD40, or LPS compared to wild-type (wt) cells *in vitro* (29–31).

Mice with impaired classical NF-κB signaling have reduced levels of circulating natural IgM and IgG3 antibodies and fail to mount antibody responses to TI-2 antigens *in vivo*. We previously described that mice lacking functional IκBNS due to a mutation in the *nfkbid* gene (*bumble* mice) also display reduced IgM and IgG3 levels and fail to respond to immunization with NP-ficoll (27, 28). Whether the impaired antibody response in mice deficient in classical NF-κB pathway signaling is a consequence of the reduced numbers of B-1 and MZB cells or is due to defects in B cell function remains unknown. Here, we demonstrate that the lack of IκBNS even at the heterozygous state (IκBNS^{+}/*bmb*) led to severely reduced antibody responses against TI-2 antigens, suggesting haploinsufficiency for IκBNS in the response to such antigens. Interestingly, unlike homozygous *bumble* mice, the heterozygous mice displayed apparently normal frequencies and numbers of MZB and B-1 cells. This indicates that the reduced responses to TI-2 antigens in these mice are due to a direct requirement of IκBNS in response to B cell receptor engagement rather than a secondary effect due to lack of responding cells.

MATERIALS AND METHODS

Mice

Mice were housed and bred at the animal research facility, MTC, Karolinska Institutet. *Bumble* mice, generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis of C57BL/6J mice, and their wt C57BL/6J counterparts were described previously (28). Animal studies were conducted with Committee for Animal Ethics (Stockholms Norra Djurförsöksetiska nämnd) approval.

Cell Preparation

Splenocytes were prepared as a single cell suspension using a 70-μm cell strainer. Peritoneal cells were isolated by flushing with cold PBS/1% FBS (5–10 ml). Peritoneal cells were discarded if contaminated with blood. Cell suspensions were diluted in RPMI-1640 supplemented with 2 mM L-glutamine, penicillin

(100 IU)–streptomycin (100 μg/ml), and 10% fetal bovine serum (complete RPMI). Splenocyte cell suspensions were washed once in Ca^{2+}-free Mg^{2+}-free PBS and treated with red blood cell lysis buffer before further processing. B cells were isolated from the spleen using the B cell isolation kit and from the peritoneal cavity using the Pan-B cell isolation kit (Stemcell Technologies). The purity of the isolated populations was ≥90%.

Immunization

Mice were immunized with 50-μg NP (40)-ficoll (Biosearch Technologies) or a 1:10 dilution of Pneumovax® (Merck & Co.) corresponding to 0.5 μg of each polysaccharide antigen. The antigens were diluted in PBS and 100 μl was injected intraperitoneally (i.p.).

Adoptive Transfer

Isolated peritoneal B cells (2×10^6 cells) from wt or heterozygous mice were mixed with 50 μg NP (40)-ficoll and injected i.p. into *bumble* mice. Splenic B cells (30×10^6 cells) were injected i.v.

ELISA

ELISA was performed by coating ELISA plates (Nunc) with polysaccharide antigens: 500 ng/well of NP (25) conjugated with BSA (Biosearch Technologies) or the pneumococcal polysaccharide antigens Type 1 161-X™ or Type 3 169-X™ (both ATCC) or 1:100 dilution of Pneumovax (corresponding to 50 ng/well of each antigen contained in the vaccine). To measure total IgM and IgG3 levels, plates were coated with unconjugated anti-IgM or anti-IgG3 (Southern Biotech). Plates were incubated overnight (4°C). Following washing (PBS + 2% Tween20) and blocking for 1 h with PBS containing 2% dry milk, serum was added in three-fold serial dilutions in blocking buffer and incubated for 1.5 h at room temperature (RT) before addition of secondary antibody HRP-coupled anti-IgM or IgG3 (Southern Biotech). The assay was developed with TMB substrate (KPL) followed by 1M H_2SO_4 and the OD was read at 450 nm using an Asys Expert 96 ELISA reader (Biochrom).

ELISpot Assay for Detection of Antibody-Secreting Cells

Detection of total IgM and NP-specific IgM producing cells was performed using enzyme-linked immunosorbent spot (ELISpot) assay. MultiScreen-IP filter plates (Millipore) were pretreated with 70% ethanol and washed in sterile PBS. Plates were coated with 5 μg/ml anti-mouse IgM (Southern Biotech) or 5 μg/ml of NP (25), conjugated with BSA (Biosearch Technologies), diluted in PBS, and incubated overnight at 4°C. The following day, plates were washed in sterile PBS, blocked in complete RPMI medium with 50-μM 2-mercaptoethanol and 10-mM HEPES for 1 h at 37°C, and the indicated cell numbers added in triplicate. Plates were incubated for 17 h at 37°C in 5% CO_2. Cells were then removed by washing in PBS and 0.1 μg/well of biotinylated anti-mouse IgM (Mabtech) diluted in PBS was added to the wells. After 2 h of incubation at RT, plates were washed and developed with 100 μl of 5-bromo-4-chloro-3-indolyl phosphate/NBT-plus substrate (Mabtech). The reaction was stopped when distinct spots could be observed, by rinsing the plates extensively in tap water. Spots were counted by ELISpot reader (CTL) and analyzed using the Biospot suite (CTL).

Flow Cytometry

Cells were incubated with Fc block (anti-CD16/32, BD) and stained with fluorochrome-conjugated monoclonal antibodies in PBS/2% FBS using the following antibodies: CD5 Brilliant Violet 421 (S3-7.3), CD19 PE (1D3), CD19 FITC (1D3), CD23 Brilliant Violet 421 (B3B4), CD21 APC (7G6), CD43 APC (S7) (all BD), B220 APC-eFluor 780 (RA3-6B2), CD93 APC (AA4.1) (all eBioscience), and IgM FITC (polyclonal) (Southern Biotech). Data were analyzed in FlowJo v9.6.4 (Treestar).

Statistics

Differences between groups were analyzed by a Mann–Whitney test (GraphPad Prism v6.0f).

RESULTS

Heterozygous *Bumble* Mice Have Reduced Serum IgM Levels and Responses to the TI-2 Antigen NP-Ficoll

In an ENU mutagenesis screen for antibody response defects, we identified a role of IκBNS for B cell development and function. We observed that mice homozygous for a specific mutation in *nfkbid* lacked responses to the TI-2 antigen NP-ficoll and had reduced levels of circulating IgM and IgG3 antibodies (28). These mice, named *bumble*, were found to have a T → G transversion in the conserved donor splice site in the fourth intron of *nfkbid*, the gene encoding IκBNS. This mutation is predicted to prevent splicing of the fourth intron from the *nfkbid* transcript, resulting in a premature stop codon after exon 4. Such a transcript would likely be targeted for nonsense-mediated decay or encode only 65 of 327 amino acids (aa) encoded by full-length *nfkbid*, and therefore not be expected to retain any function. The phenotype for *bumble* mice was copied in mice with a targeted mutation in the *nfkbid* gene (28) and was similar in IκBNS knockout mice (27). Here, we report that mice heterozygous for the *bumble* mutation displayed reduced NP-specific IgM and IgG3 antibody responses after NP-ficoll immunization (**Figure 1A**). Furthermore, when analyzing isolated splenocytes for NP-specific IgM antibody-secreting cells (ASC), we observed significantly ($p < 0.001$) lower numbers in *bumble* heterozygous compared to wt mice (**Figure 1B**). To test if the TI-2 antigen response defect in heterozygous *bumble* mice was due to a B cell intrinsic defect, we transferred isolated splenic and peritoneal B cells to *bumble* mice and immunized them with NP-ficoll. *Bumble* mice that had not received isolated B cells did not respond to immunization, whereas *bumble* mice that had received wt B cells did. *Bumble* mice that had received B cells from heterozygous *bumble* mice had an intermediate response to NP-ficoll immunization, suggesting that the defective antibody response to TI-2 antigens in heterozygous *bumble* mice is due to a B cell intrinsic defect (**Figure 1C**). Similarly to *bumble* homozygotes, mice with the heterozygous *bumble* mutation displayed significantly reduced total serum levels of IgM and IgG3

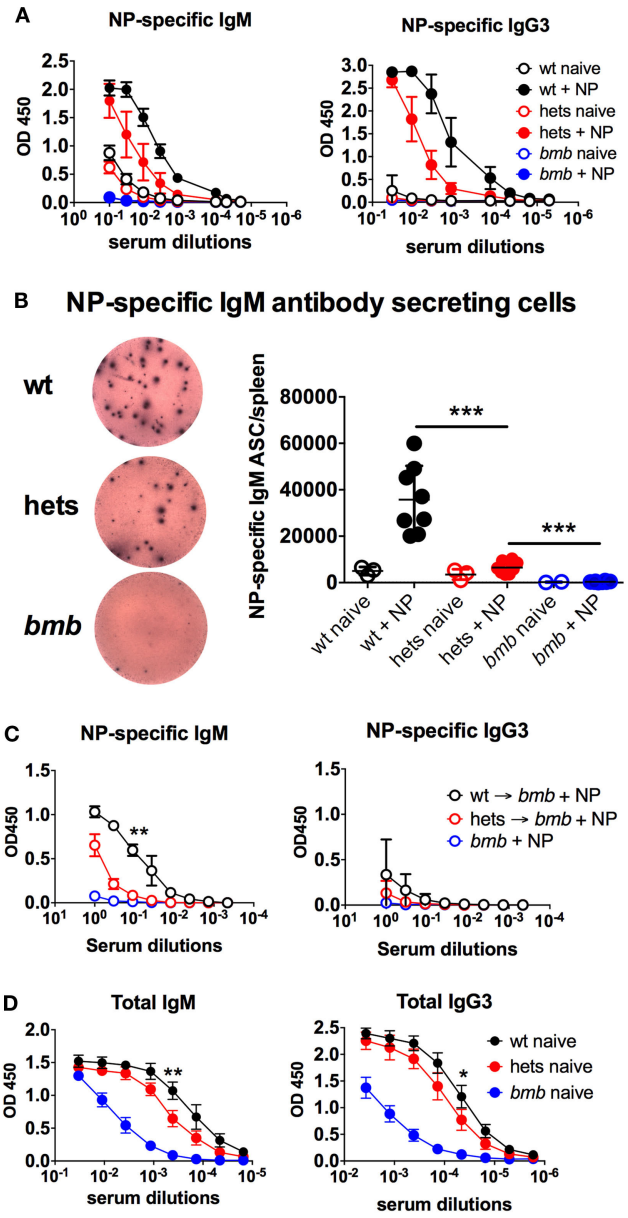


FIGURE 1 | Heterozygous *bumble* mice have reduced serum IgM levels and responses to TI-2 antigens. **(A)** Mice were immunized with 50 μg of NP-ficoll intraperitoneally and sacrificed after 6 days. NP-specific serum IgM and IgG3 were determined by ELISA. **(B)** NP-specific IgM antibody-secreting cells were determined by ELISpot. Left panel displays representative wells of wild-type (wt), heterozygous (hets), and *bumble* (*bmb*). **(C)** Isolated peritoneal B cells (2×10^6 cells) from wild-type or heterozygous *bumble* mice were mixed with 50-μg NP (40)-ficoll and injected i.p. into *bumble* mice. The recipient *bumble* mice then received splenic B cells (30×10^6 cells) from wild-type or heterozygous mice injected i.v. NP-specific serum IgM and IgG3 were determined by ELISA 6 days later. **(D)** Total serum IgM and IgG3 were determined by ELISA. Figures represent 8- to 16-week-old aged-matched mice with three to eight per group and results are representative of three **(A,B)** or two **(C)** independent experiments. Graphs display mean + SD. Statistically significant differences between *bumble*, *bumble* heterozygotes, and wt mice are indicated by $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by Mann–Whitney test.

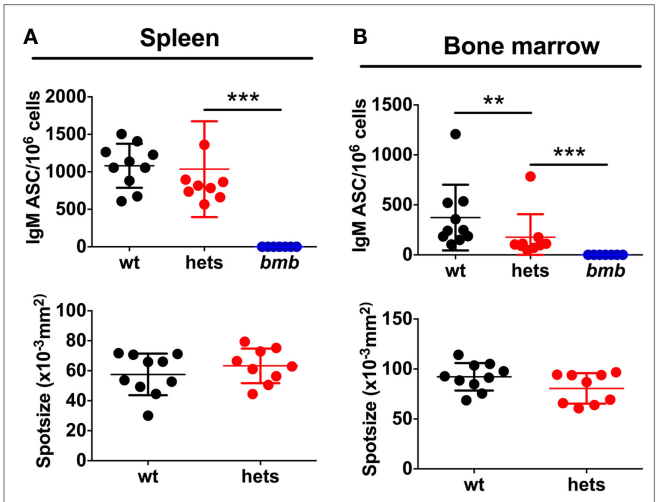


FIGURE 2 | Heterozygous *bumble* mice have fewer IgM-secreting B cells. Cell suspensions in serial dilutions were plated on ELISpot plates coated with anti-IgM. IgM antibody-secreting cells (ASC) in **(A)** spleen and **(B)** bone marrow. Upper panels display IgM ASCs per 10^6 cells and lower panels indicate mean spot size. Spot size is influenced by the number of spots per well. Therefore, wells containing similar numbers of spots across groups were used to calculate spot size (range 10–30 spots for bone marrow and 30–70 for spleen). Data were compiled from two experiments.

antibodies ($p < 0.05$) (**Figure 1D**). Thus, relative to homozygous *bumble* mice and wt mice, heterozygous *bumble* mice presented with an intermediary phenotype in terms of response to NP-ficoll and levels of circulating IgM and IgG3 antibodies.

Fewer IgM Antibody-Secreting Cells in Heterozygous *Bumble* Mice

We next asked if the reduced IgM levels in heterozygous *bumble* mice were due to fewer IgM-secreting B cells or if less IgM was produced per cell. To assess this, we performed ELISpot for total IgM producing splenic and bone marrow B cells. The same numbers of cells were plated from wt, heterozygous, and homozygous *bumble* mice. As expected, no IgM ASCs were detected from homozygous *bumble* mice. The fraction of splenic B cells spontaneously secreting IgM in heterozygous *bumble* mice was slightly lower than in wt mice, although this difference was not statistically significant ($p = 0.23$) (**Figure 2A**). The frequency of IgM ASCs in bone marrow was significantly lower in heterozygous *bumble* mice than in wt mice ($p < 0.01$). As an indication of the amount of IgM produced per IgM ASC, we evaluated the mean spot size. We observed no measurable differences in mean spot size between heterozygous and wt mice (**Figure 2B**). These data indicate that the reduced levels of natural IgM antibodies in the serum is due to fewer IgM ASCs in the heterozygous *bumble* rather than less IgM secreted per ASC.

Heterozygous *Bumble* Mice Have Normal Frequencies of the Major B Cell Subsets

The main responding B cell subsets against TI-2 antigens are B-1b cells and MZB cells (3, 7, 8), while B-1a cells are believed

to produce most of the natural IgM antibodies found in the serum at steady state (1). Homozygous *bumble* mice had normal numbers of follicular B cells, but completely lacked B-1a cells, and displayed a severe reduction in the frequencies of B-1b and MZB cells (15, 28). Transfer of wt peritoneal cells to *bumble* mice completely restored serum natural IgM levels and partly restored the antibody response to immunization with NP-ficoll (15). This suggested that the lack of B-1b cells in homozygous *bumble* mice formed the basis for the impaired response to T-independent antigens. To investigate if heterozygous *bumble* mice have defects in B cell development, we used the following strategy to phenotypically distinguish the major B cell subsets. B220 is expressed by all B-2 cells (MZB and follicular B cells), but at lower levels on most B-1 cells. CD11b and CD43 are expressed by B-1 cells, and CD5 is expressed by the B-1a cell subset. CD23 is expressed by B-2 cells, but not MZB cells, while MZB cells express high levels of CD21. When analyzing the different B cell subsets in heterozygous *bumble* mice, we found that neither overall splenic B cell nor MZB cell numbers were significantly different between wt mice and heterozygous *bumble* mice. In contrast, mice homozygous for the *bumble* mutation had significantly reduced MZB cell numbers as expected ($p < 0.01$) (**Figure 3A**). The increased surface IgM level seen in homozygous *bumble* mice (28) was not observed in the heterozygous state (**Figure 3B**). Furthermore, homozygous *bumble* mice have decreased frequencies of transitional T3 B cells (15), while in heterozygous mice, the T3 B cell frequencies were similar to those of wt mice (**Figure 3C**). No differences in bone marrow B cell progenitor populations were observed between wt, homozygous, and heterozygous *bumble* mice (**Figure 3D**). As we reported previously, homozygous *bumble* mice completely lacked B-1a cells and had significantly reduced B-1b cell frequencies ($p < 0.01$) (15). In contrast, peritoneal B-1a and B-1b cell frequencies were similar in wt and heterozygous *bumble* mice (**Figure 4A**). Similarly, bone marrow B-1a cell and spleen B-1a cell frequencies were indifferent between heterozygous *bumble* and wt mice (**Figures 4B,C**). Overall, these data indicate that the development and maintenance of all the major B cell subsets occur normally in heterozygous *bumble* mice.

Impaired Antibody Response to the TI-2 Vaccine Pneumovax in Heterozygous *Bumble* Mice

We next investigated if the impaired response to TI-2 antigens in heterozygous *bumble* mice would extend to the clinically relevant human polysaccharide vaccine Pneumovax. To this end, wt, heterozygous, and homozygous *bumble* mice were immunized with Pneumovax intraperitoneally. By 6 days post-immunization, wt mice mounted a strong antibody response against the Pneumovax vaccine antigens. No response was observed in homozygous *bumble* mice, while heterozygous *bumble* mice displayed an intermediary response (**Figure 5A**). We also investigated the Pneumovax-elicited responses to the polysaccharide antigens type 1 161-X (**Figure 5B**) and type 3 169-X (**Figure 5C**). Similarly to total Pneumovax-specific antibodies, the responses against both polysaccharides were significantly diminished in heterozygous *bumble* mice compared to in wt mice ($p < 0.01$).

DISCUSSION

The data presented here demonstrate that a heterozygous mutation in IκBNS causes a significant impairment of B cell function. Mice that express one wt allele and one allele containing the *bumble* mutation in *nfkbid* had diminished antibody responses to T-independent type II antigens and reduced levels of circulating natural IgM and IgG3 antibodies. The *bumble* mutation causes a premature stop codon after exon 4 in the *nfkbid* gene and the resulting transcript encodes only 65 of the 327 aa of the full-length IκBNS (28). The resulting mRNA transcript is likely targeted for degradation by nonsense-mediated decay. Thus, the spontaneous secretion of natural antibodies and responses to TI-2 antigens in *bumble* heterozygotes are likely impaired as a result of haploinsufficiency for the *nfkbid* gene.

Previous work has shown that $p50^{-/-}$, $BCL10^{-/-}$, and IκBα hypermorphic mice fail to respond to immunization with TI-2 antigens (32, 33), illustrating that this defect is a general feature of deficiency in the classical NF-κB signaling pathway. Similar to other proteins of the classical NF-κB pathway, IκBNS also plays an important role for maintaining normal levels of serum natural antibodies and for the antibody response against TI-2 antigens (27, 28). Notably, the major B cell subsets responsible for natural antibody production and response to TI-2 antigens, B-1 and MZB cells are lacking in mice with impaired classical NF-κB signaling, which is also similar for mice with non-functional IκBNS (15, 27, 28). It was therefore interesting that heterozygous *bumble* mice had decreased serum IgM and IgG3 levels and defective responses to TI-2 antigens, despite having apparently normal numbers and frequencies of B-1 and MZB cells. This suggested that the lack of specific B cell subsets is not the sole reason for the reduced levels of natural antibodies and response to TI-2 antigens in IκBNS-deficient mice. Rather, this indicated a more direct role of IκBNS for these aspects of immunity downstream of B cell receptor signaling. Particularly, the lack of response to TI-2 antigens of heterozygous *bumble* mice may be due to a B cell intrinsic defect in activation or ASC differentiation, although it is also possible that the BCR repertoire may be altered when IκBNS is only expressed from one allele. We did not observe any reduction in B-1a, MZB, or follicular B cell numbers in heterozygous *bumble* mice, suggesting that one functioning allele of *nfkbid* is enough to facilitate normal B cell development. However, we cannot rule out the possibility that a modest impairment of B cell development is masked by homeostatic proliferative mechanisms or altered bone marrow output to control distribution of immune cells (34).

There are only few reports of heterozygous mutations in the NF-κB pathways leading to loss of B cell function. Hypermorphic heterozygous mutations in IκBα cause impaired antibody responses (33) and CARMA1-deficient mice lacking CARD (ΔCARD) showed defective B cell proliferation at the heterozygous state (35). Since ΔCARD was found to act as a dominant-negative inhibitor of TCR-induced NF-κB activation, the impaired B cell function in heterozygous ΔCARD-deficient mice may be due to the truncated CARMA1 protein interfering with function of the wt protein (36). Protein kinase C-β (PKCβ) initiates a phosphorylation cascade that activates the CBM complex downstream BCR signaling. Heterozygous missense mutations in the gene

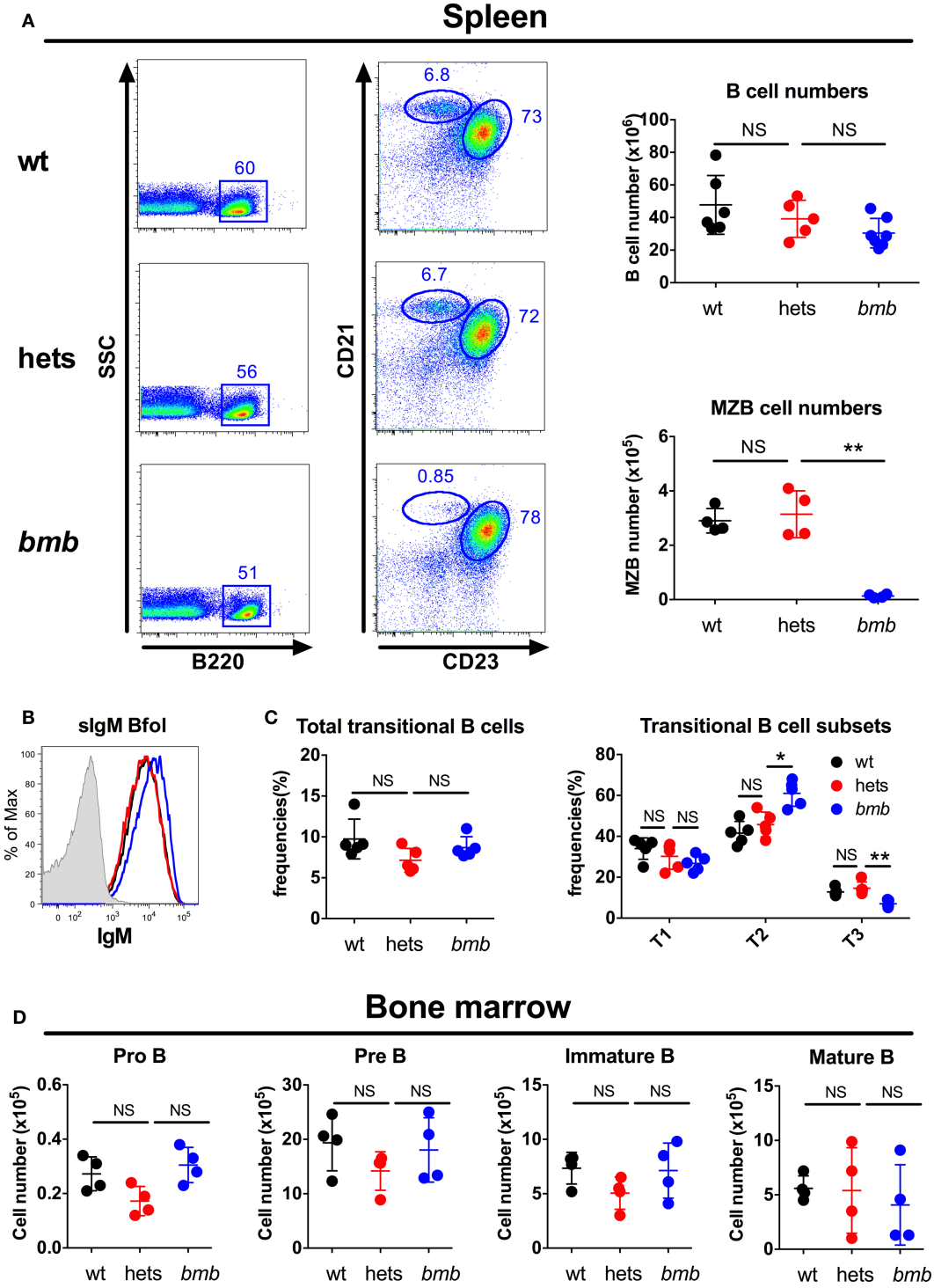


FIGURE 3 | Heterozygous *bumble* mice have normal frequencies of the major B cell subsets. Total B cells, marginal zone B cells (MZB), and transitional B cells were stained by FACS in wild-type (wt), heterozygous (hets), and *bumble* (bmb) mice. **(A)** Left panel: representative staining of marginal zone B cells (MZB, $B220^+$, $CD23^-$, and $CD21^{hi}$) and follicular B cells ($B220^+$, $CD23^+$, and $CD21^+$). Right panel: total B cell ($B220^+$) and MZB cell numbers. **(B)** Representative staining of surface IgM (sIgM) on wt (black), heterozygous (blue), and *bumble* (blue) follicular B cells. **(C)** Splenic transitional B cells ($B220^+$ and $CD93^+$), further subdivided into T1 ($CD23^-IgM^+$), T2 ($CD23^+IgM^+$), and T3 ($CD23^+IgM^{lo}$). **(D)** Bone marrow B cell progenitors, pro B ($B220^+CD43^+CD19^+CD93^+$), pre B ($B220^+CD43^-IgM^-CD93^+$), immature B ($B220^+CD43^-IgM^+CD93^+$), and mature B cells ($B220^+CD43^-IgM^+CD93^-$). For transitional and progenitor B cell gating strategy, see Figure S1 in Supplementary Material. Figures represent 8- to 16-week-old mice with four to seven mice per group, and results are representative of at least two independent experiments. Graphs display mean + SD. Statistically significant differences between *bumble*, *bumble* heterozygotes, and wt mice are indicated by $*p < 0.05$ by Mann–Whitney test.

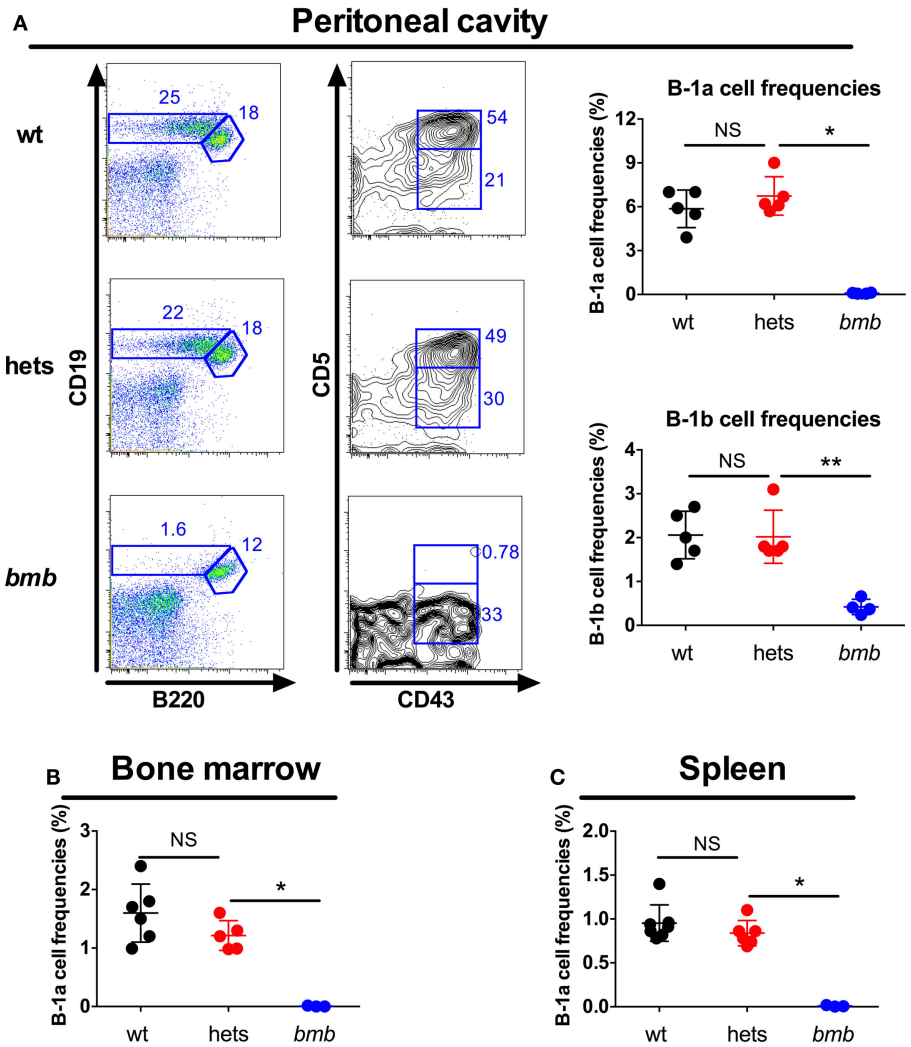


FIGURE 4 | Heterozygous bumble mice have normal frequencies of B-1 cells. Wild-type (wt), heterozygous *bumble* (hets), and *bumble* (*bmb*) cells were stained for B-1 cells. **(A)** Peritoneal B-1 cells identified as CD19^{hi}B220^{lo}CD43^{+} (B1b) and CD19^{hi}B220^{lo}CD43^{+}CD5^{+} (B1a). B-1a cells were identified as CD93-IgM^{+}C D19^{hi}B220^{lo}CD5^{+} in **(B)** bone marrow and **(C)** spleen. Representative stainings of B-1a cells in bone marrow and spleen are shown in Figure S2 in Supplementary Material. Figures represent 8- to 16-week-old mice with four to seven mice per group, and results are representative of at least two independent experiments. Graphs display mean + SD. Statistically significant differences between *bumble*, *bumble* heterozygotes, and wt mice are indicated by $^{*}p < 0.05$ and $^{**}p < 0.01$ by Mann–Whitney test.

encoding PKCβ were found to lead to impaired antipolysaccharide antibody responses and reduced natural antibody levels, despite apparently normal B cell development (37).

Several clinical cases with mutations in NF-κB proteins have demonstrated important roles of both the classical and alternative NF-κB signaling pathways for B cell development and function in humans. Hypermorphic mutations in the gene encoding IκBα lead to impaired phosphorylation-driven degradation of the mutant protein and thereby reduced NF-κB signaling (38). Hypermorphic heterozygous mutations in IκBα cause ectodermal dysplasia with immunodeficiency as evidenced by recurrent severe infections (38, 39). These patients have increased numbers of B and T cells, but display both B and T cell functional defects (39). Notably, the patient symptoms and lymphocyte functional defects could to a large extent be reproduced when introducing one of the hypermorphic IκBα mutations (S32I) to mice (33). Similar symptoms to IκBα hypermorphs are evident in patients with NEMO deficiency and B cells from these patients do not respond to CD40 ligation (40). More recently, several patients with mutations in the CBM complex have been described. Combined immunodeficiency (CID) resulting from impaired classical NF-κB signaling due to CARMA1 deficiency was associated with hypogammaglobulinaemia, impaired BAFF-R expression and a block of B cell maturation at the transitional B cell stage (41, 42). Human MALT1 deficiency, also manifested by CID, was associated with lack of MZB cells and failure to respond to Pneumovax vaccination (43, 44), a phenotype that is very similar to that seen in the corresponding mouse model (45). Rapid advances

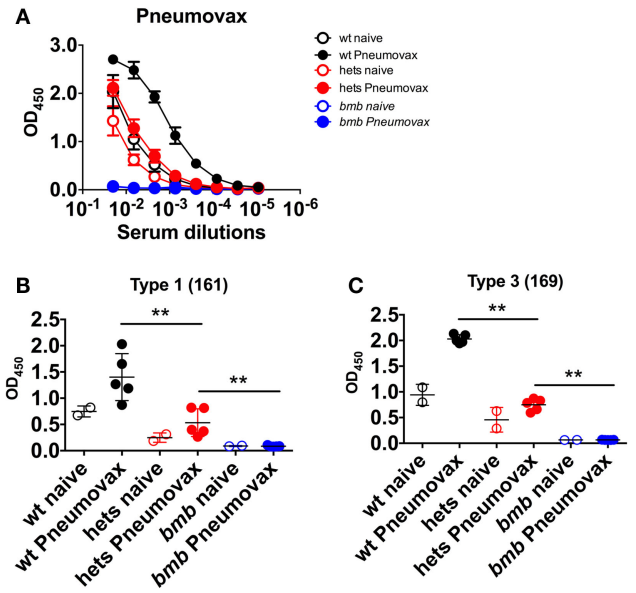


FIGURE 5 | Heterozygous *bumble* mice have reduced antibody responses to immunization with pneumococcal polysaccharide vaccine. Wild-type (wt), heterozygous *bumble* (hets), and *bumble* (bmb) mice were immunized with Pneumovax pneumococcal polysaccharide vaccine and sacrificed after 6 days. Serum antibody responses were analyzed by ELISA against **(A)** Pneumovax, **(B)** type 1 polysaccharide antigen 161-X, and **(C)** type 3 polysaccharide antigen 169-X. Figures represent 14- to 16-week-old mice with five mice per immunized group, and results are representative of two independent experiments. Graphs display mean + SD. Statistically significant differences between *bumble*, *bumble* heterozygotes, and wt mice are indicated by $^{**}p < 0.01$ by Mann–Whitney test.

in identifying genes underlying human immunodeficiencies will reveal if also IκBNS plays a role in this disease group.

So far, there are few reports of heterozygous mutations in the NF-κB pathway leading to immunodeficiency in humans. Heterozygous gain-of-function mutations in the gene encoding CARMA1 were shown to result in constitutive NF-κB activity and are manifested by lymphocytosis but impaired memory B cells and low TI-2 responses (46, 47). It is interesting to speculate if heterozygous mutations in genes encoding components of BCR signaling, including classical NF-κB pathway mediators, may contribute to the observed variability in antipolysaccharide immune responses in the human population (48, 49). We describe here that a heterozygous mutation in the *nfkbid* gene encoding the atypical IκB protein IκBNS led to reduced steady state IgM and IgG3 antibody levels and impaired response to vaccination with TI-2 antigens in mice. Heterozygous mutations in genes of the NF-κB pathway could potentially lead to haploinsufficiency for B cell function, resulting in lower antibody responses to vaccination and increased susceptibility to infection, which should be considered in future studies.

AUTHOR CONTRIBUTIONS

GKP designed and performed the experiments and wrote the manuscript. MÁ, JS, and SK performed the experiments. CA and BB designed the experiments. GKH designed the experiments and wrote the manuscript. All authors have approved the manuscript.

ACKNOWLEDGMENTS

We are grateful to the personnel at the animal facility at the Department of Microbiology, Tumor and Cell Biology at the Karolinska Institutet. This work was supported by grants from the Bill & Melinda Gates Foundation (GKH and BB), the Swedish Research Council (GKH), and David och Astrid Hageléns stiftelse (GKP).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00065

FIGURE S1 | Gating strategy for identifying transitional and progenitor B cells. (A) Representative staining of wild type splenocytes for transitional B cells ($CD93^+B220^+$) and the transitional B cell populations gated as indicated. **(B)** Representative staining of wild type bone marrow for progenitor and mature B cell populations. Pro B ($B220^+CD43^+CD19^+CD93^+$), pre B ($B220^+CD43^-IgM^-CD93^+$), immature B ($B220^+CD43^-IgM^+CD93^+$), and mature B cells ($B220^+CD43^-IgM^+CD93^-$).

FIGURE S2 | Representative stainings of bone marrow and spleen B-1a cells. B-1a cells were identified as CD93-IgM+CD19hiB220loCD5+ in (A) bone marrow and (B) spleen.

REFERENCES

1. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) **11**:34–46. doi:10.1038/nri2901
2. Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, et al. Innate response activator B cells protect against microbial sepsis. *Science* (2012) **335**:597–601. doi:10.1126/science.1215173
3. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol* (2013) **13**:118–32. doi:10.1038/nri3383
4. O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* (1992) **22**:711–7. doi:10.1002/eji.1830220314
5. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. *Immunity* (2015) **42**:607–12. doi:10.1016/j.immuni.2015.04.005
6. Maseda D, Candando KM, Smith SH, Kalampokis I, Weaver CT, Plevy SE, et al. Peritoneal cavity regulatory B cells (B10 cells) modulate IFN-gamma+CD4+ T cell numbers during colitis development in mice. *J Immunol* (2013) **191**:2780–95. doi:10.4049/jimmunol.1300649
7. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* (2001) **14**:617–29. doi:10.1016/S1074-7613(01)00129-7
8. Hsu MC, Toellner KM, Vinuesa CG, Maclennan IC. B cell clones that sustain long-term plasmablast growth in T-independent extrafollicular antibody responses. *Proc Natl Acad Sci U S A* (2006) **103**:5905–10. doi:10.1073/pnas.0601502103
9. Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J Exp Med* (1985) **161**:1554–68. doi:10.1084/jem.161.6.1554
10. Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, Porayette P, Shelley WC, Conway SJ, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci U S A* (2011) **108**:1468–73. doi:10.1073/pnas.1015841108
11. Allman D, Lindsley RC, Demuth W, Rudd K, Shinton SA, Hardy RR. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol* (2001) **167**:6834–40. doi:10.4049/jimmunol.167.12.6834

12. Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* (2003) **18**:675–85. doi:10.1016/S1074-7613(03)00111-0
13. Srivastava B, Quinn WJ III, Hazard K, Erikson J, Allman D. Characterization of marginal zone B cell precursors. *J Exp Med* (2005) **202**:1225–34. doi:10.1084/jem.20051038
14. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol* (2006) **7**:293–301. doi:10.1038/ni1301
15. Pedersen GK, Adori M, Khoenkhoen S, Dosenovic P, Beutler B, Karlsson Hedestam GB. B-1a transitional cells are phenotypically distinct and are lacking in mice deficient in IkappaBNS. *Proc Natl Acad Sci U S A* (2014) **111**(39):E4119–26. doi:10.1073/pnas.1415866111
16. Gerondakis S, Siebenlist U. Roles of the NF-kappaB pathway in lymphocyte development and function. *Cold Spring Harb Perspect Biol* (2010) **2**:a000182. doi:10.1101/cshperspect.a000182
17. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* (2002) **2**:725–34. doi:10.1038/nri968
18. Didonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* (1997) **388**:548–54. doi:10.1038/41493
19. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. *Immunol Rev* (2006) **210**:171–86. doi:10.1111/j.0105-2896.2006.00375.x
20. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* (2004) **25**:280–8. doi:10.1016/j.it.2004.03.008
21. Schuster M, Annemann M, Plaza-Sirvent C, Schmitz I. Atypical IkappaB proteins – nuclear modulators of NF-kappaB signaling. *Cell Commun Signal* (2013) **11**:23. doi:10.1186/1478-811X-11-23
22. Pedersen GK, Adori M, Karlsson Hedestam GB. NF-kappaB signaling in B-1 cell development. *Ann N Y Acad Sci* (2015) **1362**(1):39–47. doi:10.1111/nyas.12800
23. Cariappa A, Liou HC, Horwitz BH, Pillai S. Nuclear factor kappa B is required for the development of marginal zone B lymphocytes. *J Exp Med* (2000) **192**:1175–82. doi:10.1084/jem.192.8.1175
24. Tusche MW, Ward LA, Vu F, McCarthy D, Quintela-Fandino M, Ruland J, et al. Differential requirement of MALT1 for BAFF-induced outcomes in B cell subsets. *J Exp Med* (2009) **206**:2671–83. doi:10.1084/jem.20091802
25. Zhang X, Paun A, Claudio E, Wang H, Siebenlist U. The tumor promoter and NF-kappaB modulator Bcl-3 regulates splenic B cell development. *J Immunol* (2013) **191**:5984–92. doi:10.4049/jimmunol.1300611
26. Hovelmeyer N, Worns MA, Reissig S, Adams-Quack P, Leclaire J, Hahn M, et al. Overexpression of Bcl-3 inhibits the development of marginal zone B cells. *Eur J Immunol* (2014) **44**:545–52. doi:10.1002/eji.201343655
27. Touma M, Keskin DB, Shiroki F, Saito I, Koyasu S, Reinherz EL, et al. Impaired B cell development and function in the absence of IkappaBNS. *J Immunol* (2011) **187**:3942–52. doi:10.4049/jimmunol.1002109
28. Arnold CN, Pirie E, Dosenovic P, McInerney GM, Xia Y, Wang N, et al. A forward genetic screen reveals roles for Nfkbid, Zeb1, and Ruvbl2 in humoral immunity. *Proc Natl Acad Sci U S A* (2012) **109**:12286–93. doi:10.1073/pnas.1209134109
29. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* (1995) **80**:321–30. doi:10.1016/0092-8674(95)90415-8
30. Ruland J, Duncan GS, Elia A, Del Barco Barrantes I, Nguyen L, Plyte S, et al. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. *Cell* (2001) **104**:33–42. doi:10.1016/S0092-8674(01)00189-1
31. Egawa T, Albrecht B, Favier B, Sunshine MJ, Mirchandani K, O'brien W, et al. Requirement for CARMA1 in antigen receptor-induced NF-kappa B activation and lymphocyte proliferation. *Curr Biol* (2003) **13**:1252–8. doi:10.1016/S0960-9822(03)00491-3
32. Xue L, Morris SW, Orihuela C, Tuomanen E, Cui X, Wen R, et al. Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. *Nat Immunol* (2003) **4**:857–65. doi:10.1038/ni963
33. Mooster JL, Le Bras S, Massaad MJ, Jabara H, Yoon J, Galand C, et al. Defective lymphoid organogenesis underlies the immune deficiency caused by a heterozygous S32I mutation in IkappaBalpha. *J Exp Med* (2015) **212**:185–202. doi:10.1084/jem.20140979
34. Woodland RT, Schmidt MR. Homeostatic proliferation of B cells. *Semin Immunol* (2005) **17**:209–17. doi:10.1016/j.smim.2005.02.006
35. Newton K, Dixit VM. Mice lacking the CARD of CARMA1 exhibit defective B lymphocyte development and impaired proliferation of their B and T lymphocytes. *Curr Biol* (2003) **13**:1247–51. doi:10.1016/S0960-9822(03)00458-5
36. Gaide O, Favier B, Legler DF, Bonnet D, Brissoni B, Valitutti S, et al. CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-kappa B activation. *Nat Immunol* (2002) **3**:836–43. doi:10.1038/ni830
37. Teh CE, Horikawa K, Arnold CN, Beutler B, Kucharska EM, Vinuesa CG, et al. Heterozygous mis-sense mutations in Prkcb as a critical determinant of anti-polysaccharide antibody formation. *Genes Immun* (2013) **14**:223–33. doi:10.1038/gene.2013.11
38. Courtois G, Smahi A, Reichenbach J, Doffinger R, Cancrini C, Bonnet M, et al. A hypermorphic IkappaBalpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. *J Clin Invest* (2003) **112**:1108–15. doi:10.1172/JCI18714
39. Janssen R, Van Wengen A, Hoeve MA, Ten Dam M, Van Der Burg M, Van Dongen J, et al. The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes. *J Exp Med* (2004) **200**:559–68. doi:10.1084/jem.20040773
40. Jain A, Ma CA, Liu S, Brown M, Cohen J, Strober W. Specific missense mutations in NEMO result in hyper-IgM syndrome with hypohydrotic ectodermal dysplasia. *Nat Immunol* (2001) **2**:223–8. doi:10.1038/85277
41. Greil J, Rausch T, Giese T, Bandapalli OR, Daniel V, Bekeredjian-Ding I, et al. Whole-exome sequencing links caspase recruitment domain 11 (CARD11) inactivation to severe combined immunodeficiency. *J Allergy Clin Immunol* (2013) **131**:1376–83.e1373. doi:10.1016/j.jaci.2013.02.012
42. Stepensky P, Keller B, Buchta M, Kienzler AK, Elpeleg O, Somech R, et al. Deficiency of caspase recruitment domain family, member 11 (CARD11), causes profound combined immunodeficiency in human subjects. *J Allergy Clin Immunol* (2013) **131**:477–485.e471. doi:10.1016/j.jaci.2012.11.050
43. Jabara HH, Ohsumi T, Chou J, Massaad MJ, Benson H, Megarbane A, et al. A homozygous mucosa-associated lymphoid tissue 1 (MALT1) mutation in a family with combined immunodeficiency. *J Allergy Clin Immunol* (2013) **132**:151–8. doi:10.1016/j.jaci.2013.04.047
44. McKinnon ML, Rozmus J, Fung SY, Hirschfeld AF, Del Bel KL, Thomas L, et al. Combined immunodeficiency associated with homozygous MALT1 mutations. *J Allergy Clin Immunol* (2014) **133**:1458–62, 1462.e1451. doi:10.1016/j.jaci.2013.10.045
45. Ruefli-Brasse AA, French DM, Dixit VM. Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. *Science* (2003) **302**:1581–4. doi:10.1126/science.1090769
46. Snow AL, Xiao W, Stinson JR, Lu W, Chaigne-Delalande B, Zheng L, et al. Congenital B cell lymphocytosis explained by novel germline CARD11 mutations. *J Exp Med* (2012) **209**:2247–61. doi:10.1084/jem.20120831
47. Brohl AS, Stinson JR, Su HC, Badgett T, Jennings CD, Sukumar G, et al. Germline CARD11 mutation in a patient with severe congenital B cell lymphocytosis. *J Clin Immunol* (2015) **35**:32–46. doi:10.1007/s10875-014-0106-4
48. Konradsen HB, Henrichsen J, Wachmann H, Holm N. The influence of genetic factors on the immune response as judged by pneumococcal vaccination of mono- and dizygotic Caucasian twins. *Clin Exp Immunol* (1993) **92**:532–6. doi:10.1111/j.1365-2249.1993.tb03433.x
49. Schutz K, Hughes RG, Parker A, Quinti I, Thon V, Cavaliere M, et al. Kinetics of IgM and IgA antibody response to 23-valent pneumococcal polysaccharide vaccination in healthy subjects. *J Clin Immunol* (2013) **33**:288–96. doi:10.1007/s10875-012-9792-y

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 21 March 2016
doi: 10.3389/fimmu.2016.00106

The Homophilic Domain – An Immunological Archetype

Heinz Kohler[1], Jagadeesh Bayry[2,3,4,5] and Srinivas V. Kaveri[2,3,4,5]**

[1] Department of Microbiology Immunology, University of Kentucky, Lexington, KY, USA, [2] Institut National de la Santé et de la Recherche Médicale Unité 1138, Paris, France, [3] Centre de Recherche des Cordeliers, Equipe – Immunopathologie et immuno-intervention thérapeutique, Paris, France, [4] Sorbonne Universités, UPMC Univ Paris 06, UMR S 1138, Paris, France, [5] Université Paris Descartes, Sorbonne Paris Cité, UMR S 1138, Paris, France

The homophilic potential emerges as an important biological principle to boost the potency of immunoglobulins. Since homophilic antibodies in human and mouse sera exist prior environmental exposure, they are part of the natural antibody repertoire. Nevertheless, hemophilic properties are also identified in induced antibody repertoire. The use of homophilicity of antibodies in the adaptive immunity signifies an archetypic antibody structure. The unique feature of homophilicity in the antibody repertoire also highlights an important mechanism to boost the antibody potency to protect against infection and atherosclerosis as well to treat cancer patients.

Keywords: natural antibodies, homophilic, immunoglobulins, idiotype, induced antibodies, T15, 1F7

OPEN ACCESS

Edited by:
Ana María Hernández, Center of Molecular Immunology, Cuba

Reviewed by:
Mepur Hanumantha-Rao Ravindranath, Terasaki Foundation Laboratory, USA
Ronald B. Corley, Boston University School of Medicine, USA

****Correspondence:***
Heinz Kohler
heinz.kohler@uky.edu;
Srinivas V. Kaveri
srini.kaveri@crc.jussieu.fr

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *08 February 2016*
Accepted: *07 March 2016*
Published: *21 March 2016*

Citation:
Kohler H, Bayry J and Kaveri SV (2016) The Homophilic Domain – An Immunological Archetype. Front. Immunol. 7:106. doi: 10.3389/fimmu.2016.00106

NATURAL ANTIBODIES – THE VIRGIN IMMUNE RESPONSE

Natural antibodies are immunoglobulins in sera of mammalians that are neither immunologically challenged nor responding. For example, natural antibodies are in sera of neonatal or germ-free mice. Natural antibodies are of IgM, IgG, and IgA isotypes and play an important role in the immune homeostasis and protection against pathogens (1–6). B1 cells in mice produce natural antibodies (7, 8). In human, $CD20^+CD27^+CD43^+$ memory B cells were recently identified as murine B1 counterpart (9). Natural antibodies are the important components of the therapeutic intravenous immunoglobulin (IVIG) (10–12) that is widely used in the therapy of autoimmune and inflammatory diseases (13–17). These functions of natural antibodies also indicate diverse roles played by B cells in the immune homeostasis (18–22).

THE IDIOTYPIC INTERACTIONS

Oudin and Jerne are the founders of the idiotype concept (23, 24). They "discovered" that one antibody could recognize another antibody as an individual and unique member of the immune system. The uniqueness of an antibody lies in the variable region of heavy and light chains and has been named idiotype. The idiotype antibody individuality is separated from the antigen-binding site, also determined by unique sequence variability. However, both regions are linked by a unique variability and have a functional relation. Identifying an antigen-binding site also reveals the linked idiotype and *vice versa*, an idiotope reveals the associated antigen specificity. However, there are important exceptions to this linkage rule: the so-called common idiotopes (IdX) are expressed on antibodies with different antigen specificities (25). Furthermore, antigen-binding site and idiotype site can be identical or overlapping.

Studies on the functional interactions of anti-idiotype binding to antibodies have established a classification in the idiotype field. Jerne termed anti-idiotypes that do not interfere with the antigen-binding site as Ab2α and anti-idiotypes that block the antigen binding as Ab2β (26). Ab2 was further differentiated by adding the term Ab2γ to describe Ab2 that only partially inhibits antigen binding (27, 28). The Ab2β has another unique functional feature. It mimics the antigen by its perfect fit into another antigen-binding site. Ab2β has been used as an antigen to induce a specific immune response (28). Another shared idiotype has been discovered in HIV-infected primates (29). This anti-idiotype antibody has been termed as Ab2δ (30).

In mice, "prime" antibodies lack the dominant expression of certain shared idiotype, such as the so-called T15 idiotype, that develops weeks after birth without environmental challenge (31, 32). Therefore, the T15 dominance can be considered a part of the natural antibody repertoire. The T15 idiotype is also present in primates, including man (33). Perhaps a review of the idiotypic circuits is helpful for the understanding of the biological and immunological properties of the T15 archetypic idiotype. A hallmark of the T15 antibody family is their ability to self bind for producing homophilic complexes (34–36). The domain responsible for antibody self-binding (homophilicity) has been described as a region in VH of T15, extending from CDR2 to Fr3 (35, 37). Because of their preimmune presence in normal sera, homophilic antibodies are segment of the natural antibody repertoire (33, 37).

THE BIOLOGY AND IMMUNOREGULATORY FUNCTIONS OF HOMOPHILIC ANTIBODIES

The homophilic domain in antibodies was identified, and peptides that can confer the homophilic effect to other antibodies were made (38). Further studies showed that the homophilic domain can be expressed independently from the specificity of the antigen-binding site (39). This suggested that any antibody could be made homophilic by attaching the homophilic domain. In early experiments, a chemical affinity conjugation method was introduced to make the homophilic Ig (40); later, recombinant techniques were used to produce a homophilic antibody fusion protein.

Human and mouse sera contain antibodies that express the homophilic domain (33), confirming that homophilic antibodies are part of the so-called natural antibodies (41). Homophilic antibodies with specificity for phosphorylcholine (PC) are superior in protecting against *Streptococcus pneumoniae* infection (42). Homophilic anti-PC antibodies are also highly effective to reduce atherosclerotic plaque formation (43, 44) and perhaps have other housekeeping functions. Antigenicity of the homophilic domain was tested in mice, rabbits, and non-human primates by injecting the homophilic domain peptide and treating macaques with a homophilic anti-CD20 antibody (45). No antibodies could be detected against the homophilic domain. This finding suggests that homophilic antibodies could be used as therapeutic drugs to treat human diseases without allergic side effects (46).

Indeed, human natural antibodies with specificity for PC protect against atherosclerotic plaque formation by binding to oxidized LDL and inhibit macrophage activation (43, 47). Immunizing atherosclerotic mice with PC antigen reduces plaque formation (44). Silverman and colleagues showed that SLE patients with low natural antibodies develop more serious disease (48). Emerging evidence suggested that natural antibodies could control transplant rejection (49).

Another "natural" antibody in normal human sera has been described with specificity for HLA (50). This anti-HLA is not detectable in sera but is revealed after IgG purification. The homophilic T15 peptide from the complementarity-determining region/framework masked the HLA recognition of non-purified IgG. Since the T15 homophilic peptide dissociate the homophilic Ig complex, thereby reducing the polyvalency and binding potency, one can conclude that in sera, auto-anti-HLA antibodies are bound to corresponding HLA and are not detected. It follows that natural auto-anti-HLA antibodies are homophilic. Thus, the homophilic property of antibodies plays a role in the functional regulation of antibodies either by masking them or by exposing them under appropriate conditions.

A homophilic state of the Ig B-cell receptor (BCR) has been described in chronic lymphocytic leukemia (51, 52). Peptides from the VH Ig BCR bind to the BCR providing the basis for self-recognition of the BCR. Recently, we used the T15 homophilic peptide to inhibit proliferation of murine human B-cell lines (46).

THE UNIQUE BIOPHYSICAL PROPERTIES OF HOMOPHILIC ANTIBODIES

Working with homophilic-converted Trastuzumab (Herceptin), we discovered that the dose–response in inducing apoptosis in a Her2/neu-expressing human cell line was not linear but was bell shaped (53). The highest concentration tested did not induce the highest amount of apoptosis, but a lower concentration produced the most apoptosis. Similar non-linear dose effects were observed in fluorescence staining of tumor cells. This paradoxical dose effect was also observed in xenograft experiment (54).

This non-linear dose affecting the potency of homophilic Herceptin could be due to the inherent mechanic of self-binding that was observed in 1986 (37, 55). Here, self-binding of the T15 antibody decreases as the concentration of homophilic antibody increases, producing a bell-shaped pattern. This indicates that the equilibrium of self-bound antibody and free antibody is controlled by the concentration of homophilic antibody. Thus, the lattice of build up of homophilic antibodies at the tumor target cell would decrease at a higher concentration, thereby reducing apoptosis. This paradoxical effect has been observed with homophilic anti-Her2/neu (Herceptin) (54). In this study, we showed that the viscosity of homophilic antibodies increases with temperature and decreases at lower temperature, an effect not observed with the most other proteins or organic compounds. Furthermore, the binding to antigen by a homophilic antibody is also higher at physiological temperature than by the non-homophilic parental antibody. Lastly, the binding of homophilic Herceptin to tumor cells is concentration dependent, whereby a lower concentration

targets better than a higher concentration. The unique biophysical properties of homophilic antibodies represent another aspect in the natural antibody repertoire.

HOMOPHILICITY IN SHARED IDIOTYPES

We believe that we are only at the beginning to discover networks of shared idiotypes. By using polyclonal anti-idiotype antibodies, Urbain and colleagues have observed shared idiotypes expressed on antibodies with different antigen specificity in rabbits and mice (26, 56). The monoclonal 1F7 anti-idiotype recognizes antibodies against viral antigens and is first described in humans (29, 57, 58). The T15 and 1F7 idiotypic networks harbor important biological and medical properties: dominant T15 antibodies protect against pneumococcal infection (59) and perform a housekeeping function to reduce atherosclerotic plaque formation (43); the 1F7 idiotope is expressed on antibodies against HIV-1 (57, 60) and hepatitis C (61, 62) (see also contribution by Muller et al.). Furthermore, 1F7-positive human anti-HIV-1 antibodies carry the homophilic domain (Veljkovic and Kohler, unpublished). These studies on homophilic antibodies show that homophilicity is utilized in the innate immunity (natural antibodies) and by the adaptive immune response.

HOMOPHILICITY AS A GENERAL CONCEPT IN SYSTEM BIOLOGY OF NATURAL ANTIBODIES

The data on homophilic natural antibodies point to a general strategy of enhancing potency not only of antibodies but also other biological systems (63). This finding can be viewed as a paradigm in System Biology in the T15 and 1F7 idiotypic networks. Both systems utilize the antibody homophilicity. Both systems also use this property to achieve a high level of antibody potency. Antibodies with T15 expression in normal sera are close to germline encoding: (i) the T15 dominance develops late in ontogeny (31), (ii) the homophilic domain contains H2 mutations, (iii) analysis of the neonatal repertoire by Coutinho (64) shows only evidence for extensive idiotypic network and for homophilic binding, and (iv) homophilic antibodies have been detected after stimulation (38, 46). Furthermore, in support of the adaptive creation of the homophilic domain, the sequence of homophilic domains is found different (51, 52). Thus, certain homophilic antibodies are not part of the so-called natural antibodies but are induced by antigenic stimulation.

The homophilic antibody enhancement and therapeutic potential have been already demonstrated (38, 46). The native dominant expression of 1F7 idiotype on anti-HIV-1 antibodies may lead to a novel vaccine concept (65). There is one important difference between the T15 and the 1F7 system: T15 antibodies are present in preimmune serum without external antigen exposure; 1F7 antibodies recognizing anti-HIV and anti-HBC antibodies are induced after infection and are not natural antibodies. Nemazee et al. recently promoted the idea of targeting germline antibodies for inducing a maturation sequence, leading to broadly neutralizing Abs against HIV-1 (66). The use of a germline targeting immunogen is different from the concept of stimulating B-cells with idiotype expressing receptors. Prior to triggering, these B-cells do not produce detectable antibodies and do not contribute to the natural antibody repertoire (57). Using the 1F7 antibody as vaccine would not require additional immunogen(s) to trigger the antibody maturations process, since the 1F7 idiotype is expressed by potent broadly neutralizing Abs (Parsons, unpublished). HIV-1 infection is expected to stimulate the expanded clones of 1F7 expressing B-cells to produce broadly neutralizing Abs (57).

SUMMARY

The discussion of the unique feature of homophilicity as a part of the natural and induced antibody repertoires (65) highlights an important mechanism to boost the antibody potency to protect against infection and atherosclerosis (44) as well to treat cancer patients (46). The concept of homophilicity is introduced here to describe the unique coexistence and synergism of acquired immunity with innate immunity. The homophilicity of antibodies in the natural and acquired repertoires emerges as an archetypic principle in the immune system.

AUTHOR CONTRIBUTIONS

HK surveyed the literature and wrote the article. JB and SK provided suggestions. All authors approved the final version of this article for publication and accepted the responsibility for the integrity of the work.

REFERENCES

1. Avrameas S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol Today* (1991) **12**(5):154–9. doi:10.1016/S0167-5699(05)80045-3
2. Kohler H, Bayry J, Nicoletti A, Kaveri SV. Natural autoantibodies as tools to predict the outcome of immune response? *Scand J Immunol* (2003) **58**(3):285–9. doi:10.1046/j.1365-3083.2003.01314.x
3. Elluru SR, Vani J, Delignat S, Bloch MF, Lacroix-Desmazes S, Kazatchkine MD, et al. Modulation of human dendritic cell maturation and function by natural IgG antibodies. *Autoimmun Rev* (2008) **7**(6):487–90. doi:10.1016/j.autrev.2008.04.014
4. Kaveri SV, Silverman GJ, Bayry J. Natural IgM in immune equilibrium and harnessing their therapeutic potential. *J Immunol* (2012) **188**(3):939–45. doi:10.4049/jimmunol.1102107
5. Elluru SR, Kaveri SV, Bayry J. The protective role of immunoglobulins in fungal infections and inflammation. *Semin Immunopathol* (2015) **37**(2):187–97. doi:10.1007/s00281-014-0466-0
6. Cunningham AF, Flores-Langarica A, Bobat S, Dominguez Medina CC, Cook CN, Ross EA, et al. B1b cells recognize protective antigens after natural infection and vaccination. *Front Immunol* (2014) **5**:535. doi:10.3389/fimmu.2014.00535
7. Sidman CL, Shultz LD, Hardy RR, Hayakawa K, Herzenberg LA. Production of immunoglobulin isotypes by Ly-1+ B cells in viable moth-eaten and normal mice. *Science* (1986) **232**(4756):1423–5. doi:10.1126/science.3487115
8. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) **11**(1):34–46. doi:10.1038/nri2901

9. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* (2011) **208**(1):67–80. doi:10.1084/jem.20101499
10. Vani J, Elluru S, Negi VS, Lacroix-Desmazes S, Kazatchkine MD, Bayry J, et al. Role of natural antibodies in immune homeostasis: IVIg perspective. *Autoimmun Rev* (2008) **7**(6):440–4. doi:10.1016/j.autrev.2008.04.011
11. Ravindranath MH, Terasaki PI, Pham T, Jucaud V, Kawakita S. Therapeutic preparations of IVIg contain naturally occurring anti-HLA-E antibodies that react with HLA-Ia (HLA-A/-B/-Cw) alleles. *Blood* (2013) **121**(11):2013–28. doi:10.1182/blood-2012-08-447771
12. Bouhlal H, Martinvalet D, Teillaud JL, Fridman C, Kazatchkine MD, Bayry J, et al. Natural autoantibodies to Fcgamma receptors in intravenous immunoglobulins. *J Clin Immunol* (2014) **34**(Suppl 1):S4–11. doi:10.1007/s10875-014-0019-2
13. Gelfand EW. Intravenous immune globulin in autoimmune and inflammatory diseases. *N Engl J Med* (2012) **367**(21):2015–25. doi:10.1056/NEJMra1009433
14. Bayry J, Hartung HP, Kaveri SV. IVIg for relapsing-remitting multiple sclerosis: promises and uncertainties. *Trends Pharmacol Sci* (2015) **36**(7):419–21. doi:10.1016/j.tips.2015.04.012
15. Gilardin L, Bayry J, Kaveri SV. Intravenous immunoglobulin as clinical immune-modulating therapy. *CMAJ* (2015) **187**(4):257–64. doi:10.1503/cmaj.130375
16. Cantarini L, Rigante D, Vitale A, Napodano S, Sakkas LI, Bogdanos DP, et al. Intravenous immunoglobulins (IVIG) in systemic sclerosis: a challenging yet promising future. *Immunol Res* (2015) **61**(3):326–37. doi:10.1007/s12026-014-8615-z
17. Lunemann JD, Nimmerjahn F, Dalakas MC. Intravenous immunoglobulin in neurology – mode of action and clinical efficacy. *Nat Rev Neurol* (2015) **11**(2):80–9. doi:10.1038/nrneurol.2014.253
18. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol* (2012) **30**:221–41. doi:10.1146/annurev-immunol-020711-074934
19. Maddur MS, Kaveri SV, Bayry J. Regulation of human dendritic cells by B cells depends on the signals they receive. *Blood* (2012) **119**(16):3863–4. doi:10.1182/blood-2012-02-408948
20. Morva A, Lemoine S, Achour A, Pers JO, Youinou P, Jamin C. Maturation and function of human dendritic cells are regulated by B lymphocytes. *Blood* (2012) **119**(1):106–14. doi:10.1182/blood-2011-06-360768
21. Maddur MS, Sharma M, Hegde P, Stephen-Victor E, Pulendran B, Kaveri SV, et al. Human B cells induce dendritic cell maturation and favour Th2 polarization by inducing OX-40 ligand. *Nat Commun* (2014) **5**:4092. doi:10.1038/ncomms5092
22. Moulin V, Andris F, Thielemans K, Maliszewski C, Urbain J, Moser M. B lymphocytes regulate dendritic cell (DC) function in vivo: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. *J Exp Med* (2000) **192**(4):475–82. doi:10.1084/jem.192.4.475
23. Jerne NK. Towards a network theory of the immune system. *Ann Immunol (Paris)* (1974) **125C**:373–89.
24. Oudin J. [The idiotyping of antibodies]. *Ann Immunol (Paris)* (1974) **125C**:309–37.
25. Cazenave PA, Oudin J. [Idiotype of antibodies, in the serum of rabbits immunized against human fibrinogen, directed against 2 different fragments of this antigen]. *C R Acad Sci Hebd Seances Acad Sci D* (1973) **276**(2):243–5.
26. Jerne NK, Roland J, Cazenave PA. Recurrent idiotopes and internal images. *EMBO J* (1982) **1**(2):243–7.
27. Huang JH, Ward RE, Kohler H. Idiotope antigens (Ab2 alpha and Ab2 beta) can induce in vitro B cell proliferation and antibody production. *J Immunol* (1986) **137**(3):770–6.
28. Kohler H, McNamara M, Ward RE. Idiotypes as internal antigens. *Prog Clin Biol Res* (1985) **172B**:343–53.
29. Muller S, Wang HT, Kaveri SV, Chattopadhyay S, Kohler H. Generation and specificity of monoclonal anti-idiotypic antibodies against human HIV-specific antibodies. I. Cross-reacting idiotopes are expressed in subpopulations of HIV-infected individuals. *J Immunol* (1991) **147**(3):933–41.
30. Parsons MS, Muller S, Kohler H, Grant MD, Bernard NF. On the benefits of sin: can greater understanding of the 1F7-idiotypic repertoire freeze enhance HIV vaccine development? *Hum Vaccin Immunother* (2013) **9**(7):1532–8. doi:10.4161/hv.24460
31. Fung J, Kohler H. Late clonal selection and expansion of the TEPC-15 germline specificity. *J Exp Med* (1980) **152**(5):1262–73. doi:10.1084/jem.152.5.1262
32. Etlinger HM, Heusser CH. T15 dominance in BALB/c mice is not controlled by environmental factors. *J Immunol* (1986) **136**(6):1988–91.
33. Halpern R, Kaveri SV, Kohler H. Human anti-phosphorylcholine antibodies share idiotopes and are self-binding. *J Clin Invest* (1991) **88**(2):476–82. doi:10.1172/JCI115328
34. Kaveri SV, Halpern R, Kang CY, Kohler H. Self-binding antibodies (autobodies) form specific complexes in solution. *J Immunol* (1990) **145**(8):2533–8.
35. Kang CY, Brunck TK, Kieber-Emmons T, Blalock JE, Kohler H. Inhibition of self-binding antibodies (autobodies) by a VH-derived peptide. *Science* (1988) **240**(4855):1034–6. doi:10.1126/science.3368787
36. Kang CY, Cheng HL, Rudikoff S, Kohler H. Idiotypic self binding of a dominant germline idiotype (T15). Autobody activity is affected by antibody valency. *J Exp Med* (1987) **165**(5):1332–43. doi:10.1084/jem.165.5.1332
37. Kang CY, Kohler H. Immunoglobulin with complementary paratope and idiotope. *J Exp Med* (1986) **163**(4):787–96. doi:10.1084/jem.163.4.787
38. Kohler H. Discovery of homophilic antibodies as novel drugs. *Curr Trends Immunol* (2009) **10**:113–8.
39. Kaveri SV, Halpern R, Kang CY, Kohler H. Antibodies of different specificities are self-binding: implication for antibody diversity. *Mol Immunol* (1991) **28**(7):773–8. doi:10.1016/0161-5890(91)90120-9
40. Russ M, Lou D, Kohler H. Photo-activated affinity-site cross-linking of antibodies using tryptophan containing peptides. *J Immunol Methods* (2005) **304**(1–2):100–6. doi:10.1016/j.jim.2005.06.020
41. Kaveri SV, Kang CY, Kohler H. Natural mouse and human antibodies bind to a peptide derived from a germline VH chain. Evidence for evolutionary conserved self-binding locus. *J Immunol* (1990) **145**(12):4207–13.
42. Briles DE, Forman C, Hudak S, Claflin JL. The effects of idiotype on the ability of IgG1 anti-phosphorylcholine antibodies to protect mice from fatal infection with *Streptococcus pneumoniae*. *Eur J Immunol* (1984) **14**(11):1027–30. doi:10.1002/eji.1830141112
43. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* (2000) **105**(12):1731–40. doi:10.1172/JCI8472
44. Caligiuri G, Khallou-Laschet J, Vandaele M, Gaston AT, Delignat S, Mandet C, et al. Phosphorylcholine-targeting immunization reduces atherosclerosis. *J Am Coll Cardiol* (2007) **50**(6):540–6. doi:10.1016/j.jacc.2006.11.054
45. Bingaman MG, Basu GD, Golding TC, Chong SK, Lassen AJ, Kindt TJ, et al. The autophilic anti-CD20 antibody DXL625 displays enhanced potency due to lipid raft-dependent induction of apoptosis. *Anticancer Drugs* (2010) **21**(5):532–42. doi:10.1097/CAD.0b013e328337d485
46. Kohler H. Homophilic antibodies as immunotherapeutics. *Immunotherapy* (2013) **5**(3):235–46. doi:10.2217/imt.12.159
47. Shaw PX, Goodyear CS, Chang MK, Witztum JL, Silverman GJ. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells. *J Immunol* (2003) **170**(12):6151–7. doi:10.4049/jimmunol.170.12.6151
48. Silverman GJ. Targeting of B cells in SLE: rationale and therapeutic opportunities. *Bull NYU Hosp Jt Dis* (2006) **64**(1–2):51–6.
49. Porcheray F, Fraser JW, Gao B, McColl A, DeVito J, Dargon I, et al. Polyreactive antibodies developing amidst humoral rejection of human kidney grafts bind apoptotic cells and activate complement. *Am J Transplant* (2013) **13**(10):2590–600. doi:10.1111/ajt.12394
50. Ravindranath MH, Terasaki PI, Maehara CY, Jucaud V, Kawakita S, Pham T, et al. Immunoglobulin (Ig)G purified from human sera mirrors intravenous Ig human leucocyte antigen (HLA) reactivity and recognizes one's own HLA types, but may be masked by Fab complementarity-determining region peptide in the native sera. *Clin Exp Immunol* (2015) **179**(2):309–28. doi:10.1111/cei.12450
51. Binder M, Muller F, Frick M, Wehr C, Simon F, Leistler B, et al. CLL B-cell receptors can recognize themselves: alternative epitopes and structural clues for autostimulatory mechanisms in CLL. *Blood* (2013) **121**(1):239–41. doi:10.1182/blood-2012-09-454439
52. Duhren-von Minden M, Ubelhart R, Schneider D, Wossning T, Bach MP, Buchner M, et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* (2012) **489**(7415):309–12. doi:10.1038/nature11309
53. Bryan AJ, Kohler H. Physical and biological properties of homophilic therapeutic antibodies. *Cancer Immunol Immunother* (2011) **60**(4):507–14. doi:10.1007/s00262-010-0952-8

54. Kohler H, Bryan AJ. Paradoxical concentration effect of a homodimerizing antibody against a human non-small cell lung cancer cell line. *Cancer Immunol Immunother* (2009) **58**(5):749–58. doi:10.1007/s00262-008-0597-z
55. Bona CA, Kang CY, Kohler H, Monestier M. Epibody: the image of the network created by a single antibody. *Immunol Rev* (1986) **90**:115–27. doi:10.1111/j.1600-065X.1986.tb01480.x
56. Urbain J, Tasiaux N, Leuwenkroon R, Van Acker A, Mariame B. Sharing of idiotypic specificities between different antibody populations from an individual rabbit. *Eur J Immunol* (1975) **5**(8):570–5. doi:10.1002/eji.1830050813
57. Wang QL, Wang HT, Blalock E, Muller S, Kohler H. Identification of an idiotypic peptide recognized by autoantibodies in human immunodeficiency virus-1-infected individuals. *J Clin Invest* (1995) **96**(2):775–80. doi:10.1172/JCI118122
58. Muller S, Margolin DH, Nara PL, Alvord WG, Kohler H. Stimulation of HIV-1-neutralizing antibodies in simian HIV-IIIB-infected macaques. *Proc Natl Acad Sci U S A* (1998) **95**(1):276–81. doi:10.1073/pnas.95.1.276
59. Wallick S, Claflin JL, Briles DE. Resistance to *Streptococcus pneumoniae* is induced by a phosphocholine-protein conjugate. *J Immunol* (1983) **130**(6):2871–5.
60. Wang H, Muller S, Zolla-Pazner S, Kohler H. Human monoclonal and polyclonal anti-human immunodeficiency virus-1 antibodies share a common clonotypic specificity. *Eur J Immunol* (1992) **22**(7):1749–55. doi:10.1002/eji.1830220713
61. Grant MD. Antibody convergence along a common idiotypic axis in immunodeficiency virus and hepatitis C virus infections. *J Med Virol* (2002) **66**(1):13–21. doi:10.1002/jmv.2105
62. Parsons MS, Rouleau D, Routy JP, LeBlanc R, Grant MD, Bernard NF. Selection of human anti-HIV broadly neutralizing antibodies occurs within the context of frozen 1F7-idiotypic repertoire. *AIDS* (2011) **25**(10):1249–64. doi:10.1097/QAD.0b013e328347f9fa
63. Gonen S, DiMaio F, Gonen T, Baker D. Design of ordered two-dimensional arrays mediated by noncovalent protein-protein interfaces. *Science* (2015) **348**(6241):1365–8. doi:10.1126/science.aaa9897
64. Holmberg D, Forsgren S, Ivars F, Coutinho A. Reactions among IgM antibodies derived from normal, neonatal mice. *Eur J Immunol* (1984) **14**(5):435–41. doi:10.1002/eji.1830140510
65. Kohler H. Novel vaccine concept based on back-boost effect in viral infection. *Vaccine* (2015) **33**(29):3274–5. doi:10.1016/j.vaccine.2015.05.035
66. Jardine JG, Ota T, Sok D, Pauthner M, Kulp DW, Kalyuzhniy O, et al. HIV-1 Vaccines. Priming a broadly neutralizing antibody response to HIV-1 using a germline-targeting immunogen. *Science* (2015) **349**(6244):156–61. doi:10.1126/science.aac5894

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 07 April 2016
doi: 10.3389/fimmu.2016.00127

Natural Antibodies as Rheostats for Susceptibility to Chronic Diseases in the Aged

*Thomas L. Rothstein**

Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, Manhasset, NY, USA

Natural antibodies are spontaneously produced in the absence of infection or immunization, and are both anti-microbial and autoreactive. Autoreactive natural antibodies can bind noxious molecules, such as those involved in clinical situations of atherosclerosis (oxLDL), malignancy (NGcGM3), and neurodegeneration (amyloid, tau) and can affect the fate of their targets or the cells bearing them to maintain homeostasis. Clinically relevant natural antibodies have been shown to decline with advancing age in those few situations where measurements have been made. Consistent with this, human B-1 cells that are thought to be responsible for generating natural antibodies also decline with advancing age. These findings together suggest that an age-related decline in amount or efficacy of homeostatic natural antibodies is associated with relative loss of protection against molecules involved in several diseases whose incidence rises in the older age population, and that those individuals experiencing greatest loss are at greatest risk. In this view, natural antibodies act as rheostats for susceptibility to several age-related diseases. These considerations suggest that administration of natural antibodies, or of factors that maintain B-1 cells and/or enhance production of natural antibodies by B-1 cells, may serve to counteract the onset or progression of age-related chronic illness.

Keywords: human B cells, B-1 cells, natural antibody

OPEN ACCESS

Edited by:
George C. Tsokos,
Harvard Medical School, USA

Reviewed by:
Stamatis-Nick Liossis,
University of Patras Medical School, Greece
Antonio La Cava,
University of California Los Angeles, USA

***Correspondence:**
Thomas L. Rothstein
tr@nshs.edu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *19 February 2016*
Accepted: *21 March 2016*
Published: *07 April 2016*

Citation:
Rothstein TL (2016) Natural Antibodies as Rheostats for Susceptibility to Chronic Diseases in the Aged. Front. Immunol. 7:127. doi: 10.3389/fimmu.2016.00127

INTRODUCTION

Natural antibody represents immunoglobulin that is spontaneously and constitutively secreted in the absence of infection or immunization. Natural antibody is present in animals and humans, and is thought to comprise the bulk of resting IgM, along with portions of isotype-switched IgA and IgG. Natural antibody differs from adaptive antibody in many ways, importantly including repertoire and function. Much natural antibody is anti-microbial and forms a preexisting shield against infection that provides a primary layer of protection during the lag period required for germinal center formation and adaptive antibody production (1–5). Natural antibody also tends to be autoreactive (6–9) and performs a second beneficial function in housekeeping and homeostatic activity that speeds elimination of dying cell debris and noxious molecular species (4, 10–17). In this way, potentially inflammatory and/or toxic agents are removed before direct tissue injury can occur.

Natural antibody is generated for the most part by a relatively small but unique subpopulation of B cells termed B-1 cells, first recognized in 1982, that is developmentally distinct (18–20). The origin and function of B-1 cells have been most extensively studied in mice, where B-1 cells are readily identified by a clear set of phenotypic markers (B220loCD5$^+$CD23$^-$CD43$^+$IgMhiIgDlo).

For some time, the status of human B-1 cells has been uncertain, and the existence of human B-1 cells has been debated. However, a new phenotypic profile for B-1 cells in human peripheral blood was recently reported ($CD19^+CD20^+CD27^+CD38modCD43^+CD70^-$) (21–23) and, despite some controversy (24–27), this profile has gained acceptance and has been utilized by a number of investigators in translational studies of specific disease states (25, 26, 28–31).

The natural antibodies produced by B-1 cells differ in sequence from adaptive antibodies produced by conventional B (B-2) cells, which in turn dictates repertoire and function. Mouse B-1 cell antibodies are more germ line-like in comparison to mouse B-2 cell antibodies by virtue of containing little or no somatic hypermutation and much reduced, or non-existent, N-region addition (15, 32–34) both of which affect CDR3 domains that are major contributors to antigen binding. The lack of N-addition appears to derive from the absence of terminal deoxynucleotidyl transferase (TdT) during mouse hematopoietic development early in life when the bulk of B-1 cells are generated (35). However, human B-1 cell antibodies often contain N-addition, which likely reflects the presence of TdT throughout ontogeny in *Homo sapiens* (35). Like mouse B-1 cell antibodies, human B-1 cell antibodies contain little or no somatic hypermutation early in life (21), but acquire somatic mutation as time goes on, although some difference in this measure between B-1 and B-2 cell antibodies continues into adulthood (23). Because B-1 cell antibodies tend to reflect sequences delineated in the genome with little alteration, especially in mice, it has been suggested that the B-1 cell repertoire is "tuned" over evolutionary time, obeying Darwinian precepts such that sequences functioning to promote survival are retained (10). In this view, B-1 cell antibodies represent the best functioning antibodies for the roles that they fulfill.

HUMAN NATURAL ANTIBODIES RECOGNIZE MOLECULES ASSOCIATED WITH DISEASES OF AGING

Human natural antibodies directed against a variety of molecules with clinical significance have been identified. Three specific disease areas are illustrative, and these are three of the most common, distressing, and burdensome diseases associated with aging. (1) *Atherosclerosis* : healthy individuals commonly express IgM antibodies that bind oxidized low-density lipoproteins (oxLDL) (36). Oxidized LDLs arise from non-enzymatic processes, accumulate within vessel walls, and contribute to plaque formation and inflammation that together drive the disease process of atherosclerosis, resulting in cardiovascular events that can be lethal (37). One type of anti-oxLDL natural antibody binds an oxidized form of the major lipoprotein, apolipoprotein B100 (38–40). (2) *Malignancy*: healthy individuals commonly express antibodies that bind *N*-glycolylneuraminyl-lactosylceramide (NGcGM3) (41). NGcGM3 is not thought to be produced in human tissues due to an inactivating insertional mutation of cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH) that occurred evolutionarily after divergence of humankind from great apes, about 2.8 million years ago (42, 43). However, NGcGM3 is present in humans, presumably acquired exogenously by dietary intake, and for reasons that are as yet unclear is concentrated many fold in the membranes of some tumors, prominently including the malignant cells of lung cancer (44). (3) *Neurodegeneration*: healthy individuals commonly express antibodies that bind amyloid and tau proteins (45–48). Abnormal plaques (amyloid) and tangles (tau) of these proteins have been implicated in the pathogenesis of Alzheimer's Disease, in which protein aggregates result in neuronal dysfunction, and enhanced phosphorylation may play a role in this abnormal protein behavior and disease pathogenesis (49).

DISEASE-ASSOCIATED NATURAL ANTIBODIES ARE FUNCTIONAL

Natural antibodies directed against antigens associated with these three classes of disease appear to be functional. (1) In mice, a number of adoptive transfer experiments with $Apoe^{-/-}$ recipients have led to the generally accepted paradigm that B-1 cells and the IgM antibodies they produce are atheroprotective, whereas B-2 cells and the IgG antibodies they produce are atherogenic (50, 51). Less invasive studies have been carried out with people, and it has been shown that human IgM anti-oxLDL is inversely correlated with cardiovascular and carotid disease (12, 38, 39, 52–54), whereas IgG has been found to be positively correlated with atherosclerosis (12, 52, 55–60) or not correlated at all with vessel pathology (40, 61–64). The mechanism appears to involve inhibition of oxLDL uptake by macrophages (65, 66). In a recent study, human serum antibodies directed against a methylglyoxal (MGO) modified apolipoprotein B100 peptide were examined. The levels of IgM antibodies in healthy individuals aged 63–68 were found to be inversely correlated with cardiovascular events occurring during the subsequent 15 years; in contrast, the levels of IgG antibodies were not correlated with subsequent cardiovascular events (67). Thus, in both mouse and human, natural IgM antibodies against oxLDL, appear to counteract the development of atherosclerosis. (2) Human natural anti-NGcGM3 antibodies have been shown to specifically bind and eliminate malignant cells bearing NGcGM3. This tumor cell destruction by anti-NGcGM3 antibodies occurs through both a complement-dependent mechanism and an oncosis-like, complement-independent mechanism (41, 68, 69). Somewhat akin to the correlative results noted above with respect to MGO-modified apoB100 peptide, patients with lung cancer lack or have very low levels of anti-NGcGM3 antibodies (41). Separately, an anti-idiotypic antibody vaccine (racotumomab) that displays the "internal image" of NGcGM3 has been developed to stimulate production of anti-NGcGM3 antibodies (69–71). In a recent clinical trial for maintenance treatment after first line chemotherapy in non-small cell lung cancer patients, racotumomab significantly prolonged overall survival and progression free survival, and those patients experiencing the greatest antibody response had the best outcomes (69, 72). Thus, natural and elicited cytotoxic antibodies against NGcGM3 appear to protect against the onset and/or ameliorate the course of lung cancer. (3) Human natural antibodies against amyloid and tau have been proposed as agents that might oppose

and/or treat Alzheimer's neurodegeneration. As with the inverse correlation between serum levels of natural antibodies and the disease states of atherosclerosis and malignancy discussed above, natural anti-amyloid antibodies have been shown to be relatively diminished in patients with Alzheimer's Disease (46, 73–75). These natural anti-amyloid antibodies have been shown to diminish the burden of aggregated proteins and improve cell viability *in vitro* (45, 46, 76, 77). In animal studies, passive administration of antibodies against amyloid and tau has in each case depleted abnormal proteins from the brain and improved pathology and/or behavioral parameters (76, 78–81). In recent clinical trials, passive administration of monoclonal antibodies against amyloid protein failed to produce improvement in cognition or function (82–85). This failure of clinical improvement in anti-amyloid trials to date, despite preclinical data showing diminished protein aggregation, remains unexplained, but may suggest the utility of alternative anti-tau treatment. Regardless, these results indicate that circulating antibodies can affect aggregation and alter deposits of abnormal, pathological amyloid and tau proteins.

CLINICALLY RELEVANT NATURAL ANTIBODIES ARE DIMINISHED OR LESS EFFECTIVE WITH INCREASING AGE AND DISEASE

In each of the three clinical entities discussed above, natural antibodies that recognize disease-associated epitopes are diminished in affected patients. There are several potential explanations for these inverse correlations, among which is the possibility that the absence of homeostatic antibodies increases the risk of developing disease. This is perhaps most directly suggested by the prospective study of natural antibodies that recognize modified apoB100 and the associated subsequent risk of cardiovascular events, discussed above. These diseases of atherosclerosis, malignancy, and neurodegeneration are all more common with increasing age. If natural antibodies are involved in opposing disease pathogenesis and/or disease progression, it would be expected that levels of disease-related natural antibodies would be found to be diminished with advancing age. In fact, an age-related decline has been documented for natural antibodies directed against NGcGM3 (41), and for natural antibodies directed against amyloid (46). Thus far, the relationship between natural antibodies against oxidized apoB100 and age has not been examined.

B-1 CELLS GENERATE HOMEOSTATIC ANTIBODIES

In mice, natural antibodies are predominantly, if not exclusively, generated by B-1 cells. The recent phenotypic identification of human B-1 cells raises the question of whether this population is responsible for producing human natural antibodies, especially those related to disease. This has been evaluated for atherosclerosis-predictive/-protective IgM antibodies against MGO-modified apoB100. Among human B-1 cell, memory B cell, preplasmablast and plasmablast culture supernantants, natural IgM anti-MGO-apoB100 antibodies were generated predominantly by human B-1 cells (67). Similarly, B-1 cells are responsible for producing natural anti-NGcGM3 antibodies in mice (44). However, human B-1 cells have not yet been tested for production of antibodies neither against NGcGM3 nor against amyloid and tau.

AGE-RELATED CHANGES IN NATURAL ANTIBODIES LIKELY RELATE TO AGE-RELATED CHANGES IN B-1 CELLS

To the extent that human B-1 cells are the origin of disease-related homeostatic natural antibodies, then a change in B-1 cells may underlie the decline that occurs with advancing age. To address this possibility, B-1 cell and other B cell populations were enumerated in peripheral blood of healthy adult volunteers over a wide age range. This study showed an age-related decline in B-1 cells (21). Other B cell populations did not change with age. Thus, B-1 cell numbers are age-sensitive. Although some investigators have reported an age-related decline in memory B cells, others have not (86–89), but B-1 cells were not differentiated from $CD27^+$ memory B cells in earlier studies.

Beyond numbers, there is some evidence in mouse studies that the B-1 cell repertoire changes with age (90). In a careful study involving deep sequencing, Ghosn et al. showed that selection operates on the B-1 cell repertoire as mice mature (91). Consistent with this, the avidity of natural anti-amyloid antibodies is diminished in patients with Alzheimer's Disease as compared to healthy controls (75). Thus, as a result of declining B-1 cell numbers, or a change in B-1 cell repertoire, or both, natural antibody deteriorates, which appears to be accompanied by a loss of the protection, especially homeostatic protection, that natural antibody affords.

THE RHEOSTAT HYPOTHESIS FOR B-1 CELL NATURAL ANTIBODIES

Weaving these different strands of evidence together, there is reason to hypothesize, as a general paradigm, that: (1) an age-related decline in amount and/or efficacy of homeostatic natural antibodies is in turn associated with relative loss of protection against molecules involved in several diseases whose incidence rises in the older age population; and, (2) those individuals experiencing the greatest loss in amount and/or efficacy of homeostatic natural antibodies are at greatest risk. In this view, natural antibodies act as rheostats for susceptibility to several age-related diseases that are associated with accumulation of noxious molecules or involve unique molecular targets, or both. Extrapolation from this point suggests the possibility that administration of disease-opposing natural antibodies or of factors that maintain B-1 cells and/or enhance production of disease-opposing natural antibodies by B-1 cells could serve to counteract the onset or progression of age-related chronic illness.

WHY ARE NATURAL ANTIBODIES RELEVANT TO DISEASES OF THE ELDERLY PRESENT BEYOND THE AGE OF REPRODUCTION?

According to Darwinian principles, there is no advantage to counteracting diseases whose onset occurs after reproductive age. In this sense, then, results in both the mouse and human systems raise the question of why natural antibodies that protect against age-associated diseases are retained in evolution. This likely results from polyreactivity of B-1 cell natural antibodies, and antigenic mimicry of B-1 cell natural antibody targets, as illustrated graphically by mouse T15/E06 (11, 92). T15 is a completely germ-line antibody that arises in many mouse strains, first identified by its recognition of phosphorylcholine, an antigenic determinant found on pneumococci and other microbes. E06 is a completely germ-line antibody identified by its binding to oxidized LDL. T15 and E06 are, in fact, one-and-the-same; they are identical antibodies that protect against pneumococcal infection and affect the disposition of oxLDL (93). Moreover, human antibodies with these kinds of specificities appear early in life. This is highlighted by reports that IgM anti-oxLDL antibodies are found in umbilical cord blood samples and in blood samples from preterm and full-term infants (94, 95). These antibodies block the uptake of oxLDL by macrophages and are often germ line in heavy chain sequence (95). So natural antibodies capable of influencing atherosclerosis later in life appear early in ontogeny and seem to exist by virtue of a combination of similarity between antigens on bacteria and oxidized lipids and polyreactivity of B-1 cell-derived natural antibodies. The same is likely true of other natural antibodies.

DO NATURAL ANTIBODIES ARISE SPONTANEOUSLY OR ARE THEY STIMULATED BY SELF-ANTIGENS?

Mature B-1 cells secrete antibody spontaneously and constitutively, in the absence specific antigen engagement, which fails to generate typical signs of BCR signaling and activation in these cells (96). However, the BCR may play a role early on. Studies in mice indicate that B-1 cell development is enhanced by antigen engagement, the inverse of antigen-induced apoptosis in nascent B-2 cells (97, 98). The relevant antigens may be self-antigens, inasmuch as B-1 cell development is not disturbed in germ-free mice lacking foreign antigens (91). NGcGM3 would appear to contradict this paradigm because it cannot be a self-antigen in the human species that lacks CMAH and is incapable of generating this ganglioside. However, the germ-line antibody, 4ac, which binds myelin oligodendrocyte glycoprotein (MOG), a central nervous system target for EAE (experimental allergic encephalomyelitis), is identical to the germ-line anti-NGcGM3 antibody, P3, and so cross-reactivity with self components may explain the existence of natural antibodies against NGcGM3 (99). Further, potential transfer of NGcGM3 across the placenta and in mother's milk at early stages of fetal/neonatal development is unknown. Overall, the degree to which the B-1 cell repertoire is shaped by self-antigens as opposed to "foreign" antigens remains a question yet to be fully resolved. In light of the polyreactivity and antigen mimicry discussed above, the determinants of the B-1 cell repertoire are likely to have an important influence on the level of homeostatic protection provided by B-1 cell natural antibody and may be responsible, at least in part, for the variation in protective antibody noted among older individuals.

OTHER QUESTIONS REMAIN

B-1 cells in mouse and human can isotype switch and secrete natural antibodies that are IgA and IgG as well as IgM (23, 100, 101). The degree to which this happens may be important in assessing the level of homeostatic protection afforded by natural antibodies. For example, IgM anti-oxLDL antibodies protect against atherosclerosis in mice and correlate with protection against cardiovascular events in humans, whereas IgG anti-oxLDL antibodies do not. However, it is unknown at present whether IgG anti-oxLDL antibodies originate from B-1 cells and whether, if they do, they can be as protective as IgM anti-oxLDL antibodies.

In addition, other B cell populations, such as marginal zone B cells and IgM memory B cells, have been proposed as contributors to the pool of natural antibodies (102–106). At present it is unknown to what extent, if any, protective homeostatic antibodies derive from these populations but this could be relevant to the extent that enhancement of homeostatic natural antibody producing B cell populations becomes a prophylactic or therapeutic maneuver in the future.

CONCLUSION/RHEOSTAT REDUX

To summarize, it is proposed that many chronic diseases associated with aging, including atherosclerosis, cancer, and neurodegeneration, and possibly others, take place against a background of greater or lesser homeostatic protection provided by B-1 cell-derived natural antibodies that decline with advancing age due to a decrease in the B-1 cell population and/or an alteration in the B-1 cell repertoire. There is much to be learned regarding the development and function of B-1 cells and the nature of homeostatic natural antibodies, and changes that occur in both B-1 cells and natural antibodies with advancing age. As this information is acquired, it is proposed that the onset and/or course of several chronic diseases of aging might be favorably altered by therapies that maintain or enhance the native B-1 cell population and/or that replace or add exogenous natural antibodies.

AUTHOR CONTRIBUTIONS

TR conceived and wrote the perspective based on work from his laboratory and others over many years time.

ACKNOWLEDGMENTS

The author gratefully acknowledges long-term support for B-1 cell studies from the National Institutes of Health (AI029690) and more recent support from the NIH (AI099517 and AI095787) and the Lupus Research Institute. The author thanks members of his laboratory group over the years for their help in formulating the ideas expressed herein, and thanks Drs. Nichol E. Holodick, Tam D. Quach, and Gregg J. Silverman for critical review of the manuscript.

REFERENCES

1. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* (1999) **286**:2156–9. doi:10.1126/science.286.5447.2156
2. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* (2000) **192**:271–80. doi:10.1084/jem.192.2.271
3. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med* (1998) **188**:2381–6. doi:10.1084/jem.188.12.2381
4. Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *J Exp Med* (1981) **153**:694–705. doi:10.1084/jem.153.3.694
5. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity* (2005) **23**:7–18. doi:10.1016/j.immuni.2005.04.011
6. Baccala R, Quang TV, Gilbert M, Ternynck T, Avrameas S. Two murine natural polyreactive autoantibodies are encoded by nonmutated germ-line genes. *Proc Natl Acad Sci U S A* (1989) **86**:4624–8. doi:10.1073/pnas.86.12.4624
7. Hartman AB, Mallett CP, Srinivasappa J, Prabhakar BS, Notkins AL, Smith-Gill SJ. Organ reactive autoantibodies from non-immunized adult BALB/c mice are polyreactive and express non-biased VH gene usage. *Mol Immunol* (1989) **26**:359–70. doi:10.1016/0161-5890(89)90125-9
8. Lalor PA, Morahan G. The peritoneal Ly-1 (CD5) B cell repertoire is unique among murine B cell repertoires. *Eur J Immunol* (1990) **20**:485–92. doi:10.1002/eji.1830200305
9. Klinman DM, Holmes KL. Differences in the repertoire expressed by peritoneal and splenic Ly-1 (CD5)+ B cells. *J Immunol* (1990) **144**:4520–5.
10. Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against *Streptococcus pneumoniae*. *J Exp Med* (1982) **156**:1177–85. doi:10.1084/jem.156.4.1177
11. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* (2000) **105**:1731–40. doi:10.1172/JCI8472
12. Tsimikas S, Brilakis ES, Lennon RJ, Miller ER, Witztum JL, McConnell JP, et al. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *J Lipid Res* (2007) **48**:425–33. doi:10.1194/jlr.M600361-JLR200
13. Soto Y, Conde H, Aroche R, Brito V, Luaces P, Nasiff A, et al. Autoantibodies to oxidized low density lipoprotein in relation with coronary artery disease. *Hum Antibodies* (2009) **18**:109–17. doi:10.3233/HAB-2009-0202
14. Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, et al. B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. *Circ Res* (2011) **109**:830–40. doi:10.1161/CIRCRESAHA.111.248542
15. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J Immunol* (1989) **142**:3643–51.
16. Pennell CA, Sheehan KM, Brodeur PH, Clarke SH. Organization and expression of VH gene families preferentially expressed by Ly-1+ (CD5) B cells. *Eur J Immunol* (1989) **19**:2115–21. doi:10.1002/eji.1830191122
17. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* (2009) **182**:6031–43. doi:10.4049/jimmunol.0804191
18. Rothstein TL, Griffin DO, Holodick NE, Quach TD, Kaku H. Human B-1 cells take the stage. *Ann N Y Acad Sci* (2013) **1285**:97–114. doi:10.1111/nyas.12137
19. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol* (2006) **7**:293–301. doi:10.1038/ni1301
20. Ghosn EE, Waters J, Phillips M, Yamamoto R, Long BR, Yang Y, et al. Fetal hematopoietic stem cell transplantation fails to fully regenerate the B-lymphocyte compartment. *Stem Cell Rep* (2015) **6**:137–49. doi:10.1016/j.stemcr.2015.10.011
21. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+CD27+CD43+CD70. *J Exp Med* (2011) **208**:67–80. doi:10.1084/jem.201014992084c
22. Griffin DO, Rothstein TL. Human B1 cell frequency: isolation and analysis of human B1 cells. *Front Immunol* (2012) **3**:122. doi:10.3389/fimmu.2012.00122
23. Quach TD, Rodriguez-Zhurbenko N, Hopkins TJ, Guo X, Hernandez AM, Li W, et al. Distinctions among circulating antibody-secreting cell populations, including B-1 cells, in human adult peripheral blood. *J Immunol* (2016) **196**(3):1060–9. doi:10.4049/jimmunol.1501843
24. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells are CD3-: a reply to "A human equivalent of mouse B-1 cells?" and "The nature of circulating CD27+CD43+ B cells." *J Exp Med* (2011) **208**:2566–9. doi:10.1084/jem.20111761
25. Kraljevic K, Wong S, Fulcher DA. Circulating phenotypic B-1 cells are decreased in common variable immunodeficiency and correlate with immunoglobulin M levels. *Clin Exp Immunol* (2013) **171**:278–82. doi:10.1111/cei.12008
26. Suchanek O, Sadler R, Bateman EA, Patel SY, Ferry BL. Immunophenotyping of putative human B1 B cells in healthy controls and common variable immunodeficiency (CVID) patients. *Clin Exp Immunol* (2012) **170**:333–41. doi:10.1111/j.1365-2249.2012.04656.x
27. Li W, Batliwalla F, Rothstein TL. Human B-1 cells are not preplasmablasts: analysis of microarray data and other issues. *Blood* (2013) **122**:3691–3. doi:10.1182/blood-2013-08-520031
28. Verbinnen B, Covens K, Moens L, Meyts I, Bossuyt X. Human CD20+CD43+CD27+CD5- B cells generate antibodies to capsular polysaccharides of *Streptococcus pneumoniae*. *J Allergy Clin Immunol* (2012) **130**:272–5. doi:10.1016/j.jaci.2012.04.040
29. Leggat DJ, Khaskhely NM, Iyer AS, Mosakowski J, Thompson RS, Weinandy JD, et al. Pneumococcal polysaccharide vaccination induces polysaccharide-specific B cells in adult peripheral blood expressing CD19CD20CD3CD70CD27IgMCD43CD5. *Vaccine* (2013) **31**:4632–40. doi:10.1016/j.vaccine.2013.07.030
30. Torring C, Petersen CC, Bjerg L, Kofod-Olsen E, Petersen T, Hollsberg P. The B1-cell subpopulation is diminished in patients with relapsing-remitting multiple sclerosis. *J Neuroimmunol* (2013) **262**:92–9. doi:10.1016/j.jneuroim.2013.06.002
31. Moins-Teisserenc H, Busson M, Herda A, Apete S, Peffault de Latour R, Robin M, et al. CD19+CD5+ B cells and B1-like cells following allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2013) **19**:988–91. doi:10.1016/j.bbmt.2013.03.006
32. Gu H, Forster I, Rajewsky K. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. *EMBO J* (1990) **9**:2133–40.
33. Forster I, Gu H, Rajewsky K. Germline antibody V regions as determinants of clonal persistence and malignant growth in the B cell compartment. *EMBO J* (1988) **7**:3693–703.
34. Pennell CA, Mercolino TJ, Grdina TA, Arnold LW, Haughton G, Clarke SH. Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. *Eur J Immunol* (1989) **19**:1289–95. doi:10.1002/eji.1830190721
35. Payne KJ, Crooks GM. Immune-cell lineage commitment: translation from mice to humans. *Immunity* (2007) **26**:674–7. doi:10.1016/j.immuni.2007.05.011
36. Binder CJ. Natural IgM antibodies against oxidation-specific epitopes. *J Clin Immunol* (2010) **30**(Suppl 1):S56–60. doi:10.1007/s10875-010-9396-3
37. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* (2005) **352**:1685–95. doi:10.1056/NEJMra043430
38. McLeod O, Silveira A, Fredrikson GN, Gertow K, Baldassarre D, Veglia F, et al. Plasma autoantibodies against apolipoprotein B-100 peptide 210 in subclinical atherosclerosis. *Atherosclerosis* (2014) **232**:242–8. doi:10.1016/j.atherosclerosis.2013.11.041
39. Engelbertsen D, Anand DV, Fredrikson GN, Hopkins D, Corder R, Shah PK, et al. High levels of IgM against methylglyoxal-modified apolipoprotein B100 are associated with less coronary artery calcification in patients with type 2 diabetes. *J Intern Med* (2012) **271**:82–9. doi:10.1111/j.1365-2796.2011.02411.x

40. Fredrikson GN, Hedblad B, Berglund G, Alm R, Nilsson JA, Schiopu A, et al. Association between IgM against an aldehyde-modified peptide in apolipoprotein B-100 and progression of carotid disease. *Stroke* (2007) **38**:1495–500. doi:10.1161/STROKEAHA.106.474577
41. Rodriguez-Zhurbenko N, Martinez D, Blanco R, Rondon T, Grinan T, Hernandez AM. Human antibodies reactive to NeuGcGM3 ganglioside have cytotoxic anti-tumor properties. *Eur J Immunol* (2013) **43**:826–37. doi:10.1002/eji.201242693
42. Springer SA, Diaz SL, Gagneux P. Parallel evolution of a self-signal: humans and new world monkeys independently lost the cell surface sugar Neu5Gc. *Immunogenetics* (2014) **66**:671–4. doi:10.1007/s00251-014-0795-0
43. Chou HH, Hayakawa T, Diaz S, Krings M, Indriati E, Leakey M, et al. Inactivation of CMP-N-acetylneuraminic acid hydroxylase occurred prior to brain expansion during human evolution. *Proc Natl Acad Sci U S A* (2002) **99**:11736–41. doi:10.1073/pnas.182257399
44. Rodriguez-Zhurbenko N, Rabade-Chediak M, Martinez D, Grinan T, Hernandez AM. Anti-NeuGcGM3 reactivity: a possible role of natural antibodies and B-1 cells in tumor immunosurveillance. *Ann N Y Acad Sci* (2015) **1362**:224–38. doi:10.1111/nyas.12827
45. Szabo P, Relkin N, Weksler ME. Natural human antibodies to amyloid beta peptide. *Autoimmun Rev* (2008) **7**:415–20. doi:10.1016/j.autrev.2008.03.007
46. Britschgi M, Olin CE, Johns HT, Takeda-Uchimura Y, LeMieux MC, Rufibach K, et al. Neuroprotective natural antibodies to assemblies of amyloidogenic peptides decrease with normal aging and advancing Alzheimer's disease. *Proc Natl Acad Sci U S A* (2009) **106**:12145–50. doi:10.1073/pnas.0904866106
47. Rosenmann H, Meiner Z, Geylis V, Abramsky O, Steinitz M. Detection of circulating antibodies against tau protein in its unphosphorylated and in its neurofibrillary tangles-related phosphorylated state in Alzheimer's disease and healthy subjects. *Neurosci Lett* (2006) **410**:90–3. doi:10.1016/j.neulet.2006.01.072
48. Terryberry JW, Thor G, Peter JB. Autoantibodies in neurodegenerative diseases: antigen-specific frequencies and intrathecal analysis. *Neurobiol Aging* (1998) **19**:205–16. doi:10.1016/S0197-4580(98)00049-9
49. Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E. Alzheimer's disease. *Lancet* (2011) **377**:1019–31. doi:10.1016/S0140-6736(10)61349-9
50. Tsiantoulas D, Sage AP, Mallat Z, Binder CJ. Targeting B cells in atherosclerosis: closing the gap from bench to bedside. *Arterioscler Thromb Vasc Biol* (2015) **35**:296–302. doi:10.1161/ATVBAHA.114.303569
51. Morris-Rosenfeld S, Lipinski MJ, McNamara CA. Understanding the role of B cells in atherosclerosis: potential clinical implications. *Expert Rev Clin Immunol* (2014) **10**:77–89. doi:10.1586/1744666X.2014.857602
52. Mayr M, Kiechl S, Tsimikas S, Miller E, Sheldon J, Willeit J, et al. Oxidized low-density lipoprotein autoantibodies, chronic infections, and carotid atherosclerosis in a population-based study. *J Am Coll Cardiol* (2006) **47**:2436–43. doi:10.1016/j.jacc.2006.03.024
53. Karvonen J, Paivansalo M, Kesaniemi YA, Horkko S. Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. *Circulation* (2003) **108**:2107–12. doi:10.1161/01.CIR.0000092891.55157.A7
54. Su J, Georgiades A, Wu R, Thulin T, de Faire U, Frostegard J. Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension. *Atherosclerosis* (2006) **188**:160–6. doi:10.1016/j.atherosclerosis.2005.10.017
55. Bui MN, Sack MN, Moutsatsos G, Lu DY, Katz P, McCown R, et al. Autoantibody titers to oxidized low-density lipoprotein in patients with coronary atherosclerosis. *Am Heart J* (1996) **131**:663–7. doi:10.1016/S0002-8703(96)90268-9
56. Inoue T, Uchida T, Kamishirado H, Takayanagi K, Hayashi T, Morooka S. Clinical significance of antibody against oxidized low density lipoprotein in patients with atherosclerotic coronary artery disease. *J Am Coll Cardiol* (2001) **37**:775–9. doi:10.1016/S0735-1097(00)01199-2
57. Lehtimaki T, Lehtinen S, Solakivi T, Nikkila M, Jaakkola O, Jokela H, et al. Autoantibodies against oxidized low density lipoprotein in patients with angiographically verified coronary artery disease. *Arterioscler Thromb Vasc Biol* (1999) **19**:23–7. doi:10.1161/01.ATV.19.1.23
58. Maiolino G, Pedon L, Cesari M, Frigo AC, Barisa M, Rossitto G, et al. Antibodies to malondialdehyde oxidized low-density lipoproteins predict long term cardiovascular mortality in high risk patients. *Int J Cardiol* (2013) **168**:484–9. doi:10.1016/j.ijcard.2012.09.165
59. Puurunen M, Manttari M, Manninen V, Tenkanen L, Alfthan G, Ehnholm C, et al. Antibody against oxidized low-density lipoprotein predicting myocardial infarction. *Arch Intern Med* (1994) **154**:2605–9. doi:10.1001/archinte.1994.00420220105012
60. Inoue T, Uchida T, Kamishirado H, Takayanagi K, Morooka S. Antibody against oxidized low density lipoprotein may predict progression or regression of atherosclerotic coronary artery disease. *J Am Coll Cardiol* (2001) **37**:1871–6. doi:10.1016/S0735-1097(00)01199-2
61. Boullier A, Hamon M, Walters-Laporte E, Martin-Nizart F, Mackereel R, Fruchart JC, et al. Detection of autoantibodies against oxidized low-density lipoproteins and of IgG-bound low density lipoproteins in patients with coronary artery disease. *Clin Chim Acta* (1995) **238**:1–10. doi:10.1016/0009-8981(95)06054-H
62. Uusitupa MI, Niskanen L, Luoma J, Vilja P, Mercuri M, Rauramaa R, et al. Autoantibodies against oxidized LDL do not predict atherosclerotic vascular disease in non-insulin-dependent diabetes mellitus. *Arterioscler Thromb Vasc Biol* (1996) **16**:1236–42. doi:10.1161/01.ATV.16.10.1236
63. van de Vijver LP, Steyger R, van Poppel G, Boer JM, Kruijssen DA, Seidell JC, et al. Autoantibodies against MDA-LDL in subjects with severe and minor atherosclerosis and healthy population controls. *Atherosclerosis* (1996) **122**:245–53. doi:10.1016/0021-9150(95)05759-5
64. Rossi GP, Cesari M, De Toni R, Zanchetta M, Maiolino G, Pedon L, et al. Antibodies to oxidized low-density lipoproteins and angiographically assessed coronary artery disease in white patients. *Circulation* (2003) **108**:2467–72. doi:10.1161/01.CIR.0000097122.19430.48
65. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, et al. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest* (1999) **103**:117–28. doi:10.1172/JCI4533
66. Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A* (1999) **96**:6353–8. doi:10.1073/pnas.96.11.6353
67. Engelbertsen D, Vallejo J, Quach TD, Fredrikson GN, Alm R, Hedblad B, et al. Low levels of IgM antibodies against an advanced glycation end-product-modified apolipoprotein B100 peptide predict cardiovascular events in nondiabetic subjects. *J Immunol* (2015) **195**:3020–5. doi:10.4049/jimmunol.1402869
68. Hernandez AM, Rodriguez N, Gonzalez JE, Reyes E, Rondon T, Grinan T, et al. Anti-NeuGcGM3 antibodies, actively elicited by idiotypic vaccination in nonsmall cell lung cancer patients, induce tumor cell death by an oncosis-like mechanism. *J Immunol* (2011) **186**:3735–44. doi:10.4049/jimmunol.1000609
69. Hernandez AM, Toledo D, Martinez D, Grinan T, Brito V, Macias A, et al. Characterization of the antibody response against NeuGcGM3 ganglioside elicited in non-small cell lung cancer patients immunized with an anti-idiotype antibody. *J Immunol* (2008) **181**:6625–34. doi:10.4049/jimmunol.181.9.6625
70. Vazquez AM, Hernandez AM, Macias A, Montero E, Gomez DE, Alonso DF, et al. Racotumomab: an anti-idiotype vaccine related to N-glycolyl-containing gangliosides – preclinical and clinical data. *Front Oncol* (2012) **2**:150. doi:10.3389/fonc.2012.00150
71. Vazquez AM, Rodreguez-Zhurbenko N, Lopez AM. Anti-ganglioside anti-idiotypic vaccination: more than molecular mimicry. *Front Oncol* (2012) **2**:170. doi:10.3389/fonc.2012.00170
72. Alfonso S, Valdes-Zayas A, Santiesteban ER, Flores YI, Areces F, Hernandez M, et al. A randomized, multicenter, placebo-controlled clinical trial of racotumomab-alum vaccine as switch maintenance therapy in advanced non-small cell lung cancer patients. *Clin Cancer Res* (2014) **20**:3660–71. doi:10.1158/1078-0432.CCR-13-1674
73. Weksler ME, Relkin N, Turkenich R, LaRusse S, Zhou L, Szabo P. Patients with Alzheimer disease have lower levels of serum anti-amyloid peptide antibodies than healthy elderly individuals. *Exp Gerontol* (2002) **37**:943–8. doi:10.1016/S0531-5565(02)00029-3
74. Du Y, Dodel R, Hampel H, Buerger K, Lin S, Eastwood B, et al. Reduced levels of amyloid beta-peptide antibody in Alzheimer disease. *Neurology* (2001) **57**:801–5. doi:10.1212/WNL.57.5.801

75. Jianping L, Zhibing Y, Wei Q, Zhikai C, Jie X, Jinbiao L. Low avidity and level of serum anti-Abeta antibodies in Alzheimer disease. *Alzheimer Dis Assoc Disord* (2006) **20**:127–32. doi:10.1097/00002093-200607000-00001
76. Dodel R, Balakrishnan K, Keyvani K, Deuster O, Neff F, Andrei-Selmer LC, et al. Naturally occurring autoantibodies against beta-amyloid: investigating their role in transgenic animal and in vitro models of Alzheimer's disease. *J Neurosci* (2011) **31**:5847–54. doi:10.1523/JNEUROSCI.4401-10.2011
77. Congdon EE, Gu J, Sait HB, Sigurdsson EM. Antibody uptake into neurons occurs primarily via clathrin-dependent Fcgamma receptor endocytosis and is a prerequisite for acute tau protein clearance. *J Biol Chem* (2013) **288**:35452–65. doi:10.1074/jbc.M113.491001
78. Chai X, Wu S, Murray TK, Kinley R, Cella CV, Sims H, et al. Passive immunization with anti-tau antibodies in two transgenic models: reduction of tau pathology and delay of disease progression. *J Biol Chem* (2011) **286**:34457–67. doi:10.1074/jbc.M111.229633
79. Boutajangout A, Ingadottir J, Davies P, Sigurdsson EM. Passive immunization targeting pathological phospho-tau protein in a mouse model reduces functional decline and clears tau aggregates from the brain. *J Neurochem* (2011) **118**:658–67. doi:10.1111/j.1471-4159.2011.07337.x
80. Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, et al. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat Med* (2000) **6**:916–9. doi:10.1038/78682
81. DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* (2001) **98**:8850–5. doi:10.1073/pnas.151261398
82. Siemers ER, Friedrich S, Dean RA, Gonzales CR, Farlow MR, Paul SM, et al. Safety and changes in plasma and cerebrospinal fluid amyloid beta after a single administration of an amyloid beta monoclonal antibody in subjects with Alzheimer disease. *Clin Neuropharmacol* (2010) **33**:67–73. doi:10.1097/WNF.0b013e3181cb577a
83. Doody RS, Thomas RG, Farlow M, Iwatsubo T, Vellas B, Joffe S, et al. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med* (2014) **370**:311–21. doi:10.1056/NEJMoa1312889
84. Salloway S, Sperling R, Fox NC, Blennow K, Klunk W, Raskind M, et al. Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *N Engl J Med* (2014) **370**:322–33. doi:10.1056/NEJMoa1304839
85. Anand K, Sabbagh M. Early investigational drugs targeting tau protein for the treatment of Alzheimer's disease. *Expert Opin Investig Drugs* (2015) **24**:1355–60. doi:10.1517/13543784.2015.1075002
86. Caraux A, Klein B, Paiva B, Bret C, Schmitz A, Fuhler GM, et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. *Haematologica* (2010) **95**:1016–20. doi:10.3324/haematol.2009.018689
87. Chong Y, Ikematsu H, Yamaji K, Nishimura M, Nabeshima S, Kashiwagi S, et al. CD27(+) (memory) B cell decrease and apoptosis-resistant CD27(-) (naive) B cell increase in aged humans: implications for age-related peripheral B cell developmental disturbances. *Int Immunol* (2005) **17**:383–90. doi:10.1093/intimm/dxh218
88. Colonna-Romano G, Bulati M, Aquino A, Pellicano M, Vitello S, Lio D, et al. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech Ageing Dev* (2009) **130**:681–90. doi:10.1016/j.mad.2009.08.003
89. Veneri D, Ortolani R, Franchini M, Tridente G, Pizzolo G, Vella A. Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages. *Blood Transfus* (2009) **7**:29–34. doi:10.2450/2008.0007-08
90. Holodick NE, Rothstein TL. B cells in the aging immune system: time to consider B-1 cells. *Ann N Y Acad Sci* (2015) **1362**:176–87. doi:10.1111/nyas.12825
91. Yang Y, Wang C, Yang Q, Kantor AB, Chu H, Ghosn EE, et al. Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *eLife* (2015) **4**:e09083. doi:10.7554/eLife.09083
92. Witztum JL, Lichtman AH. The influence of innate and adaptive immune responses on atherosclerosis. *Annu Rev Pathol* (2014) **9**:73–102. doi:10.1146/annurev-pathol-020712-163936
93. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* (2003) **9**:736–43. doi:10.1038/nm876
94. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* (2009) **119**:1335–49. doi:10.1172/JCI36800
95. Wang C, Turunen SP, Kummu O, Veneskoski M, Lehtimaki J, Nissinen AE, et al. Natural antibodies of newborns recognize oxidative stress-related malondialdehyde acetaldehyde adducts on apoptotic cells and atherosclerotic plaques. *Int Immunol* (2013) **25**:575–87. doi:10.1093/intimm/dxt022
96. Morris DL, Rothstein TL. Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes. *J Exp Med* (1993) **177**:857–61. doi:10.1084/jem.177.3.857
97. Hayakawa K, Asano M, Shinton SA, Gui M, Wen LJ, Dashoff J, et al. Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development. *J Exp Med* (2003) **197**:87–99. doi:10.1084/jem.20021459
98. Hardy RR. B-1 B cell development. *J Immunol* (2006) **177**:2749–54. doi:10.4049/jimmunol.177.5.2749
99. Libbey JE, Peterson LK, Tsunoda I, Fujinami RS. Monoclonal MOG-reactive autoantibody from progressive EAE has the characteristics of a natural antibody. *J Neuroimmunol* (2006) **173**:135–45. doi:10.1016/j.jneuroim.2005.12.010
100. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) **11**:34–46. doi:10.1038/nri2901
101. Herzenberg LA. Layered evolution in the immune system: a view from history. *Ann N Y Acad Sci* (2015) **1362**:1–5. doi:10.1111/nyas.12795
102. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* (2001) **14**:617–29. doi:10.1016/S1074-7613(01)00129-7
103. Bagnara D, Squillario M, Kipling D, Mora T, Walczak AM, Da Silva L, et al. A reassessment of IgM memory subsets in humans. *J Immunol* (2015) **195**:3716–24. doi:10.4049/jimmunol.1500753
104. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* (2004) **104**:3647–54. doi:10.1182/blood-2004-01-0346
105. Capolunghi F, Rosado MM, Sinibaldi M, Aranburu A, Carsetti R. Why do we need IgM memory B cells? *Immunol Lett* (2013) **152**:114–20. doi:10.1016/j.imlet.2013.04.007
106. Hardy RR, Hayakawa K. Positive and negative selection of natural autoreactive B cells. *Adv Exp Med Biol* (2012) **750**:227–38. doi:10.1007/978-1-4614-3461-0_17

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 14 March 2017
doi: 10.3389/fimmu.2017.00275

Monomeric Immunoglobulin A from Plasma Inhibits Human Th17 Responses *In Vitro* Independent of FcαRI and DC-SIGN

Chaitrali Saha[1†], Mrinmoy Das[1,2†], Veerupaxagouda Patil[1†], Emmanuel Stephen-Victor[1,2], Meenu Sharma[1], Sandra Wymann[3], Monika Jordi[3], Cédric Vonarburg[3], Srini V. Kaveri[1,2,4] and Jagadeesh Bayry[1,2,4*]*

[1] Institut National de la Santé et de la Recherche Médicale, Paris, France, [2] Sorbonne Universités, UPMC Univ Paris 06, Centre de Recherche des Cordeliers, Paris, France, [3] Research Department, CSL Behring AG, Bern, Switzerland, [4] Université Paris Descartes, Sorbonne Paris Cité, Paris, France

OPEN ACCESS

Edited by:
Ana María Hernández, Center of Molecular Inmunology, Cuba

Reviewed by:
Jocelyne Demengeot, Instituto Gulbenkian de Ciência, Portugal
Lubka T. Roumenina, INSERM UMRS 1138, France

***Correspondence:**
Srini V. Kaveri
srini.kaveri@crc.jussieu.fr;
Jagadeesh Bayry
jagadeesh.bayry@crc.jussieu.fr

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 03 November 2016
Accepted: 24 February 2017
Published: 14 March 2017

Citation:
Saha C, Das M, Patil V, Stephen-Victor E, Sharma M, Wymann S, Jordi M, Vonarburg C, Kaveri SV and Bayry J (2017) Monomeric Immunoglobulin A from Plasma Inhibits Human Th17 Responses In Vitro Independent of FcαRI and DC-SIGN. Front. Immunol. 8:275. doi: 10.3389/fimmu.2017.00275

Circulating immunoglobulins including immunoglobulin G (IgG) and IgM play a critical role in the immune homeostasis by modulating functions of immune cells. These functions are mediated in part by natural antibodies. However, despite being second most abundant antibody in the circulation, the immunoregulatory function of IgA is relatively unexplored. As Th17 cells are the key mediators of a variety of autoimmune, inflammatory, and allergic diseases, we investigated the ability of monomeric IgA (mIgA) isolated from pooled plasma of healthy donors to modulate human Th17 cells. We show that mIgA inhibits differentiation and amplification of human Th17 cells and the production of their effector cytokine IL-17A. mIgA also suppresses IFN-γ responses under these experimental conditions. Suppressive effect of mIgA on Th17 responses is associated with reciprocal expansion of FoxP3-positive regulatory T cells. The effect of mIgA on Th17 cells is dependent on $F(ab')_2$ fragments and independent of FcαRI (CD89) and DC-SIGN. Mechanistically, the modulatory effect of mIgA on Th17 cells implicates suppression of phosphorylation of signal transducer and activator of transcription 3. Furthermore, mIgA binds to $CD4^+$ T cells and recognizes in a dose-dependent manner the receptors for cytokines (IL-6Rα and IL-1RI) that mediate Th17 responses. Our findings thus reveal novel anti-inflammatory functions of IgA and suggest potential therapeutic utility of mIgA in autoimmune and inflammatory diseases that implicate Th17 cells.

Keywords: monomeric IgA, Th17, IL-17, natural antibodies, IVIG, FcαRI, CD89, DC-SIGN, Treg

INTRODUCTION

$CD4^+$ T-helper (Th) cells play an important role in the immune responses against both pathogens and self-antigens. Based on distinct cytokine and transcription factor profiles, several subsets of $CD4^+$ Th cells have been elucidated. These include Th1, Th2, and $CD4^+CD25^+$ regulatory T cells (Tregs). In addition, Th17 cells that are characterized by lineage-specific transcription factor retinoic acid-related orphan receptor C (RORC) and the secretion of signature cytokine IL-17A are also identified (1). Phosphorylation of signal transducer and activator of transcription 3 (STAT3) is

essential for the differentiation and amplification of Th17 cells. Th17 cells have been shown to play an important role in the defense against extracellular pathogens and in the pathogenesis of a variety of autoimmune, inflammatory, and allergic diseases (1, 2). Therefore, modulation of Th17 responses represents one of the fast evolving therapeutic options for the treatment of autoimmune and systemic inflammatory diseases.

Immunoglobulin A (IgA) is the most prevalent antibody at mucosal sites and the second most abundant antibody in the circulation. Secretory IgA at mucosal sites is dimeric in nature. It has been proposed that high affinity secretory IgA prevents mucosal colonization of invading pathogens and low affinity secretory IgA regulates commensal bacteria (3, 4). In the circulation, IgA is mostly monomeric although nearly 10–20% of circulating IgA are presented as dimeric or polymeric in nature (5). Currently, three bona fide receptors have been identified for IgA. FcαRI (CD89) expressed on various innate cells such as monocytes, macrophages, dendritic cells (DCs), and neutrophils is specific for IgA and mediates effector functions of IgA. The other two receptors, polymeric Ig receptor and Fcα/μR recognize both IgA and IgM and are therefore not specific for IgA. In addition to the aforementioned receptors, two alternative IgA receptors have also been identified. They are the asialoglycoprotein receptor that is implicated in the catabolism of IgA, and the transferrin receptor that mediates deposition of IgA in tissues (6).

Although triggering of FcαRI by IgA immune complexes or polymers induces activation of immune cells (7–9) *via* complete phosphorylation of tyrosine residues of immunoreceptor tyrosine-based activation motif (ITAM) within the associated FcRγ adaptors, naturally occurring monomeric IgA (mIgA) in the plasma was found to exert inhibitory effects on the activation of immune cells by triggering inhibitory ITAM (ITAMi) signaling through the associated FcRγ chain and recruitment of tyrosine phosphatase Src homology 2 domain-containing phosphatase-1 (SHP-1) (10–13). mIgA also induces death in activated neutrophils (14) and inhibits complement deposition mediated by anti-ganglioside antibodies (15). The anti-inflammatory effects of mIgA have been explored in various experimental models (10–13, 16).

Thus, so far anti-inflammatory effects of mIgA have been elucidated mainly in the context of innate immune cells and FcαRI. It is not known whether anti-inflammatory effects of mIgA observed in various experimental models are solely due to the modulation of innate cells or also due to anti-inflammatory effects on the cells of adaptive immune compartment and particularly $CD4^+$ T cells that are critical players in the pathogenesis of autoimmune and inflammatory diseases. Therefore, in view of emerging roles of Th17 cells in the pathogenesis of autoimmune, allergy, and inflammatory diseases, we explored the immunomodulatory role of mIgA isolated from the pooled plasma of healthy donors on the human Th17 cell differentiation, amplification, and secretion of effector cytokine IL-17A. Our data indicate that mIgA binds to $CD4^+$ T cells independent of FcαRI (CD89), and reciprocally regulates human Th17 and FoxP3-positive Treg cells. The effect of mIgA on Th17 cells is dependent on $F(ab')_2$ fragments and implicates suppression of phosphorylation of STAT3. Our data thus reveal FcαRI-independent immunomodulatory functions of naturally occurring mIgA and potential therapeutic utility of mIgA in autoimmune and inflammatory diseases that implicate Th17 cells.

MATERIALS AND METHODS

Cell-Culture Reagents and Antibodies

Anti-CD3 (clone UCHT1), anti-CD28 mAbs (clone 37407), and TGF-β1 were procured from R&D Systems (Lille, France). IL-1β, IL-6, and IL-21 were purchased from Immuno Tools (Friesoythe, Germany). Plasma-derived human serum albumin (HSA) was from Laboratoire Française de Biotechnologies (Les Ulis, France).

Immunoglobulins

Monomeric IgA and $F(ab')_2$ fragments of mIgA and IVIG (Privigen®) were provided by CSL Behring AG (Bern, Switzerland).

Monomeric IgA was derived from the AIEX chromatographic step of the IVIG manufacture process of CSL Behring AG. Fraction F4 was obtained after a post-wash of the Macro-Prep High Q (BioRad, Hercule, CA, USA) column with 10 mM phosphate/30 mM acetate at pH 6.5 by elution with 55 mM tartrate/5 mM acetate at pH 7.6. Fraction F4 was then brought to approximately 1 mg/ml in PBS by ultra-/diafiltration and then depleted of IgG by affinity chromatography using an IgSelect resin (GE Healthcare, Glattbrugg, Switzerland). mIgA was directly harvested from the flow through fraction of the IgSelect chromatography and brought to its final formulation *via* ultra-/diafiltration of 48.5 g/l in PBS.

$F(ab')_2$ fragments from IgA were generated by solid phase pepsin digestion using pepsin-coupled beads (Thermo Fisher Scientific, Allschwil, Switzerland). The $F(ab')_2$ fragments were recovered by centrifugation. The supernatant was sterile filtered (0.45 μm) and formulated in PBS using ultrafiltration centrifugal devices (30,000 Da MWCO; Sartorius, Tagelswangen, Switzerland). Purity and integrity were controlled by SDS-PAGE and SE chromatography.

The labeling of mIgA and IVIG was done with the Lightning-Link® Rapid DyLight® 650 kit (Innova Biosciences, Cambridge, UK) according to manufacturer's instructions.

Cell Purification

Buffy coats from the healthy donors were processed to purify peripheral blood mononuclear cells (PBMCs). Ethics committee approval for the use of such material (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012) was obtained and experiments were performed in accordance with the approved guidelines of INSERM. The $CD4^+$ T cell isolation kit-II (Miltenyi Biotec, Paris, France) was used to isolate untouched total $CD4^+$ T cells by negative selection. Subsequently, $CD45RA^+$ and $CD45RO^+$ $CD4^+$ T cells were separated by using CD45RO microbeads (Miltenyi Biotec). Furthermore, $CD25^+$ cells were depleted from the $CD45RA^+$ fraction by using CD25 microbeads (Miltenyi Biotec) to obtain $CD4^+CD25^-CD45RO^-$ naïve T cells. The purity of all subpopulations was more than 96%.

Monocytes were isolated from PBMC by using CD14 microbeads (Miltenyi Biotec) and were cultured for 5 days

with GM-CSF (1,000 IU/million cells) and IL-4 (500 IU/million cells) (both from Miltenyi Biotec) for the differentiation into DCs (17).

T-Cell Stimulation and Culture

Forty-eight well flat bottom plates were coated with 1.5 µg/ml anti-CD3 mAb for at least 5 h at 37°C. At the end of incubation, the wells were rinsed once with RPMI-1640 medium. A total of 5×10^4 $CD4^+$ T cells/well/500 µl were stimulated in serum-free X-VIVO 15 medium with soluble anti-CD28 mAb (1.0 µg/ml) in presence of cytokines including acid-treated TGF-β1 (5 ng/ml), IL-21 (25 ng/ml) for naïve T cells, and IL-1β (12.5 ng/ml) and IL-6 (25 ng/ml) for memory T cells (18). Indicated concentrations of IgA, $F(ab')_2$ fragments of IgA, IVIG, or HSA were added to the cells 12 h after the initiation of culture. The cells were cultured for 6 days at 37°C in 5% CO_2. The supernatants were collected at the end of experiments for cytokine analysis and the cells were used for intracellular staining.

Intracellular Staining for $CD4^+$ T Cells and Cytokine Assays

Cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml, Sigma-Aldrich, Saint Quentin Fallavier, France) and ionomycin (500 ng/ml, Sigma-Aldrich) at 37°C for 6 h with GolgiStop (BD Biosciences Le Pont de Claix, France) for the last 3 h. Cells were surface stained with BV421-conjugated CD4 mAb (clone RPA-T4, BD Biosciences). Cells were fixed, permeabilized (Fix/Perm, eBioscience, Paris, France), and incubated with APC-conjugated anti-human Foxp3 (clone 236A/E7, eBioscience), PE-conjugated anti-human IL-17A (clone eBio64CAP17, eBioscience), and FITC-conjugated anti-human IFN-γ (Clone 4S.B3, BD Biosciences) at 4°C. Ten thousand cells were acquired for each sample and data were analyzed by using FACS DIVA and Flowjo softwares.

Intracellular staining for the phosphorylated STAT3 (pSTAT3) was carried out on stimulated $CD4^+$ T cells at indicated time points. Cells were harvested and fixed in pre-warmed BD Cytofix buffer by incubation for 10 min at 37°C. The cells were washed twice with staining buffer (1% fetal calf serum/PBS), permeabilized with chilled BD Phosflow Perm Buffer III on ice for 30 min. At the end of incubation, the cells were washed twice with staining buffer, incubated with PE-conjugated anti-STAT3 pY705 (clone 4/P-STAT3, BD Biosciences) at room temperature for 45–60 min. Cells were washed and resuspended in staining buffer before acquisition.

Amounts of IL-17A in the cell-free supernatants were quantified by ELISA (DuoSet ELISA kit, R&D Systems).

Analysis of Expression of CD89 and DC-SIGN

$CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 24 h. Cells were surface stained with BV421-conjugated CD4 mAb and APC-conjugated CD89 (clone A59, BioLegend, London, UK) or FITC-conjugated DC-SIGN (clone DCN46, BD Biosciences) mAbs for 30 min at 4°C. Monocytes were stained with FITC-conjugated HLA-DR (clone TU36, BD Biosciences) and APC-conjugated CD89, while DCs were stained with APC-conjugated HLA-DR (clone G46-6, BD Biosciences) and FITC-conjugated DC-SIGN mAbs. The expression of markers was analyzed by flow cytometry.

IgA- and IgG (IVIG)-Binding Assay

Total $CD4^+$ T cells were stimulated with anti-CD3/CD28 mAbs for 24 hrs. To analyze the binding of IgA or IgG (IVIG) to $CD4^+$ T cells, either resting or anti-CD3, anti-CD28-stimulated T cells were incubated with BV421-conjugated anti-human CD4, FITC-conjugated CD45RA (clone HI100, BD Biosciences), and BV510-conjugated CD45RO (clone UCHL1, BD Biosciences) antibodies and DyLight 650-conjugated-IgA or IgG (IVIG) (3 µg/10^6 cells) for 30 min. Cells were analyzed by flow cytometry.

Immunoglobulin A and IgG binding to IL-1RI and IL-6Rα was measured by ELISA. ELISA plates were coated with recombinant IL-1RI (human IL-1Ra, expressed in 293E cells) and IL-6Rα (human IL-6Rα, expressed in insect cells) (1 µg/ml) (BioLegend) overnight at 4°C. After blocking with 5% BSA in PBS, 0.02% Tween 20 for 1 h at 37°C, wells were incubated with different concentrations of IgA, or IVIG for 2 h at 37°C. Following washings, the plates were incubated with horseradish peroxidase-labeled anti-human IgA or IgG for 2 h, followed by development with 3,3′,5,5′-tetramethylbenzidine. The absorbance was measured at 450 and 570 nm. The data were analyzed by subtracting the absorbance of 570 nm from those of 450 nm.

Statistical Analysis

The data were analyzed by one-way ANOVA (repeated measures with Tukey's multiple comparison test) or two-tailed Student's *t*-test using GraphPad Prism software.

RESULTS

mIgA Inhibits Differentiation of Human Th17 Cells

$CD45RA^+CD25^-$ naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence TGF-β and IL-21 for 6 days to differentiate Th17 cells. TGF-β is required to induce both FoxP3 and RORC in naïve T cells and to inhibit IFN-γ while IL-21 relives RORC from the FoxP3 by inducing STAT3 activation (2, 18). Twelve hours after the initiation of culture, mIgA was added to the cells at various concentrations (5, 15, and 25 mg/ml). We observed that mIgA significantly inhibits the differentiation of human Th17 cells (**Figures 1A,B**) as analyzed by intracellular staining for IL-17A. Significant inhibitory effect was observed even at low concentrations (5 mg/ml) of mIgA. In addition, mIgA also inhibited the production of IL-17A, the signature cytokine of Th17 cells (**Figure 1C**). The suppressive effect of mIgA was similar to that of therapeutic intravenous immunoglobulin IgG (IVIG) (**Figures 1A–C**) that is used in the therapy of various autoimmune, inflammatory, and infectious diseases (19–23) and was previously shown to inhibit Th17 responses both in experimental models and in patients with autoimmune diseases (24–29).

The effect of mIgA on the inhibition of Th17 differentiation was specific, as equimolar concentration of HSA (10 mg,

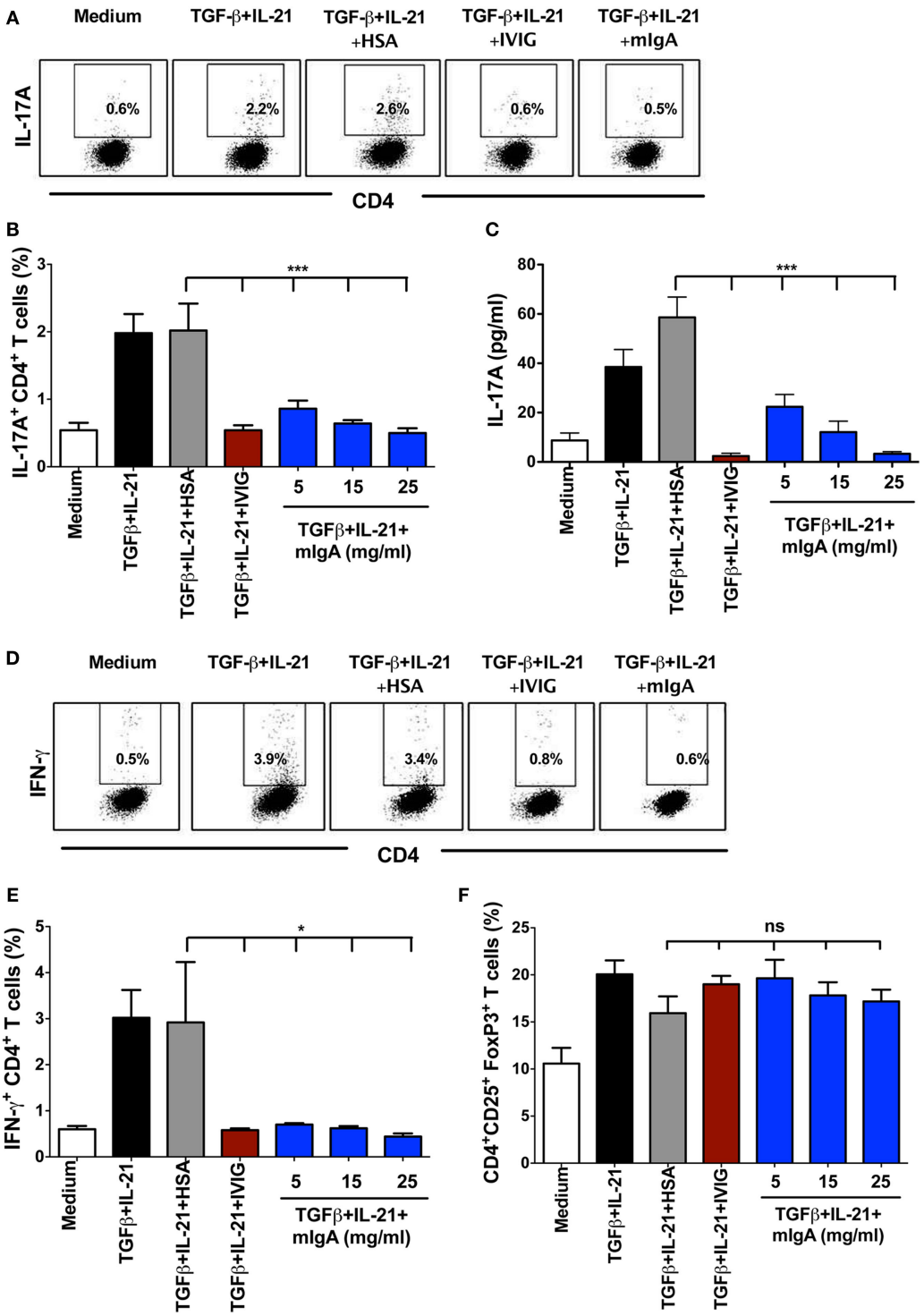


FIGURE 1 | Monomeric IgA (mIgA) inhibits differentiation of human Th17 cells and affects the generation of IFN-γ$^+$CD4$^+$ T cells under Th17 differentiation conditions without modulating FoxP3$^+$ T cells. **(A)** Flow cytometry analysis of intracellular IL-17A in the naïve CD4$^+$ T cells cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (medium) or stimulated with TGFβ and IL-21 for 6 days. mIgA (25 mg/ml), IVIG (25 mg/ml), or human serum albumin (HSA) (10 mg/ml) (0.15mM) were added to the T cell cultures after 12 h of cytokine stimulation. Data from one of five independent experiments are presented. **(B)** Percentage of IL-17A$^+$CD4$^+$ T cells (mean ± SEM, $n = 5$ donors) and **(C)** amount of secreted IL-17A (mean ± SEM, $n = 9$ donors) in T cell cultures differentiated under above conditions. mIgA was added at three different concentrations (5, 15, and 25 mg/ml). **(D)** Flow cytometry analysis of intracellular IFN-γ in the naïve CD4$^+$ T cells under Th17 differentiation conditions. Data from one of five independent experiments are presented. **(E)** Percentage of IFN-γ$^+$CD4$^+$ T cells and **(F)** CD4$^+$CD25$^+$FoxP3$^+$ T cells (mean ± SEM, $n = 5$ donors) among CD4$^+$ T cells cultured under above conditions. Statistical significance as determined by one-way ANOVA is indicated (*$P < 0.05$; ***$P < 0.001$; ns, not significant).

0.15 mM), used as protein control, did not alter Th17 differentiation (**Figures 1A–C**). Also, the inhibitory effect of mIgA on Th17 responses was not due to toxic effects of the immunoglobulins as we did not observe differences in the yield of cells in mIgA-treated conditions as compared to cytokine-treated control cells or HSA-treated cells.

We also analyzed the effect of mIgA on frequency of IFN-γ-secreting $CD4^+$ T cells in the culture. We found that mIgA was equally effective to inhibit IFN-γ responses under Th17 differentiation conditions (**Figures 1D,E**). Further, similar to our previous report on IVIG (24), inhibition of Th17 differentiation by mIgA was not associated with the reciprocal enhancement of $CD4^+CD25^{high}$ $FoxP3^+$ Tregs (**Figure 1F**). Thus, these results demonstrate that mIgA exhibits inhibitory effects on the differentiation of human Th17 cells.

mIgA Suppresses Amplification of Human Th17 Cells

For the amplification of human Th17 cells, $CD45RO^+$ memory $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of IL-1β and IL-6 for 6 days. By signaling through corresponding cytokine receptors, IL-1β and IL-6 induce phosphorylation of STAT3 and amplify Th17 cells in the memory $CD4^+$ T cell pool (2, 18). Similar to Th17 cell differentiation conditions, various concentrations of mIgA (5, 15, and 25 mg/ml) were added to the cells 12 h post initiation of culture. The pro-inflammatory cytokines IL-1β and IL-6 significantly enhanced the frequency of IL-17A-producing cells as compared to memory $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 mAbs alone (**Figures 2A,B**). Remarkably, mIgA significantly suppressed the amplification of Th17 cells (**Figures 2A,B**) and the amount of IL-17A (**Figure 2C**) produced by these cells. Its inhibitory effect was similar to that of high-dose IVIG (**Figures 2A–C**). Of note, the inhibitory effect of mIgA on IFN-γ responses under Th17 amplification conditions was observed only at the highest immunoglobulin concentration (**Figure 2D**).

Under Th17 amplification conditions, the inhibitory effect of mIgA on Th17 cells was associated with a reciprocal enhancement of $FoxP3^+$ Tregs (**Figure 2E**). The effect of mIgA on Tregs was prominent at the highest concentration (25 mg/ml). These results thus suggest that both mIgA and IVIG exert similar modulatory effects on Th17 amplification.

FcαRI (CD89) and DC-SIGN Are Dispensable for the Inhibition of Th17 Response by mIgA

Recent reports have indicated that FcαRI (CD89) plays a major role in mediating anti-inflammatory effects of mIgA on innate immune cells (10–13, 16). In addition, SIGN-R1 on DCs was also implicated in the immunoregulatory functions of secretory IgA (30). To explore if FcαRI and DC-SIGN (human counterpart of SIGN-R1) receptors are implicated in the inhibitory effect of mIgA, we investigated the expression of these two receptors on activated $CD4^+$ T cells. We found that human $CD4^+$ T cells were negative for both, FcαRI and DC-SIGN (**Figures 3A,B**), thus ruling out their implication in the inhibition of Th17 responses by mIgA. Importantly, monocytes and DCs that were used as positive controls stained with specific anti-FcαRI and anti-DC-SIGN fluorescent antibodies, respectively (**Figures 3A,B**).

Inhibition of Th17 Response by mIgA Implicates $F(ab')_2$ Fragments

Lack of expression of FcαRI and DC-SIGN on $CD4^+$ T cells raised an important prospect that suppressive effects of mIgA on Th17 cell responses might be mediated *via* $F(ab')_2$ fragments. Indeed, we found that $F(ab')_2$ fragments of mIgA significantly inhibited the frequency of IL-17A-positive T cells under Th17 differentiation conditions (**Figure 3C**). This effect was also associated with significant downregulation of IFN-γ-secreting $CD4^+$T cells in the culture (**Figure 3D**).

mIgA Binds to $CD4^+$ T Cells

Natural antibodies that recognize various self-motifs have been identified (31–34). Therefore, to further understand the mechanisms underlying the inhibitory effect of IgA on Th17 responses, we analyzed the binding of mIgA to $CD4^+$ T cells by flow cytometry. We found that nearly 45% of the stimulated $CD4^+$ T cells ($n = 9$ donors) were positive for mIgA binding (**Figures 4A,B**). mIgA however did not bind to resting $CD4^+$ T cells ($n = 3$ donors) suggesting that activation signals license T cells for immunoglobulin binding. Further, the extent of binding of mIgA was similar for $CD45RA^+$ naïve and $CD45RO^+$ memory $CD4^+$ T cells (**Figures 4C,D**). This finding indicates that naturally occurring mIgA exerts its immunomodulatory effects by binding to $CD4^+$ T cells.

mIgA and IVIG Recognize $CD4^+$ T Cells to a Similar Extent

As both mIgA and IVIG reciprocally regulated Th17 and Tregs at equivalent concentration, rises the possibility that they recognize $CD4^+$ T cells to a similar extent. Confirming our proposition, we observed that both immunoglobulin fractions bind $CD4^+$ T cells to a similar magnitude (**Figures 5A–C**). To further substantiate these results, we investigated the surface molecules on $CD4^+$ T cells that could be recognized by mIgA and IVIG. As mIgA and IVIG inhibited cytokine-mediated Th17 differentiation and amplification, we hypothesized that the corresponding cytokine receptors on $CD4^+$ T cells are the targets for these immunoglobulin fractions. Therefore, we analyzed binding of mIgA and IVIG to IL-6Rα and IL-1RI, the receptors for IL-6 and IL-1β that are implicated in the amplification of Th17 cells (1, 2, 18, 35). mIgA recognized both IL-6Rα and IL-1RI in a dose-dependent manner (**Figures 5D,E**). The binding of mIgA was however stronger for IL-6Rα. Importantly, IVIG and mIgA showed similar pattern of recognition of IL-6Rα and IL-1RI (**Figures 5D,E**).

mIgA Interferes with STAT3 Activation

STAT3 has a key role in the Th17 cell programming by relieving RORC from FoxP3-mediated inhibition. Together with RORC, it facilitates the secretion of effector cytokines of

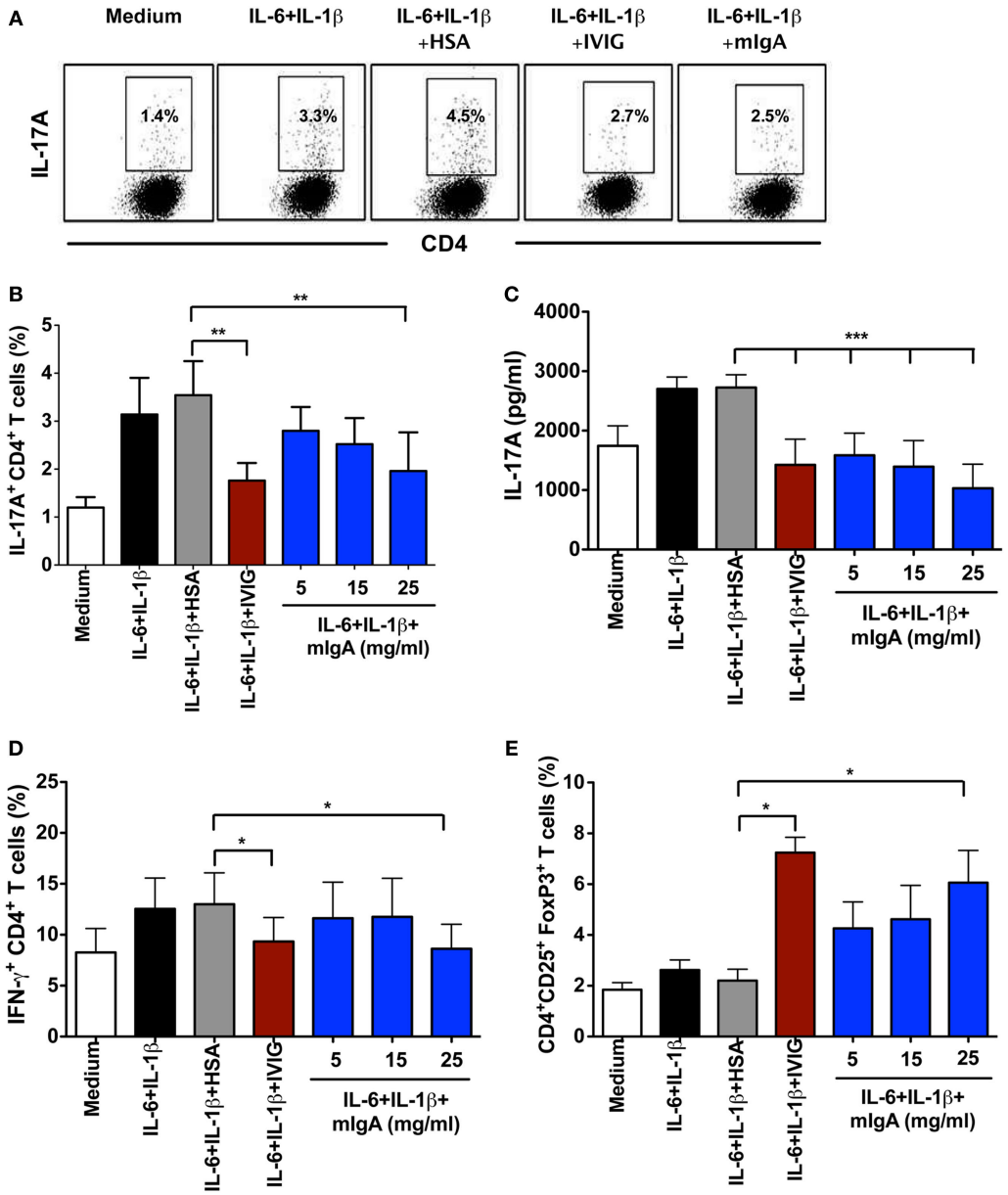


FIGURE 2 | **Monomeric IgA (mIgA) reciprocally regulates Th17 cells and FoxP3⁺ T cells under Th17 amplification conditions**. **(A)** Flow cytometry analysis of intracellular IL-17A in the memory CD4⁺ T cells cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (medium) or stimulated with IL-6 and IL-1β for 6 days. mIgA (25 mg/ml), IVIG (25 mg/ml), or human serum albumin (HSA) (10 mg/ml) (0.15mM) were added to the T cell cultures after 12 h of cytokine stimulation. Data from one of five independent experiments are presented. **(B)** Percentage of IL-17A⁺CD4⁺ T cells (mean ± SEM, $n = 5$ donors) and **(C)** amount of secreted IL-17A (mean ± SEM, $n = 9$ donors) in T cells cultured under above conditions. mIgA was added at three different concentrations (5, 15, and 25 mg/ml). **(D)** Percentage of IFN-γ⁺CD4⁺ T cells (mean ± SEM, $n = 5$ donors) and **(E)** CD4⁺CD25⁺FoxP3⁺ T cells (mean ± SEM, $n = 5$ donors) among CD4⁺ T cells cultured under above conditions. Statistical significance as determined by one-way ANOVA is indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Th17 cells. STAT3 is activated by Th17-polarizing cytokines (1, 2). As we found that mIgA recognizes cytokine receptors implicated in Th17 responses, we aimed at exploring if this binding of mIgA on CD4⁺ T cells has a repercussion on STAT3 phosphorylation and hence interferes with early signaling events of Th17 cells. In line with our proposition, we uncovered that mIgA significantly suppresses the phosphorylation of STAT3 at Y705 both in Th17 differentiation and amplification conditions (**Figures 6A–E**).

DISCUSSION

Patients with selective IgA deficiency not only exhibit enhanced predisposition to mucosal infections but also to

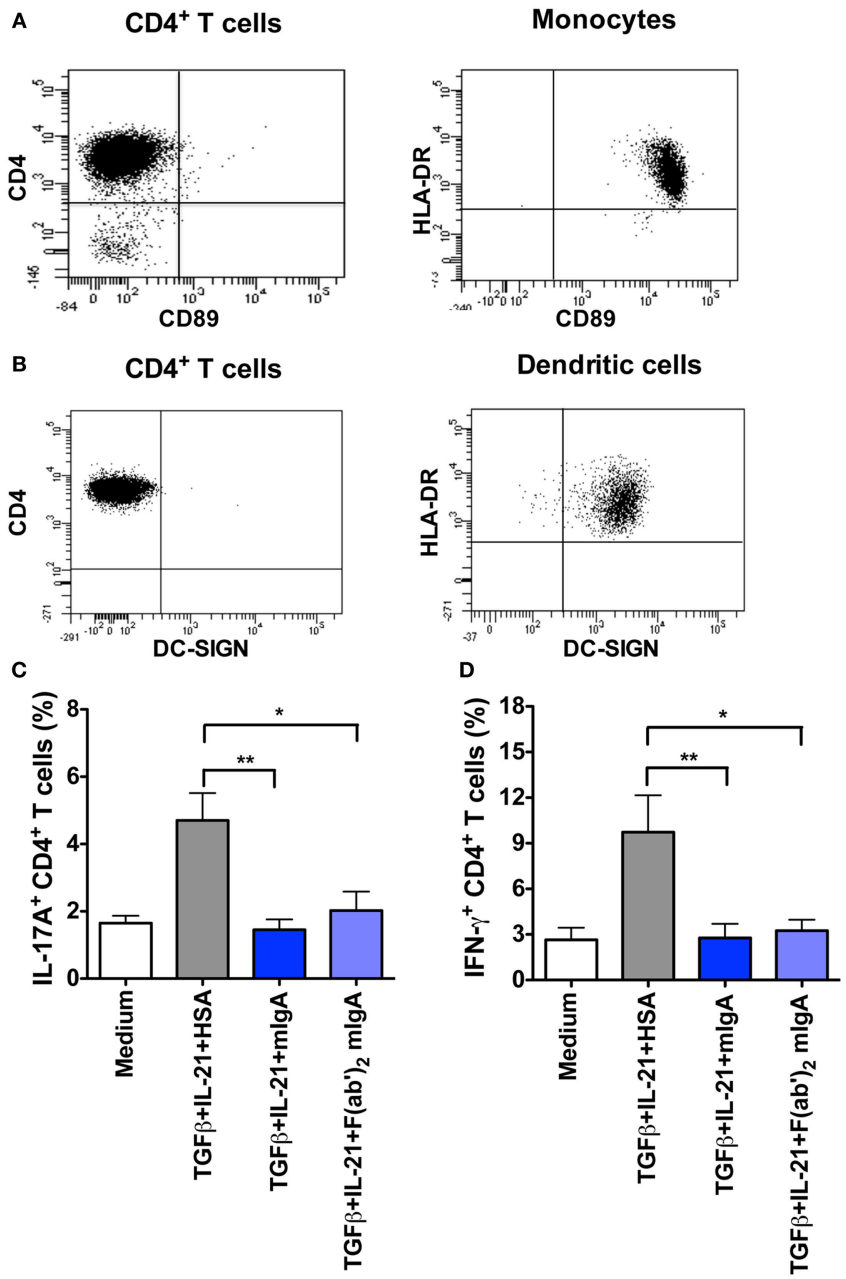


FIGURE 3 | **Inhibition of Th17 response by monomeric IgA (mIgA) implicates F(ab′)$_2$ fragments while CD89 and DC-SIGN are dispensable**. **(A,B)** Flow cytometric analysis human CD4$^+$ T cells, monocytes, and dendritic cells for the expression of CD89 and DC-SIGN. **(C,D)** Percentage of **(C)** IL-17A$^+$CD4$^+$ T cells and **(D)** IFN-γ$^+$CD4$^+$ T cells (mean ± SEM, n = 4 donors) in T cells cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (Medium) or stimulated with TGFβ and IL-21 for 6 days. mIgA (25 mg/ml), F(ab′)$_2$ fragments of mIgA (15 mg/ml), or human serum albumin (HSA) (10 mg/ml) (0.15mM) were added to the T cell cultures after 12 h of cytokine stimulation. Statistical significance as determined by one-way ANOVA is indicated (*P < 0.05; **P < 0.01).

several autoimmune and allergic conditions including arthritis, autoimmune endocrinopathies, and intestinal inflammatory diseases such as ulcerative colitis and Crohn's disease (36). These observations support the role of IgA in the immune regulation and homeostasis. The immunoregulatory functions of IgA are mediated mainly *via* FcαRI, expressed on various innate immune cells. Whether such an interaction is inflammatory or anti-inflammatory is determined by nature of the IgA. Thus, IgA immune complexes and polymeric IgA were reported to induce activation of innate immune cells while mIgA was found to be anti-inflammatory. The anti-inflammatory effects of mIgA include its inhibitory effect on the chemotaxis of immune

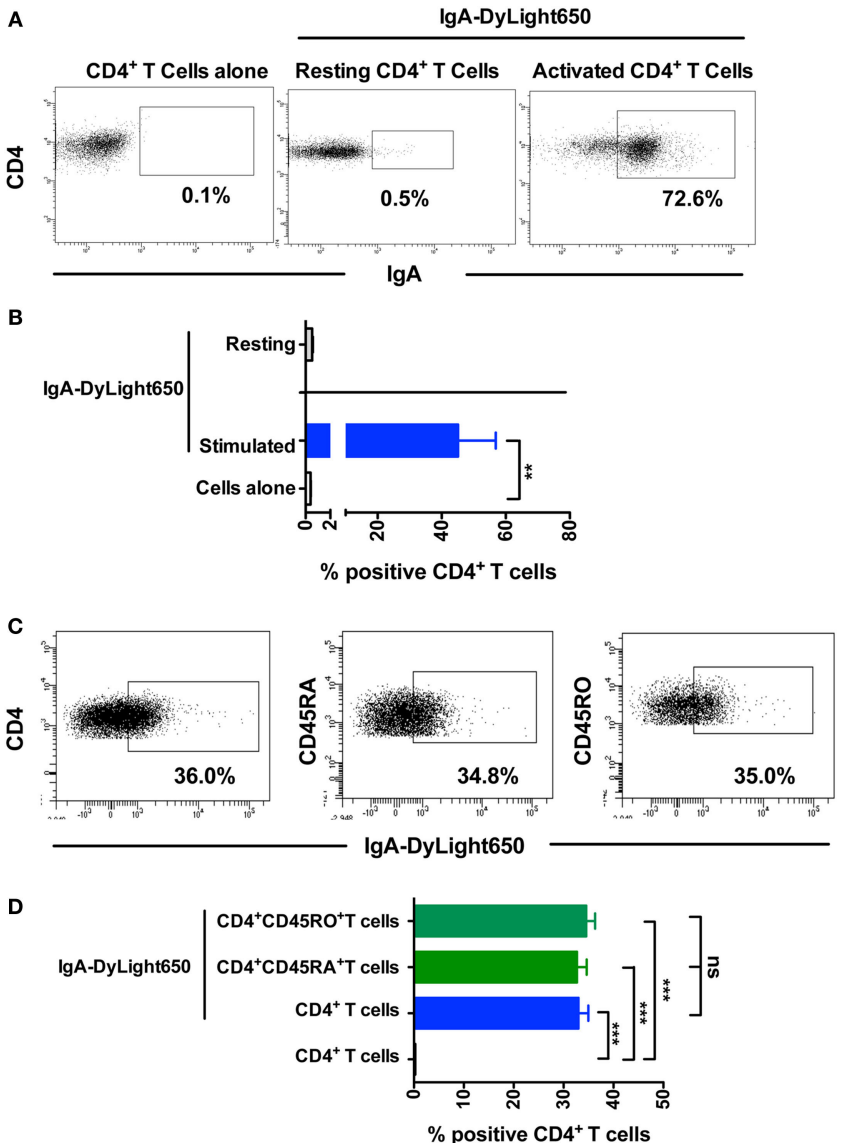


FIGURE 4 | Monomeric IgA (mIgA) binds to CD4⁺ T cells. (A,B) Representative dot plots and percentage (mean ± SEM, n = 3–9 donors) of binding of DyLight650-conjugated mIgA to CD4⁺ T cells. Statistical significance as determined by two-tailed Student's t-test is indicated (**$P < 0.01$). **(C,D)** Representative dot plots and percentage (mean ± SEM, n = 4) of binding of DyLight650-conjugated mIgA to CD4⁺ T cells, CD4⁺CD45RA⁺ naïve T cells, and CD4⁺CD45RO⁺ memory T cells. Statistical significance as determined by one-way ANOVA is indicated (**$P < 0.01$; ***$P < 0.001$; ns, not significant).

cells, IgG-mediated phagocytosis and bactericidal activity of polymorphonuclear cells, and secretion of inflammatory cytokines such as TNF and IL-6 (37–43). Furthermore, mIgA also induces the production of IL-10 by human monocytes and monocyte-derived DCs (44). Of note, recognition of secretory IgA *via* carbohydrate-recognizing receptors on innate cells such as SIGN-R1 has also been suggested (30, 45, 46). This interaction rendered DC tolerogenic characterized by the secretion of IL-10 and gaining the ability to expand Tregs. Our current results show that mIgA exerts direct anti-inflammatory functions on effector T cells, independent of FcαRI, DC-SIGN, and innate cells. These data thus further expand the landscape of immunoregulatory functions of IgA and of natural immunoglobulins.

Aberrant activation of Th17 cells and their effector cytokines IL-17A and GM-CSF are implicated in the pathogenesis of various autoimmune and inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, psoriasis, dermatomyositis, allergy, asthma, and others (2). Importantly, targeting Th17 responses have given promising results in experimental models of autoimmune diseases and in patients (47). Recent data from ours and others show that beneficial effects of therapeutic IVIG containing IgG from pooled plasma of thousands of healthy

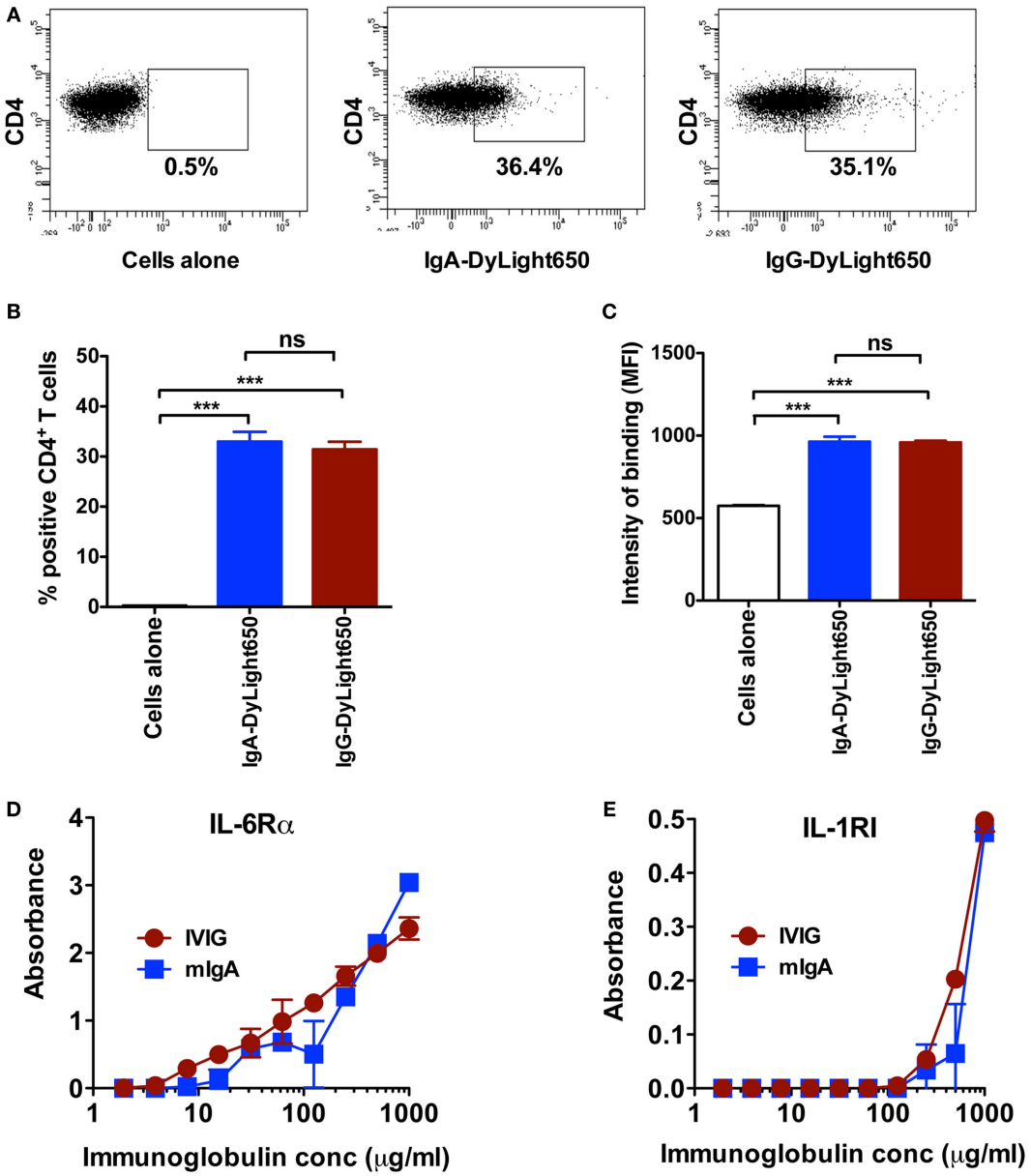


FIGURE 5 | Monomeric IgA (mIgA) and IVIG recognize CD4⁺ T cells and cytokine receptors to a similar extent. (A–C) Representative dot plots, percentage (mean ± SEM, *n* = 4), and intensity (mean ± SEM, *n* = 4) of binding [as shown by median fluorescence intensity (MFI)] of DyLight650-conjugated mIgA and IVIG to CD4⁺ T cells. **(D,E)** Binding of mIgA and IVIG to recombinant IL-6Rα and IL-1RI as analyzed by ELISA. Immunoglobulins were tested at serial concentrations (1–0.002 mg/ml). Statistical significance as determined by one-way ANOVA is indicated (***$P < 0.001$; ns, not significant).

donors is associated with inhibition of Th17 responses (24–29, 48–50), indicating that immunoglobulins have regulatory functions on Th17 cells. The data from current report show that in addition to IgG, mIgA also exerts modulatory effects on Th17 responses. In fact, mIgA was recently demonstrated to attenuate experimental arthritis in human CD89 transgenic mice (11), a disease where Th17 cells have a key role in the pathogenesis. It should be noted that the serum levels of IgA ranges from 2 to 3 mg/ml, but we observed consistent inhibitory effect of mIgA both on differentiation and amplification of Th17 cells at higher doses (25 mg) and was analogous to what is observed with IVIG (24). The lower concentration of mIgA (5 mg) although shown inhibitory effects on Th17 differentiation, significant effects were not observed on all parameters of Th17 amplification. Immunoglobulins exert their anti-inflammatory effects *via* several mutually non-excusive mechanisms and it might explain requisite of higher concentrations of immunoglobulins for the therapeutic purposes to inhibit inflammation.

Recent reports show that Th17 cells are required for the production of high affinity secretory IgA at intestinal mucosal

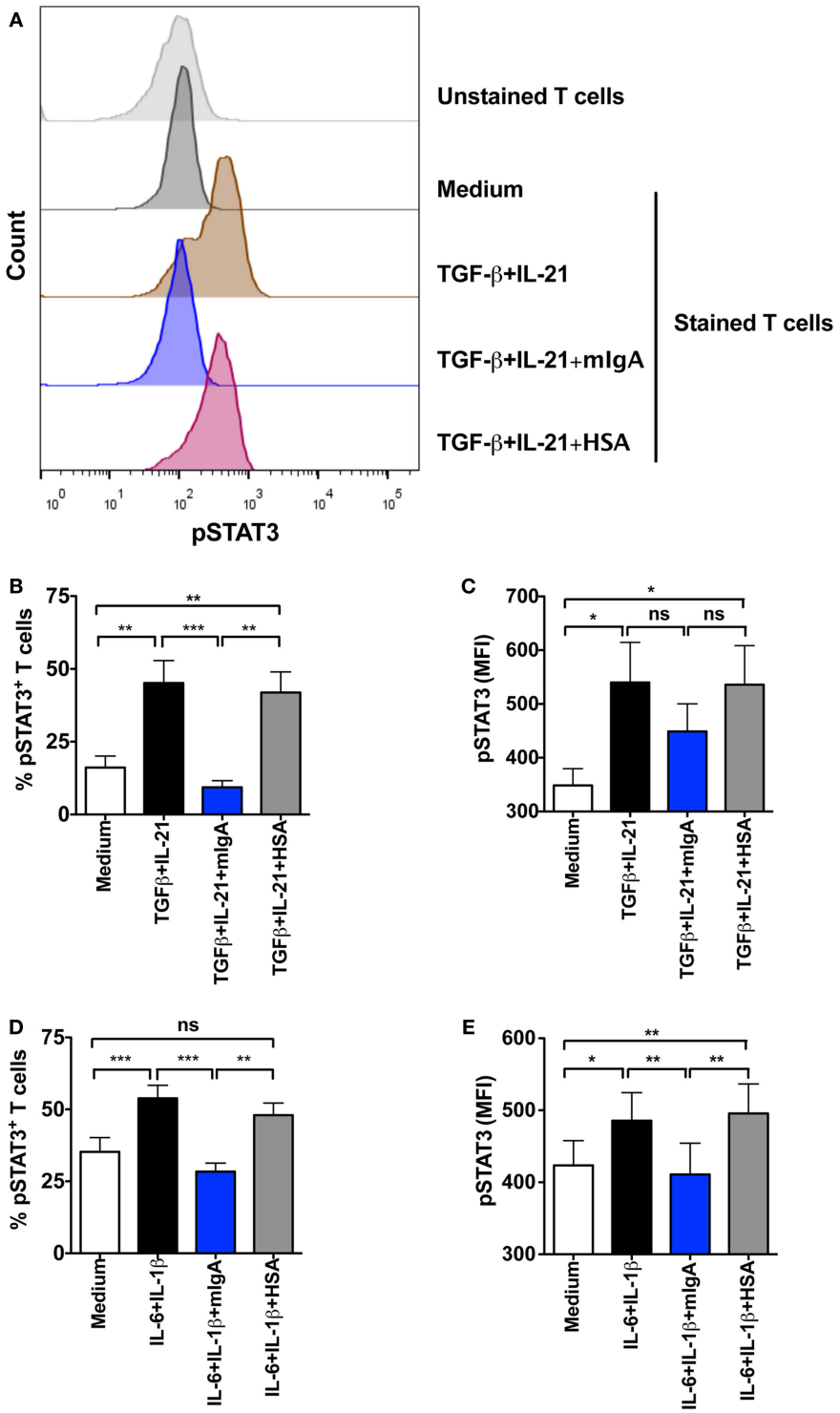


FIGURE 6 | **Monomeric IgA (mIgA) interferes with signal transducer and activator of transcription 3 (STAT3) activation**. CD4+ T cells were cultured in serum-free X-vivo medium in the presence of anti-CD3 and anti-CD28 mAbs alone (medium) or stimulated with cytokines for the differentiation of Th17 cells [TGFβ and IL-21, panels **(A–C)**] or for the amplification of Th17 cells [IL-1β and IL-6, panels **(D,E)**]. mIgA (25 mg/ml) or human serum albumin (HSA) (10 mg/ml) (0.15 mM) were added to the T cell cultures. Phosphorylation of STAT3 was analyzed after 72 h. **(A)** Representative histograms showing pSTAT3 in different experimental conditions for differentiating Th17 cells. **(B–E)** Percentage of pSTAT3+CD4+ T cells **(B,D)** and median fluorescence intensity (MFI) of pSTAT3 **(C,E)** under Th17 differentiation **(B,C)** (mean ± SEM, $n = 5$ donors) and amplification **(D,E)** (mean ± SEM, $n = 5$ donors) conditions. Statistical significance as determined by one-way ANOVA is indicated (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant).

surfaces (51, 52). Payer patch-homing Th17 cells induce IgA-producing germinal center B cells by acquiring the phenotype of follicular T cells and producing IL-21. A similar mechanism was also reported for the promotion of local IgA responses in lungs by vaccine-induced Th17 cells (53). Thus, Th17 cells and secretory IgA work in cooperation to protect mucosal surfaces against microbial invasion and regulating the microbiota. Although Th17 cells were reported to provide help for B cells to produce systemic IgG response *via* IL-21 and IL-17 (54), their role for circulating IgA response is not known. As we uncovered that mIgA inhibits Th17 responses, these data together suggest that under the conditions where Th17 cells are hyperactivated, IgA has the ability to control its own helpers to keep the immune response at check.

Natural antibodies (immunoglobulins) that are produced in the absence of deliberate immunization and independent of external antigens constitute an integral part of immunoglobulin repertoire (55–64). A major fraction of these natural antibodies recognize self-motifs and are termed as natural autoantibodies. These natural autoantibodies have important role in the therapeutic benefits of IVIG in autoimmune and inflammatory conditions (23, 31, 65). Our results indicate that inhibitory effect of mIgA on Th17 cells is mediated in part *via* natural IgA autoantibodies that recognize cytokine receptors on $CD4^+$ T cells and interfere with Th17 programming. In fact, we found that both mIgA and IVIG recognize $CD4^+$ T cells as well as IL-6Rα and IL-1RI to a similar extent. Also, significant downregulation of STAT3 phosphorylation by mIgA support our proposition.

Although we found that mIgA directly inhibits Th17 responses independent of FcαRI and DC-SIGN, we believe that the effect of mIgA on Th17 cells *in vivo* also implicates innate cells such as DCs, monocytes, and macrophages, which are known to provide signals for Th17 responses (2). In fact, several reports have now shown that mIgA exerts FcαRI-mediated anti-inflammatory effects on innate cells by prompting ITAMi configuration (10–13, 16). It should be noted that IVIG (IgG) and IgA display distinct differences in their glycosylation pattern. IgG is glycosylated at Asn297 of the Fc-fragment and about 15–25% of IgG are glycosylated at the Fab region (66). IgA on the other hand is the most glycosylated form of immunoglobulin and over 6% of IgA content are represented by sugars (67). In addition to *N*-linked glycans at asparagine 263 and asparagine 459 of Fc region, up to five *O*-linked glycan chains containing of *N*-acetylgalactosamine with β1,3-linked galactose and sialic acids can be found at the hinge region serine and threonine residues of IgA1, the predominant IgA subclass in the circulation (68, 69). Similar to IgG, about 30% of Fab fragments of IgA1 also contain *N*-linked glycans (69). Further, IgA1 and IgG display significant differences in the sialylation content of *N*-glycans. In contrast to IgG that contains sialic acid in less than 10% *N*-glycans, nearly 90% of the *N*-glycans in IgA1 are sialylated mainly with α2,6-configuration (69). Whether differences in the glycosylation patterns of IgA and IgG impact modulation of innate cells and innate cell-mediated Th17 responses remains to be investigated. Due to heterogeneous composition of glycan chains, further work is also necessary with mIgA preparations containing defined glycosylation patterns to finely dissect the role of IgA in modulating Th17 responses. Since $F(ab')_2$ fragments of mIgA could inhibit Th17 responses similar to intact mIgA and that $F(ab')_2$ fragments of IVIG were previously reported to inhibit Th17 responses both *in vitro* and *in vivo* (24, 70) imply that *N*-linked glycans (and hence sialylation) at Fc region of mIgA might not have a role in regulating Th17 responses.

In conclusion, our data highlight the promise of plasma-derived mIgA as therapeutic molecule for autoimmune and inflammatory diseases and hence represents an innovative plasma-derived therapeutic product (71). Although shorter half-life of IgA as compared to IgG is the major drawback, the efficacy of mIgA on Th17 cells should instigate the therapeutic development of monomeric plasma IgA as an analog to IVIG.

AUTHOR CONTRIBUTIONS

SK and JB designed the research. CS, MD, VP, ES-V, and MS performed the research. SW, MJ, and CV provided research tools. CS, MD, VP, SW, MJ, CV, SK, and JB contributed to data analyses and data interpretation. JB wrote the manuscript. CS, MD, VP, ES-V, MS, SW, MJ, CV, SK, and JB revised the manuscript critically for important intellectual content and approved the final version.

ACKNOWLEDGMENTS

The authors thank Dr. M.S. Maddur and Ms. A. Karnam for the support.

FUNDING

The study was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie, Université Paris Descartes, Paris, France; and CSL Behring, Bern, Switzerland.

REFERENCES

1. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol* (2009) 27:485–517. doi:10.1146/annurev.immunol.021908.132710
2. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *Am J Pathol* (2012) 181:8–18. doi:10.1016/j.ajpath.2012.03.044
3. Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. The immune geography of IgA induction and function. *Mucosal Immunol* (2008) 1:11–22. doi:10.1038/mi.2007.6
4. Slack E, Balmer ML, Fritz JH, Hapfelmeier S. Functional flexibility of intestinal IgA – broadening the fine line. *Front Immunol* (2012) 3:100. doi:10.3389/fimmu.2012.00100
5. Oortwijn BD, Roos A, van der Boog PJ, Klar-Mohamad N, van Remoortere A, Deelder AM, et al. Monomeric and polymeric IgA show a similar association with the myeloid FcalphaRI/CD89. *Mol Immunol* (2007) 44:966–73. doi:10.1016/j.molimm.2006.03.014
6. Monteiro R, Van De Winkel JG. IgA Fc receptors. *Annu Rev Immunol* (2003) 21:177–204. doi:10.1146/annurev.immunol.21.120601.141011
7. Pasquier B, Lepelletier Y, Baude C, Hermine O, Monteiro RC. Differential expression and function of IgA receptors (CD89 and CD71) during

maturation of dendritic cells. *J Leukoc Biol* (2004) 76:1134–41. doi:10.1189/jlb.0204101
8. Geissmann F, Launay P, Pasquier B, Lepelletier Y, Leborgne M, Lehuen A, et al. A subset of human dendritic cells expresses IgA Fc receptor (CD89), which mediates internalization and activation upon cross-linking by IgA complexes. *J Immunol* (2001) 166:346–52. doi:10.4049/jimmunol.166.1.346
9. Kanamaru Y, Arcos-Fajardo M, Moura IC, Tsuge T, Cohen H, Essig M, et al. Fc alpha receptor I activation induces leukocyte recruitment and promotes aggravation of glomerulonephritis through the FcR gamma adaptor. *Eur J Immunol* (2007) 37:1116–28. doi:10.1002/eji.200636826
10. Pasquier B, Launay P, Kanamaru Y, Moura IC, Pfirsch S, Ruffie C, et al. Identification of FcalphaRI as an inhibitory receptor that controls inflammation: dual role of FcRgamma ITAM. *Immunity* (2005) 22:31–42. doi:10.1016/j.immuni.2004.11.017
11. Rossato E, Ben Mkaddem S, Kanamaru Y, Hurtado-Nedelec M, Hayem G, Descatoire V, et al. Reversal of arthritis by human monomeric IgA through the receptor-mediated SH2 domain-containing phosphatase 1 inhibitory pathway. *Arthritis Rheumatol* (2015) 67:1766–77. doi:10.1002/art.39142
12. Kanamaru Y, Pfirsch S, Aloulou M, Vrtovsnik F, Essig M, Loirat C, et al. Inhibitory ITAM signaling by Fc alpha RI-FcR gamma chain controls multiple activating responses and prevents renal inflammation. *J Immunol* (2008) 180:2669–78. doi:10.4049/jimmunol.180.4.2669
13. Watanabe T, Kanamaru Y, Liu C, Suzuki Y, Tada N, Okumura K, et al. Negative regulation of inflammatory responses by immunoglobulin A receptor (FcalphaRI) inhibits the development of toll-like receptor-9 signalling-accelerated glomerulonephritis. *Clin Exp Immunol* (2011) 166:235–50. doi:10.1111/j.1365-2249.2011.04452.x
14. Wehrli M, Cortinas-Elizondo F, Hlushchuk R, Daudel F, Villiger PM, Miescher S, et al. Human IgA Fc receptor FcalphaRI (CD89) triggers different forms of neutrophil death depending on the inflammatory microenvironment. *J Immunol* (2014) 193:5649–59. doi:10.4049/jimmunol.1400028
15. Sudo M, Miyaji K, Spath PJ, Morita-Matsumoto K, Yamaguchi Y, Yuki N. Polyclonal IgM and IgA block in vitro complement deposition mediated by anti-ganglioside antibodies in autoimmune neuropathies. *Int Immunopharmacol* (2016) 40:11–5. doi:10.1016/j.intimp.2016.08.019
16. Ben Mkaddem S, Rossato E, Heming N, Monteiro RC. Anti-inflammatory role of the IgA Fc receptor (CD89): from autoimmunity to therapeutic perspectives. *Autoimmun Rev* (2013) 12:666–9. doi:10.1016/j.autrev.2012.10.011
17. Maddur MS, Sharma M, Hegde P, Stephen-Victor E, Pulendran B, Kaveri SV, et al. Human B cells induce dendritic cell maturation and favour Th2 polarization by inducing OX-40 ligand. *Nat Commun* (2014) 5:4092. doi:10.1038/ncomms5092
18. Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, et al. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. *Nature* (2008) 454:350–2. doi:10.1038/nature07021
19. Gilardin L, Bayry J, Kaveri SV. Intravenous immunoglobulin as clinical immune-modulating therapy. *CMAJ* (2015) 187:257–64. doi:10.1503/cmaj.130375
20. Arnson Y, Shoenfeld Y, Amital H. Intravenous immunoglobulin therapy for autoimmune diseases. *Autoimmunity* (2009) 42:553–60. doi:10.1080/08916930902785363
21. Gelfand EW. Intravenous immune globulin in autoimmune and inflammatory diseases. *N Engl J Med* (2012) 367:2015–25. doi:10.1056/NEJMra1009433
22. Shopsin B, Kaveri SV, Bayry J. Tackling difficult *Staphylococcus aureus* infections: antibodies show the way. *Cell Host Microbe* (2016) 20:555–7. doi:10.1016/j.chom.2016.10.018
23. Seite JF, Shoenfeld Y, Youinou P, Hillion S. What is the contents of the magic draft IVIg? *Autoimmun Rev* (2008) 7:435–9. doi:10.1016/j.autrev.2008.04.012
24. Maddur MS, Vani J, Hegde P, Lacroix-Desmazes S, Kaveri SV, Bayry J. Inhibition of differentiation, amplification, and function of human TH17 cells by intravenous immunoglobulin. *J Allergy Clin Immunol* (2011) 127:823–30. doi:10.1016/j.jaci.2010.12.1102
25. Maddur MS, Kaveri SV, Bayry J. Comparison of different IVIg preparations on IL-17 production by human Th17 cells. *Autoimmun Rev* (2011) 10:809–10. doi:10.1016/j.autrev.2011.02.007
26. Othy S, Hegde P, Topçu S, Sharma M, Maddur MS, Lacroix-Desmazes S, et al. Intravenous gammaglobulin inhibits encephalitogenic potential of pathogenic T cells and interferes with their trafficking to the central nervous system, implicating sphingosine-1 phosphate receptor 1-mammalian target of rapamycin axis. *J Immunol* (2013) 190:4535–41. doi:10.4049/jimmunol.1201965
27. Lee SY, Jung YO, Ryu JG, Kang CM, Kim EK, Son HJ, et al. Intravenous immunoglobulin attenuates experimental autoimmune arthritis by inducing reciprocal regulation of Th17 and Treg cells in an interleukin-10-dependent manner. *Arthritis Rheumatol* (2014) 66:1768–78. doi:10.1002/art.38627
28. Maddur MS, Rabin M, Hegde P, Bolgert F, Guy M, Vallat JM, et al. Intravenous immunoglobulin exerts reciprocal regulation of Th1/Th17 cells and regulatory T cells in Guillain-Barre syndrome patients. *Immunol Res* (2014) 60:320–9. doi:10.1007/s12026-014-8580-6
29. Guo MM, Tseng WN, Ko CH, Pan HM, Hsieh KS, Kuo HC. Th17- and Treg-related cytokine and mRNA expression are associated with acute and resolving Kawasaki disease. *Allergy* (2015) 70:310–8. doi:10.1111/all.12558
30. Diana J, Moura IC, Vaugier C, Gestin A, Tissandie E, Beaudoin L, et al. Secretory IgA induces tolerogenic dendritic cells through SIGNR1 dampening autoimmunity in mice. *J Immunol* (2013) 191:2335–43. doi:10.4049/jimmunol.1300864
31. Schwartz-Albiez R, Monteiro R, Rodriguez M, Binder C, Shoenfeld Y. Natural antibodies, intravenous immunoglobulin and their role in autoimmunity, cancer and inflammation. *Clin Exp Immunol* (2009) 158:43–50. doi:10.1111/j.1365-2249.2009.04026.x
32. Le Pottier L, Bendaoud B, Dueymes M, Daridon C, Youinou P, Shoenfeld Y, et al. BAFF, a new target for intravenous immunoglobulin in autoimmunity and cancer. *J Clin Immunol* (2007) 27:257–65. doi:10.1007/s10875-007-9082-2
33. von Gunten S, Simon HU. Natural anti-Siglec autoantibodies mediate potential immunoregulatory mechanisms: implications for the clinical use of intravenous immunoglobulins (IVIg). *Autoimmun Rev* (2008) 7:453–6. doi:10.1016/j.autrev.2008.03.015
34. Bouhlal H, Martinvalet D, Teillaud JL, Fridman C, Kazatchkine MD, Bayry J, et al. Natural autoantibodies to Fcgamma receptors in intravenous immunoglobulins. *J Clin Immunol* (2014) 34(Suppl 1):S4–11. doi:10.1007/s10875-014-0019-2
35. Stephen-Victor E, Sharma VK, Das M, Karnam A, Saha C, Lecerf M, et al. IL-1β, but not programed death-1 and programed death ligand pathway, is critical for the human Th17 response to *Mycobacterium tuberculosis*. *Front Immunol* (2016) 7:465. doi:10.3389/fimmu.2016.00465
36. Monteiro RC. The role of IgA and IgA Fc receptors as anti-inflammatory agents. *J Clin Immunol* (2010) 30(Suppl 1):S61–4. doi:10.1007/s10875-010-9397-2
37. Van Epps DE, Williams RC Jr. Suppression of leukocyte chemotaxis by human IgA myeloma components. *J Exp Med* (1976) 144:1227–42. doi:10.1084/jem.144.5.1227
38. Van Epps DE, Reed K, Williams RC Jr. Suppression of human PMN bactericidal activity by human IgA paraproteins. *Cell Immunol* (1978) 36:363–76. doi:10.1016/0008-8749(78)90280-0
39. Wilton JM. Suppression by IgA of IgG-mediated phagocytosis by human polymorphonuclear leucocytes. *Clin Exp Immunol* (1978) 34:423–8.
40. Wolf HM, Fischer MB, Puhringer H, Samstag A, Vogel E, Eibl MM. Human serum IgA downregulates the release of inflammatory cytokines (tumor necrosis factor-alpha, interleukin-6) in human monocytes. *Blood* (1994) 83:1278–88.
41. Nikolova EB, Russell MW. Dual function of human IgA antibodies: inhibition of phagocytosis in circulating neutrophils and enhancement of responses in IL-8-stimulated cells. *J Leukoc Biol* (1995) 57:875–82.
42. Wolf HM, Hauber I, Gulle H, Samstag A, Fischer MB, Ahmad RU, et al. Anti-inflammatory properties of human serum IgA: induction of IL-1 receptor antagonist and Fc alpha R (CD89)-mediated down-regulation of tumour necrosis factor-alpha (TNF-alpha) and IL-6 in human monocytes. *Clin Exp Immunol* (1996) 105:537–43. doi:10.1046/j.1365-2249.1996.d01-793.x
43. Olas K, Butterweck H, Teschner W, Schwarz HP, Reipert B. Immunomodulatory properties of human serum immunoglobulin A: anti-inflammatory and pro-inflammatory activities in human monocytes and peripheral blood mononuclear cells. *Clin Exp Immunol* (2005) 40:478–90. doi:10.1111/j.1365-2249.2005.02779.x

44. Pilette C, Detry B, Guisset A, Gabriels J, Sibille Y. Induction of interleukin-10 expression through Fcalpha receptor in human monocytes and monocyte-derived dendritic cells: role of p38 MAPKinase. *Immunol Cell Biol* (2010) 88:486–93. doi:10.1038/icb.2009.120
45. Heystek HC, Moulon C, Woltman AM, Garonne P, van Kooten C. Human immature dendritic cells efficiently bind and take up secretory IgA without the induction of maturation. *J Immunol* (2002) 168:102–7. doi:10.4049/jimmunol.168.1.102
46. Baumann J, Park CG, Mantis NJ. Recognition of secretory IgA by DC-SIGN: implications for immune surveillance in the intestine. *Immunol Lett* (2010) 131:59–66. doi:10.1016/j.imlet.2010.03.005
47. Yang J, Sundrud MS, Skepner J, Yamagata T. Targeting Th17 cells in autoimmune diseases. *Trends Pharmacol Sci* (2014) 35:493–500. doi:10.1016/j.tips.2014.07.006
48. Kim DJ, Lee SK, Kim JY, Na BJ, Hur SE, Lee M, et al. Intravenous immunoglobulin G modulates peripheral blood Th17 and Foxp3(+) regulatory T cells in pregnant women with recurrent pregnancy loss. *Am J Reprod Immunol* (2014) 71:441–50. doi:10.1111/aji.12208
49. Li S, Jin T, Zhang HL, Yu H, Meng F, Concha Quezada H, et al. Circulating Th17, Th22, and Th1 cells are elevated in the Guillain-Barre syndrome and downregulated by IVIg treatments. *Mediators Inflamm* (2014) 2014:740947. doi:10.1155/2014/740947
50. Figueiredo CA, Drohomyrecky PC, McCarthy SD, Leontyev D, Ma XZ, Branch DR, et al. Optimal attenuation of experimental autoimmune encephalomyelitis by intravenous immunoglobulin requires an intact interleukin-11 receptor. *PLoS One* (2014) 9:e101947. doi:10.1371/journal.pone.0101947
51. Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, et al. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. *Nat Immunol* (2013) 14:372–9. doi:10.1038/ni.2552
52. Cao AT, Yao S, Gong B, Nurieva RI, Elson CO, Cong Y. Interleukin (IL)-21 promotes intestinal IgA response to microbiota. *Mucosal Immunol* (2015) 8:1072–82. doi:10.1038/mi.2014.134
53. Christensen D, Mortensen R, Rosenkrands I, Dietrich J, Andersen P. Vaccine-induced Th17 cells are established as resident memory cells in the lung and promote local IgA responses. *Mucosal Immunol* (2017) 10:260–70. doi:10.1038/mi.2016.28
54. Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK, et al. Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proc Natl Acad Sci U S A* (2010) 107:14292–7. doi:10.1073/pnas.1009234107
55. Schwartz-Albiez R, Laban S, Eichmuller S, Kirschfink M. Cytotoxic natural antibodies against human tumours: an option for anti-cancer immunotherapy? *Autoimmun Rev* (2008) 7:491–5. doi:10.1016/j.autrev.2008.03.012
56. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* (2010) 10:778–86. doi:10.1038/nri2849
57. Kaveri SV, Silverman GJ, Bayry J. Natural IgM in immune equilibrium and harnessing their therapeutic potential. *J Immunol* (2012) 188:939–45. doi:10.4049/jimmunol.1102107
58. Grönwall C, Vas J, Silverman G. Protective roles of natural IgM antibodies. *Front Immunol* (2012) 3:66. doi:10.3389/fimmu.2012.00066
59. Tsiantoulas D, Gruber S, Binder C. B-1 cell immunoglobulin directed against oxidation-specific epitopes. *Front Immunol* (2013) 3:415. doi:10.3389/fimmu.2012.00415
60. Kohler H, Bayry J, Kaveri SV. The homophilic domain – an immunological archetype. *Front Immunol* (2016) 7:106. doi:10.3389/fimmu.2016.00106
61. Rothstein T. Natural antibodies as rheostats for susceptibility to chronic diseases in the aged. *Front Immunol* (2016) 7:127. doi:10.3389/fimmu.2016.00127
62. Baumgarth N. B-1 cell heterogeneity and the regulation of natural and antigen-induced IgM production. *Front Immunol* (2016) 7:324. doi:10.3389/fimmu.2016.00324
63. Lobo PI. Role of natural autoantibodies and natural IgM anti-leucocyte autoantibodies in health and disease. *Front Immunol* (2016) 7:198. doi:10.3389/fimmu.2016.00198
64. Gunti S, Messer RJ, Xu C, Yan M, Coleman WG Jr, Peterson KE, et al. Stimulation of toll-like receptors profoundly influences the titer of polyreactive antibodies in the circulation. *Sci Rep* (2015) 5:15066. doi:10.1038/srep15066
65. Kaveri SV. Intravenous immunoglobulin: exploiting the potential of natural antibodies. *Autoimmun Rev* (2012) 11:792–4. doi:10.1016/j.autrev.2012.02.006
66. Holland M, Yagi H, Takahashi N, Kato K, Savage CO, Goodall DM, et al. Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochim Biophys Acta* (2006) 1760:669–77. doi:10.1016/j.bbagen.2005.11.021
67. Monteiro RC. Role of IgA and IgA fc receptors in inflammation. *J Clin Immunol* (2010) 30:1–9. doi:10.1007/s10875-009-9338-0
68. Mestecky J, Tomana M, Moldoveanu Z, Julian BA, Suzuki H, Matousovic K, et al. Role of aberrant glycosylation of IgA1 molecules in the pathogenesis of IgA nephropathy. *Kidney Blood Press Res* (2008) 31:29–37. doi:10.1159/000112922
69. Mattu TS, Pleass RJ, Willis AC, Kilian M, Wormald MR, Lellouch AC, et al. The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fcα receptor interactions. *J Biol Chem* (1998) 273:2260–72. doi:10.1074/jbc.273.4.2260
70. Othy S, Topcu S, Saha C, Kothapalli P, Lacroix-Desmazes S, Kasermann F, et al. Sialylation may be dispensable for reciprocal modulation of helper T cells by intravenous immunoglobulin. *Eur J Immunol* (2014) 44:2059–63. doi:10.1002/eji.201444440
71. Engert A, Balduini C, Brand A, Coiffier B, Cordonnier C, Dohner H, et al. The European hematology association roadmap for european hematology research: a consensus document. *Haematologica* (2016) 101:115–208. doi:10.3324/haematol.2015.136739

Conflict of Interest Statement: SW, MJ, and CV are employees of CSL Behring.

The reviewer LR declared a shared affiliation and a past coauthorship with several of the authors (JB and SK) to the handling editor, who ensured that the process met the standards of a fair and objective review.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 11 April 2016
doi: 10.3389/fimmu.2016.00138

An Overview of B-1 Cells as Antigen-Presenting Cells

Ana F. Popi, Ieda M. Longo-Maugéri and Mario Mariano*

Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

The role of B cells as antigen-presenting cells (APCs) has been extensively studied, mainly in relation to the activation of memory T cells. Considering the B cell subtypes, the role of B-1 cells as APCs is beginning to be explored. Initially, it was described that B-1 cells are activated preferentially by T-independent antigens. However, some reports demonstrated that these cells are also involved in a T-dependent response. The aim of this review is to summarize information about the ability of B-1 cells to play a role as APCs and to briefly discuss the role of the BCR and toll-like receptor signals in this process. Furthermore, some characteristics of B-1 cells, such as natural IgM production and phagocytic ability, could interfere in the participation of these cells in the onset of an adaptive response.

Keywords: B-1 cells, APCs, BCR, TLR, autoimmune diseases

OPEN ACCESS

Edited by:
Nichol E. Holodick, The Feinstein Institute for Medical Research, USA

Reviewed by:
Adam Cunningham, University of Birmingham, UK
Sylvie Bertholet, GlaxoSmithKline Vaccines, Italy

****Correspondence:***
Ana F. Popi
afpopi@unifesp.br

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *13 November 2015*
Accepted: *28 March 2016*
Published: *11 April 2016*

Citation:
Popi AF, Longo-Maugéri IM and Mariano M (2016) An Overview of B-1 Cells as Antigen-Presenting Cells. Front. Immunol. 7:138. doi: 10.3389/fimmu.2016.00138

INTRODUCTION

Murine B-1 cells are known as innate B lymphocytes. However, the classification of these cells as B cells raised many questions, including whether this B cell subtype works in a similar or different way than its counterpart, conventional B-2 cells. Several reviews discuss differences between these two cell populations, regarding to their ontogeny, anatomical localization, antibody repertoire, antigen stimulus, and role in the immune response (1–6). The two major subsets of B cells, B-2 and B-1 cells, are defined by the differential expression of CD5 (7). Along with the presence of CD5 on their surface, B-1 cells are further differentiated from B-2 cells by the expression of CD11b, IgM^{high}, IgD^{low}, and the absence of CD23 (8). Furthermore, CD5 expression also subdivides the B-1 cells into two different subsets, B-1a cells, which are $CD5^+$, and B-1b cells, which are $CD5^-$ (2, 8). B-2 cells are produced in the bone marrow from hematopoietic stem cells and migrate to the secondary lymphoid organs as immature B cells. In these organs, they differentiate into follicular and marginal zone B cells (9, 10). B-1 cells are mainly present in the peritoneal and pleural cavities and constitute only a small fraction of the B cells in the spleen (11). B-1 cell origin and development occur primarily during the fetal stage from distinct precursors from B-2 cells (12, 13). Recently, Ghosn et al. (14) described that HSCs sorted from adult bone marrow and transferred to lethally irradiated recipients clearly give rise to B-2 and B-1b cells but do not detectably reconstitute B-1a cells (14). Furthermore, it has been postulated that the B-1a lineage derives independently from a hematopoietic stem cell (14). These B cell subsets specialize in the recognition of diverse antigens; consequently, they provide distinct immune effector functions. B-1a cells play a role in innate immunity *via* their contribution to natural antibodies, whereas B-1b cells are critical in the development of IgM memory cells (1). B-1a cells respond rapidly to T-cell-independent antigen (15). B-1a cells are also known to produce most of the natural antibodies in the serum (16, 17). Despite this, B-1 cell antibodies have been found to be reactive to self-antigens, and hyperplasia

of the B-1 cell population has been found in some autoimmune diseases (18, 19). The antibody production by B-1b cells has been poorly investigated. By contrast, the B-2 cell response to protein antigens is well described and elicits a T-cell-dependent immune response.

There are few reports about the possible roles each B cell subtype exerts in the immune response by acting as APCs. Although the majority of articles indicate the participation of B-1 cells in recognizing the T-cell-independent antigen, some reports demonstrate their role as antigen-presenting cells (APCs) (20–25). This role is extremely important because it could be one of the functions that have allowed the maintenance of B-1 cells through phylogenetic evolution. Furthermore, a more comprehensive status regarding this function could provide explanations concerning the role of B-1 cells in the immune response and in some diseases, such as autoimmune diseases.

ANTIGEN-PRESENTING B-1 CELLS

Ron et al. (26) first demonstrated evidence of the role of B-2 cells in the $CD4^+$ T cell response by showing a failure of proliferative T cell responses to protein antigens in B cell-depleted mice. To determine whether B cell deficiency caused the T cell response impairment in these mice, the authors showed that splenic cells and peritoneal macrophages were able to stimulate T cell response *in vitro*. Although the first description of this occurred in the beginning of the 80s, the priming of T cells by B lymphocytes remains controversial (26–30), and scarce information exists about the role of other B cell subtypes. It has been postulated that the antigen-presentation capacity of antigen-specific B-2 cells is reserved for the re-activation of memory T cells (29, 31, 32). This could be, in part, attributed to the kinetics of the response of B-2 cells, considering that the activation of these cells by a specific antigen takes several days. In this case, activated B-2 cells might encounter their cognate primed T cell and then play a role in their function as APCs (33–35). In this condition, activated B-2 cells are optimal APCs because they drive the T lymphocyte response to specific antigens (36, 37) and are able to modulate the polarization of T cells by cytokines.

As mentioned before, B-1 cells respond to T-independent antigens and are responsible for the production of natural antibodies, mostly IgM. These cells do not effectively participate in T-dependent responses [reviewed by Berland and Wortis (1)]. Despite this, some reports provide evidence that B-1 cells also stimulate T cells and respond to T-dependent antigens (20). Combined adoptive transfer experiments of OVA-pulsed peritoneal-derived B-1 cells and CFSE-labeled T cells demonstrated that B-1 cells are able to stimulate T cell proliferation. Furthermore, antigen presentation by B-1 cells to OVA-specific T cells was also demonstrated *in vitro* (20). Constitutive expression of MHC class-II, CD80, and CD86 by B-1 cells validated these findings (22). Furthermore, the presence of an inflammatory stimulus or a specific antigen augments these molecules on the surface of B-1 cells (22, 38, 39). Zimecki and Kapp (24) and Zimecki et al. (25) showed that B-1 cells present Ags to Ag-specific T cells and induced more efficient proliferation than conventional B cells.

BCR AND TLR AS ANTIGEN UPTAKE PLAYERS ON B-1 CELLS

B cells have two primary pathways for their activation as APCs, which occurs through BCR or the germline-encoded PAMP receptors (40–42). BCR plays a dual role in B-2 cell activation: (1) the ligation of specific antigens in the BCR induces a signaling cascade that leads to the activation and proliferation of B-2 cells (43) and (2) the BCR–antigen interaction results in internalization and processing of the antigen. Although they are not completely elucidated, the BCR signals in B-1 cells are quite different than in B-2 cells (44–46). B-1 cells show a failure to be activated after BCR engagement, and multiple mechanisms appear to be involved in maintaining B-1 cells in an anergic state. One such mechanism involves Lyn, which acts by phosphorylating ITIMs on inhibitory receptors, leading to the recruitment of PTPs that antagonize the BCR-mediated activation of PTKs. IL-10 also plays a key role in controlling the expansion of self-reactive B-1 cells. CD5 was also indicated as a negative regulator of BCR signals in B-1 cells. Defects in the negative regulatory mechanisms may account for the accumulation of B-1 cells and autoantibodies in autoimmune diseases. However, in an infectious disease, signals from CD40 and high-dose TLR ligands can overcome the anergic state of B-1 cells, enabling their activation during infection (44–46).

Interestingly, in addition to the fact that a non-functional BCR results in a defect in the activation of B-2 cells, it also causes a failure in the T cell response (26). This information supports the idea that internalization of the antigen by the BCR is important to the APC function of B-2 cells. It has been demonstrated that the absence of B cell antigen presentation, due to the lack of MHC expression or a non-functional BCR, results in a defect in the memory CD4 response. Barr et al. (40) demonstrated that the TLR activation of B-2 cells is important for the generation of the primary Th1 response in an antigen presentation-independent process. However, BCR recognition and B cell antigen presentation are absolutely required for the development of Th1 memory cells and hence confer protective immunity to *Salmonella*. With respect to B-1 cells, Gao et al. (47) demonstrated that the antigen specificity of the BCR was involved in the uptake of *Salmonella* by B-1 cells, and the number of phagocytic peritoneal B-1 cells from TgVH3B4 mice was almost threefold higher than that observed in the littermate control mice. Based on these results, it could be suggested that the BCR is essential for the phagocytosis of bacteria by B-1 cells and is also important for enabling these cells to exert APC functions.

Conversely, specific antigen uptake by the BCR and the activation of B cells by the TLRs connect them to an innate phase of the immune response. B-2 cells express many of the TLRs (42, 48, 49), and TLR4 and TLR9 are the most studied. TLR expression is quite different among the B cells subsets. B-2 cells express less TLR2, 6, and 7 and equal amounts of TLR1, 4, 5, and 9 compared to other B cell subsets. Interestingly, MZ B cells and B-1 cells show a clear similarity in TLR expression, displaying increased levels of TLR2, 6, and 7, along with decreased levels of TLR8 (50).

Intriguingly, differences regarding their response to TLR agonists were found between naive murine B cell subsets. Triggering of the TLR induces B-1, but not B-2, cells to differentiate into

fully mature plasma cells (51). Based on these data, the authors proposed that during a natural infection, the TLR stimulation leads to the production of protective natural antibodies by the B-1 cells, which could control the microbial load until the adaptive arm of the immune response becomes fully functional.

It is well known that LPS functions as a B-2 cell mitogen, leading to the activation and polyclonal expansion of these cells, independent of BCR reactivity. *In vitro*, B-1 cells proliferate and produce antibodies in response to TLR engagement. Certain TLR ligands, including Pam3CSK (TLR1/TLR2), MALP2 (TLR2/TLR6), LPS (TLR4), and R848 (TLR7/TLR8), are able to induce B-1 cell proliferation, whereas others, including flagellin (TLR5) and polyIC (TLR3), are not (51). The proliferation of B-1 cells in response to LPS is stronger than in B-2 cells; however, it is less intense in response to CpG.

Some reports show evidence that stimulation of the TLRs in B cells impacts in the T cell response *via* not only the secretion of diverse cytokines but also the increased expression of several costimulatory molecules involved in the T:B cell interaction, such as MHC-II, CD40, CD80, CD86, and others (42, 49, 52–54). Interestingly, our group has described the adjuvant effect of *Propionibacterium acnes* on the expression of the TLR by B-1 cells (39) (Gambero et al., submitted). *P. acnes*, a Gram-positive bacillus, is the major constituent of the normal human adult skin microflora (55). As already demonstrated in clinical and experimental models, a dead *P. acnes* suspension modulates the innate and acquired immune responses, including an increase in antibody responses and phagocytic and tumoricidal macrophage functions (56–63). An important effect of *P. acnes* is its capacity to direct the immune response toward a Th1 or Th2 response (64, 65). *P. acnes* interferes in the B-1 cell APC activity through the toll-like receptors (TLRs), principally TLR2 and TLR9 (66–68). Interestingly, the bacterium increased the expression of MHC-II, CD80, CD86, CD40, TLR2, TLR4, and TLR9 by the B-1 cells (39). It was also confirmed that the adjuvant effect induced by *P. acnes* on the B-1 cells is mediated by TLR2 (Gambero et al., submitted). *P. acnes* increases the number of B-1 cells *in vitro* and *in vivo*, induces their early differentiation into phagocytes *in vitro*, and increases the phagocytic ability of these cells (39) (Gambero et al., submitted). The direct influence of *P. acnes* on antigen presentation by B-1 cells is under investigation. Furthermore, LPS stimulus also increases the differentiation of B-1 cells into phagocytes *in vitro* and *in vivo* (69, 70); however, whether the stimulus increases the APC activity is not yet clear.

Another important point in TLR signaling in B cells is cytokine production. Barr et al. (41) demonstrated that the binding of TLR2, TLR4, and TLR9 in B cells induces B-2 cells to secrete IFN-gamma and IL-6. Interestingly, TLR engagement in B-1 cells augmented the secretion of the proinflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 (71), which have antagonistic effects in the course of the immune response. However, both cytokines are important to B-1 cell proliferation and viability. IL-6 is related to antibody production by B-1 cells in autoimmune disease, and IL-10 plays a negative role in the activation of the BCR. It could be speculated that the secretion of both cytokines after TLR signals could be important in the control of the B-1 cell response in the onset of inflammation and could prevent the development of an exacerbated response to self-antigens.

THE ROLE OF CD80/CD86 IN B-1 CELLS

Some studies point to the role of certain molecules in the induction of $CD4^+$ T cell polarization by B-1 cells. B-1 cells express elevated levels of CD80 and CD86 in comparison to B-2 cells (72). Zhong et al. (73) demonstrated that CD86 blockage markedly reduced the capacity of B-1 cells to stimulate alloreactive T cells. Interestingly, the same treatment increased the generation of Treg cells after antigen presentation by B-1 cells. However, anti-CD80 antibody treatment had much less of an effect on the capacity of B-1 cells to stimulate alloreactive T cells and on the generation of Treg cells than did anti-CD86 antibody treatment (73). Anti-CD86 treatment also partially inhibited the induction of Th17 cells (23). In agreement with these findings, De Lorenzo et al. (38) demonstrated that OVA-presensitized B-1 cells were less able to induce T cell proliferation than naive B-1 cells. Additionally, these authors described that OVA-presensitized B-1 cells express high levels of MHC-II molecules compared to naive B-1 cells and that the expression of CD86 was lower.

Interestingly, the CD86 molecule may also provide the costimulatory signal in fish B cell, similar to B-1 cells (74). These results indicate that even in primitive vertebrates, B cells may act as pivotal initiating APCs in priming naive T cells, similar to DCs and macrophages in mammals. Elucidating how antigen presentation occurs in ancient vertebrates and in different B cell subtypes in mammals may contribute to the understanding of the evolutionary history of B cell populations, such as the origin of the B-1 subset, and of the evolution of the adaptive immune response.

T CELL POLARIZATION BY B-1 CELLS

Although some authors have described the production of cytokines by B-1 cells, scarce literature exists about the polarization of T cells by them. Some reports about this pointed out that B-1 cells favor Th17 polarization (23, 73).

Comparing the APC activity of B-2 cells and peritoneal B-1 cells, Margry et al. (20) demonstrated that peritoneal B-1a cells increase the percentage of IL-10-, IFN-γ-, and IL-4-producing T cells. Furthermore, peritoneal B-1a cells present antigens to $CD4^+$ T cells in the peritoneal cavity, which is totally distinct from the milieu, where antigen presentation by conventional B cells occurs. The authors also show that the activation of $CD4^+$ T cells by peritoneal B-1a cells promotes a more intense proliferation compared to conventional B cells. Although peritoneal B-1a cells induce activated T cells to produce larger amounts of IL-10, IFN-γ, and IL-4 *in vitro* (20), B-1a cells derived from the spleen preferentially induce IL-17 production by T cells. In agreement with this, other authors also demonstrated that B-1 cells preferentially induce Th1 and Th17 differentiation *in vivo* (73). This strong inflammatory response elicited by the B-1 cells could be related to their role in the autoimmune diseases (21). Interestingly, in cultures for the generation of Treg cells, when B-1 cells were used as the APCs, fewer $Foxp3^+$ Treg cells were

generated in comparison to cultures where splenic B-2 cells were used (73). The failure to generate Treg cells, in addition to the induction of a Th1/Th17 profile by the B-1 cells, could be one of the mechanisms of the autoimmune profile of B-1 cells. However, this hypothesis is only a speculation, because the induction of the Th profile by the B-1 cells needs to be investigated in more detail and in different infection/disease models.

Other important point to be discussed is about the localization of antigen presentation by B-1 cells. Despite the migration of B-1 cells to inflammatory milieu and other organs (75–77), it was not well documented if it is necessary that these cells migrate to secondary lymphoid organs to exert APC role. Interestingly, Margry et al. (20) demonstrated that peripheral T cells visit the peritoneal cavity and engage with antigen presented by B-1 cells in this location. In this context, we could postulate that APC role of B-1 cells could be more direct to reactivate memory T cells than priming naive T cell since that the majority of T cells found in the peritoneal cavity is memory T cells.

THE ROLE OF B-1 CELL ANTIGEN PRESENTATION IN AUTOIMMUNE DISEASES

As mentioned before and in the literature, the TLR and BCR signals are important in the assembly of the adaptive response. In an early stage, TLR signals drive the secretion of cytokines by the APCs and contribute to the expression of some molecules that are important in the antigen-presenting process. Later, BCR-mediated uptake is important to the antigen presentation by B cells and the maintenance of memory T cells. In addition to its importance in the secondary response during an infection, this also could be a mechanism that induces autoimmune disease. In fact, the involvement of BCR/TLR signals in driving T cells in lupus (78) and EAE has already been described (21, 79).

Sato et al. (21) described an increase in the B-1 cell population in the disease target organs of a murine model of lupus. These authors demonstrated that B-1 cells aberrantly migrate into the thymus during the development of lupus nephritis and that B-1, but not B-2, cells induce the activation and expansion of thymic $CD4^+$ T cells in the presence of IL-2. Considering that B-1 cells often recognize self-antigens and express higher levels of costimulatory molecules, their role in activating the autoreactive TCR should be considered. These findings may provide a novel understanding of the mechanism for the loss of immunological tolerance in the development of autoimmune disease.

CONCLUDING REMARKS

B-1 cells have peculiar features, such as a mixture of innate and lymphoid cell properties. As mentioned here, some authors describe similarities between B-1 cells and primordial B cells in ancient vertebrates (74). As described for teleost fish B cells, B-1 cells are phagocytic cells (6, 69, 70, 75) and have microbicidal activity (80, 81), similar to DCs and macrophages in mammals. Furthermore, several reports indicate that B-1 cells may play a role as initiating APCs, not only in the maintenance of memory T cells, similar to conventional B cells, but also in priming $CD4^+$ T cells. It is important to reinforce that B-1 cells produce a majority of the IgM natural antibodies. In this context, we postulate that these antibodies could form immunocomplexes with self-antigens and that B-1 cells could internalize these, leading to intense antigen presentation to T cells. A speculative scenario was proposed, in a continuous inflammatory stimulus, elevated levels of IL-6 increased induce expansion of B-1 cell population, and also increased the B-1-cell antibody production. Furthermore, the CD86 expression by B-1 cells is also augmented, and it favors the B-1 cell APCs function and also the induction of Th17 profile. Considering that B-1 cells produce mainly self-reactive antibodies, the increased levels of the B-1 cell-derived antibodies could leave to an increase in the antigen uptake by these cells and also augment in antigen presentation to self-reactive TCR, which could lead finally to an induction of autoimmune diseases.

In conclusion, further detailed investigations should be conducted to elucidate the role of B-1 cells in priming T cells during an infectious disease. Several strategies have been developed considering the use of APCs in therapies for which amplification of the immune response is necessary; thus, B-1 cells might also be considered in this context. Furthermore, the participation of these cells in the breakdown of self-tolerance should also be considered.

AUTHOR CONTRIBUTIONS

AFP conceived the idea and topic of the review, wrote and discussed the text with other authors. IMLM and MM discussed the ideas, read and made suggestions in the final text.

FUNDING

This review was supported by FAPESP grant number 2008/58561-0 and 2015/01986-2.

REFERENCES

1. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* (2002) **20**:253–300. doi:10.1146/annurev.immunol.20.100301.064833
2. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med* (1983) **157**(1):202–18. doi:10.1084/jem.157.1.202
3. Herzenberg LA. Toward a layered immune system. *Cell* (1989) **59**(6):953–4. doi:10.1016/0092-8674(89)90748-4
4. Montecino-Rodriguez E, Dorshkind K. New perspectives in B-1 B cell development and function. *Trends Immunol* (2006) **27**(9):428–33. doi:10.1016/j.it.2006.07.005
5. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol* (2006) **7**(3):293–301. doi:10.1038/ni1301
6. Popi AF. B-1 phagocytes: the myeloid face of B-1 cells. *Ann N Y Acad Sci* (2015) **1362**:86–97. doi:10.1111/nyas.12814
7. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR. The Ly-1 B cell lineage. *Immunol Rev* (1986) **93**:81–102. doi:10.1111/j.1600-065X.1986.tb01503.x

8. Tung JW, Mrazek MD, Yang Y, Herzenberg LA, Herzenberg LA. Phenotypically distinct B cell development pathways map to the three B cell lineages in the mouse. *Proc Natl Acad Sci U S A* (2006) **103**(16):6293–8. doi:10.1073/pnas.0511305103
9. Chung JB, Silverman M, Monroe JG. Transitional B cells: step by step towards immune competence. *Trends Immunol* (2003) **24**(6):343–9. doi:10.1016/S1471-4906(03)00119-4
10. Miller JP, Stadanlick JE, Cancro MP. Space, selection, and surveillance: setting boundaries with BLyS. *J Immunol* (2006) **176**(11):6405–10. doi:10.4049/jimmunol.176.11.6405
11. Deenen GJ, Kroese FG. Kinetics of peritoneal B-1a cells (CD5 B cells) in young adult mice. *Eur J Immunol* (1993) **23**(1):12–6. doi:10.1002/eji.1830230104
12. Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA. Adoptive transfer of murine B-cell lineages. *Ann N Y Acad Sci* (1992) **651**:168–9. doi:10.1111/j.1749-6632.1992.tb24610.x
13. Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. *Proc Natl Acad Sci U S A* (1992) **89**(8):3320–4. doi:10.1073/pnas.89.8.3320
14. Ghosn EE, Yamamoto R, Hamanaka S, Yang Y, Herzenberg LA, Nakauchi H, et al. Distinct B-cell lineage commitment distinguishes adult bone marrow hematopoietic stem cells. *Proc Natl Acad Sci U S A* (2012) **109**(14):5394–8. doi:10.1073/pnas.1121632109
15. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* (2001) **14**(5):617–29. doi:10.1016/S1074-7613(01)00129-7
16. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med* (2008) **205**(13):3053–64. doi:10.1084/jem.20080979
17. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity* (2005) **23**(1):7–18. doi:10.1016/j.immuni.2005.04.011
18. Duan B, Croker BP, Morel L. Lupus resistance is associated with marginal zone abnormalities in an NZM murine model. *Lab Invest* (2007) **87**(1):14–28. doi:10.1038/labinvest.3700497
19. Duan B, Morel L. Role of B-1a cells in autoimmunity. *Autoimmun Rev* (2006) **5**(6):403–8. doi:10.1016/j.autrev.2005.10.007
20. Margry B, Wieland WH, van Kooten PJ, van Eden W, Broere F. Peritoneal cavity B-1a cells promote peripheral CD4+ T-cell activation. *Eur J Immunol* (2013) **43**(9):2317–26. doi:10.1002/eji.201343418
21. Sato T, Ishikawa S, Akadegawa K, Ito T, Yurino H, Kitabatake M, et al. Aberrant B1 cell migration into the thymus results in activation of CD4 T cells through its potent antigen-presenting activity in the development of murine lupus. *Eur J Immunol* (2004) **34**(12):3346–58. doi:10.1002/eji.200425373
22. Vigna AF, Godoy LC, Rogerio de Almeida S, Mariano M, Lopes JD. Characterization of B-1b cells as antigen presenting cells in the immune response to gp43 from *Paracoccidioides brasiliensis* in vitro. *Immunol Lett* (2002) **83**(1):61–6. doi:10.1016/S0165-2478(02)00070-6
23. Wang Y, Rothstein TL. Induction of Th17 cell differentiation by B-1 cells. *Front Immunol* (2012) **3**:281. doi:10.3389/fimmu.2012.00281
24. Zimecki M, Kapp JA. Presentation of antigen by B cell subsets. II. The role of CD5 B cells in the presentation of antigen to antigen-specific T cells. *Arch Immunol Ther Exp (Warsz)* (1994) **42**(5–6):349–53.
25. Zimecki M, Whiteley PJ, Pierce CW, Kapp JA. Presentation of antigen by B cells subsets. I. Lyb-5+ and Lyb-5- B cells differ in ability to stimulate antigen specific T cells. *Arch Immunol Ther Exp (Warsz)* (1994) **42**(2):115–23.
26. Ron Y, De Baetselier P, Gordon J, Feldman M, Segal S. Defective induction of antigen-reactive proliferating T cells in B cell-deprived mice. *Eur J Immunol* (1981) **11**(12):964–8. doi:10.1002/eji.1830111203
27. Barr TA, Gray M, Gray D. B cells: programmers of CD4 T cell responses. *Infect Disord Drug Targets* (2012) **12**(3):222–31. doi:10.2174/187152612800564446
28. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* (2006) **176**(6):3498–506. doi:10.4049/jimmunol.176.6.3498
29. Epstein MM, Di Rosa F, Jankovic D, Sher A, Matzinger P. Successful T cell priming in B cell-deficient mice. *J Exp Med* (1995) **182**(4):915–22. doi:10.1084/jem.182.4.915
30. Rivera A, Chen CC, Ron N, Dougherty JP, Ron Y. Role of B cells as antigen-presenting cells in vivo revisited: antigen-specific B cells are essential for T cell expansion in lymph nodes and for systemic T cell responses to low antigen concentrations. *Int Immunol* (2001) **13**(12):1583–93. doi:10.1093/intimm/13.12.1583
31. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. *Science* (1992) **258**(5085):1156–9. doi:10.1126/science.1439825
32. Lassila O, Alanen A, Vainio O, Houssaint E, Pink JR, Weber WT. B cell precursors in chick embryos surgically bursectomized at 72 h of incubation. *Eur J Immunol* (1988) **18**(11):1867–70. doi:10.1002/eji.1830181136
33. Kurt-Jones EA, Liano D, HayGlass KA, Benacerraf B, Sy MS, Abbas AK. The role of antigen-presenting B cells in T cell priming in vivo. Studies of B cell-deficient mice. *J Immunol* (1988) **140**(11):3773–8.
34. Liu Y, Wu Y, Ramarathinam L, Guo Y, Huszar D, Trounstine M, et al. Gene-targeted B-deficient mice reveal a critical role for B cells in the CD4 T cell response. *Int Immunol* (1995) **7**(8):1353–62. doi:10.1093/intimm/7.8.1353
35. Topham DJ, Tripp RA, Hamilton-Easton AM, Sarawar SR, Doherty PC. Quantitative analysis of the influenza virus-specific CD4+ T cell memory in the absence of B cells and Ig. *J Immunol* (1996) **157**(7):2947–52.
36. Malynn BA, Romeo DT, Wortis HH. Antigen-specific B cells efficiently present low doses of antigen for induction of T cell proliferation. *J Immunol* (1985) **135**(2):980–8.
37. Townsend SE, Goodnow CC. Abortive proliferation of rare T cells induced by direct or indirect antigen presentation by rare B cells in vivo. *J Exp Med* (1998) **187**(10):1611–21. doi:10.1084/jem.187.10.1611
38. De Lorenzo BH, Brito RR, Godoy LC, Lopes JD, Mariano M. Tolerogenic property of B-1b cells in a model of allergic reaction. *Immunol Lett* (2007) **114**(2):110–8. doi:10.1016/j.imlet.2007.09.013
39. Mussalem JS, Squaiella-Baptistao CC, Teixeira D, Yendo TM, Thies FG, Popi AF, et al. Adjuvant effect of killed *Propionibacterium acnes* on mouse peritoneal B-1 lymphocytes and their early phagocyte differentiation. *PLoS One* (2012) **7**(3):e33955. doi:10.1371/journal.pone.0033955
40. Barr TA, Brown S, Mastroeni P, Gray D. TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to *Salmonella enterica*. *J Immunol* (2010) **185**(5):2783–9. doi:10.4049/jimmunol.1001431
41. Barr TA, Brown S, Ryan G, Zhao J, Gray D. TLR-mediated stimulation of APC: distinct cytokine responses of B cells and dendritic cells. *Eur J Immunol* (2007) **37**(11):3040–53. doi:10.1002/eji.200636483
42. Gray D, Gray M, Barr T. Innate responses of B cells. *Eur J Immunol* (2007) **37**(12):3304–10. doi:10.1002/eji.200737728
43. Wang LD, Clark MR. B-cell antigen-receptor signalling in lymphocyte development. *Immunology* (2003) **110**(4):411–20. doi:10.1111/j.1365-2567.2003.01756.x
44. Alhakeem SS, Sindhava VJ, McKenna MK, Gachuki BW, Byrd JC, Muthusamy N, et al. Role of B cell receptor signaling in IL-10 production by normal and malignant B-1 cells. *Ann N Y Acad Sci* (2015) **1362**:239–49. doi:10.1111/nyas.12802
45. Holodick NE, Rothstein TL. Atypical response of B-1 cells to BCR ligation: a speculative model. *Front Immunol* (2013) **4**:457. doi:10.3389/fimmu.2013.00457
46. Sindhava VJ, Bondada S. Multiple regulatory mechanisms control B-1 B cell activation. *Front Immunol* (2012) **3**:372. doi:10.3389/fimmu.2012.00372
47. Gao J, Ma X, Gu W, Fu M, An J, Xing Y, et al. Novel functions of murine B1 cells: active phagocytic and microbicidal abilities. *Eur J Immunol* (2012) **42**(4):982–92. doi:10.1002/eji.201141519
48. Booth J, Wilson H, Jimbo S, Mutwiri G. Modulation of B cell responses by toll-like receptors. *Cell Tissue Res* (2011) **343**(1):131–40. doi:10.1007/s00441-010-1031-3
49. Fillatreau S, Manz RA. Tolls for B cells. *Eur J Immunol* (2006) **36**(4):798–801. doi:10.1002/eji.200636040
50. Meyer-Bahlburg A, Rawlings DJ. Differential impact of toll-like receptor signaling on distinct B cell subpopulations. *Front Biosci (Landmark Ed)* (2012) **17**:1499–516. doi:10.2741/4000
51. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J Immunol* (2007) **178**(12):7779–86. doi:10.4049/jimmunol.178.12.7779
52. Avalos AM, Busconi L, Marshak-Rothstein A. Regulation of autoreactive B cell responses to endogenous TLR ligands. *Autoimmunity* (2010) **43**(1):76–83. doi:10.3109/08916930903374618

53. Crampton SP, Voynova E, Bolland S. Innate pathways to B-cell activation and tolerance. *Ann N Y Acad Sci* (2010) **1183**:58–68. doi:10.1111/j.1749-6632.2009.05123.x
54. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* (2004) **5**(10):987–95. doi:10.1038/ni1112
55. Webster GF. Inflammatory acne. *Int J Dermatol* (1990) **29**(5):313–7. doi:10.1111/j.1365-4362.1990.tb04749.x
56. Abath FG, Coutinho EM, Montenegro SM, Gomes YM, Carvalho AB. The use of non-specific immunopotentiators in experimental *Trypanosoma cruzi* infection. *Trans R Soc Trop Med Hyg* (1988) **82**(1):73–6. doi:10.1016/0035-9203(88)90268-4
57. Brener Z, Cardoso JE. Nonspecific resistance against *Trypanosoma cruzi* enhanced by *Corynebacterium parvum*. *J Parasitol* (1976) **62**(4):645–6. doi:10.2307/3279437
58. Mussalem JS, Vasconcelos JR, Squaiella CC, Ananias RZ, Braga EG, Rodrigues MM, et al. Adjuvant effect of the *Propionibacterium acnes* and its purified soluble polysaccharide on the immunization with plasmidial DNA containing a *Trypanosoma cruzi* gene. *Microbiol Immunol* (2006) **50**(4):253–63. doi:10.1111/j.1348-0421.2006.tb03791.x
59. Squaiella CC, Ananias RZ, Mussalem JS, Braga EG, Rodrigues EG, Travassos LR, et al. In vivo and in vitro effect of killed *Propionibacterium acnes* and its purified soluble polysaccharide on mouse bone marrow stem cells and dendritic cell differentiation. *Immunobiology* (2006) **211**(1–2):105–16. doi:10.1016/j.imbio.2005.10.013
60. Halpern BN, Biozzi G, Stiffel C, Mouton D. Inhibition of tumour growth by administration of killed *Corynebacterium parvum*. *Nature* (1966) **212**(5064):853–4. doi:10.1038/212853a0
61. Nussenzweig RS. Increased nonspecific resistance to malaria produced by administration of killed *Corynebacterium parvum*. *Exp Parasitol* (1967) **21**(2):224–31. doi:10.1016/0014-4894(67)90084-7
62. Smith SR, Calzetta A, Bankowski J, Kenworthy-Bott L, Terminelli C. Lipopolysaccharide-induced cytokine production and mortality in mice treated with *Corynebacterium parvum*. *J Leukoc Biol* (1993) **54**(1):23–9.
63. Warr GW, James K. Effect of *Corynebacterium parvum* on the class and subclass of antibody produced in the response of different strains of mice to sheep erythrocytes. *Immunology* (1975) **28**(3):431–42.
64. Braga EG, Ananias RZ, Mussalem JS, Squaiella CC, Longhini AL, Mariano M, et al. Treatment with *Propionibacterium acnes* modulates the late phase reaction of immediate hypersensitivity in mice. *Immunol Lett* (2003) **88**(2):163–9. doi:10.1016/S0165-2478(03)00079-8
65. Squaiella CC, Longhini AL, Braga EG, Mussalem JS, Ananias RZ, Yendo TM, et al. Modulation of the type I hypersensitivity late phase reaction to OVA by *Propionibacterium acnes*-soluble polysaccharide. *Immunol Lett* (2008) **121**(2):157–66. doi:10.1016/j.imlet.2008.10.005
66. Kalis C, Gumenscheimer M, Freudenberg N, Tchaptchet S, Fejer G, Heit A, et al. Requirement for TLR9 in the immunomodulatory activity of *Propionibacterium acnes*. *J Immunol* (2005) **174**(7):4295–300. doi:10.4049/jimmunol.174.7.4295
67. Kim J, Ochoa MT, Krutzik SR, Takeuchi O, Uematsu S, Legaspi AJ, et al. Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses. *J Immunol* (2002) **169**(3):1535–41. doi:10.4049/jimmunol.169.3.1535
68. Squaiella-Baptistao CC, Teixeira D, Mussalem JS, Ishimura ME, Longo-Maugeri IM. Modulation of Th1/Th2 immune responses by killed *Propionibacterium acnes* and its soluble polysaccharide fraction in a type I hypersensitivity murine model: induction of different activation status of antigen-presenting cells. *J Immunol Res* (2015) **2015**:132083. doi:10.1155/2015/132083
69. Popi AF, Motta FLT, Mortara RA, Schenkman S, Lopes JD, Mariano M. Co-ordinated expression of lymphoid and myeloid specific transcription factors during B-1b cell differentiation into mononuclear phagocytes in vitro. *Immunology* (2009) **126**(1):114–22. doi:10.1111/j.1365-2567.2008.02883.x
70. Popi AF, Osugui L, Perez KR, Longo-Maugeri IM, Mariano M. Could a B-1 cell derived phagocyte "be one" of the peritoneal macrophages during LPS-driven inflammation? *PLoS One* (2012) **7**(3):e34570. doi:10.1371/journal.pone.0034570
71. Ng LG, Ng CH, Woehl B, Sutherland AP, Huo J, Xu S, et al. BAFF costimulation of toll-like receptor-activated B-1 cells. *Eur J Immunol* (2006) **36**(7):1837–46. doi:10.1002/eji.200635956
72. Tumang JR, Hastings WD, Bai C, Rothstein TL. Peritoneal and splenic B-1 cells are separable by phenotypic, functional, and transcriptomic characteristics. *Eur J Immunol* (2004) **34**(8):2158–67. doi:10.1002/eji.200424819
73. Zhong X, Gao W, Degauque N, Bai C, Lu Y, Kenny J, et al. Reciprocal generation of Th1/Th17 and T(reg) cells by B1 and B2 B cells. *Eur J Immunol* (2007) **37**(9):2400–4. doi:10.1002/eji.200737296
74. Zhu LY, Lin AF, Shao T, Nie L, Dong WR, Xiang LX, et al. B cells in teleost fish act as pivotal initiating APCs in priming adaptive immunity: an evolutionary perspective on the origin of the B-1 cell subset and B7 molecules. *J Immunol* (2014) **192**(6):2699–714. doi:10.4049/jimmunol.1301312
75. Almeida SR, Aroeira LS, Frymuller E, Dias MA, Bogsan CS, Lopes JD, et al. Mouse B-1 cell-derived mononuclear phagocyte, a novel cellular component of acute non-specific inflammatory exudate. *Int Immunol* (2001) **13**(9):1193–201. doi:10.1093/intimm/13.9.1193
76. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* (2002) **16**(1):67–76. doi:10.1016/S1074-7613(01)00257-6
77. Labadi A, Balogh P. Differential preferences in serosal homing and distribution of peritoneal B-cell subsets revealed by in situ CFSE labeling. *Int Immunol* (2009) **21**(9):1047–56. doi:10.1093/intimm/dxp071
78. Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderon Gomez E, Sweenie CH, et al. TLR-activated B cells suppress T cell-mediated autoimmunity. *J Immunol* (2008) **180**(7):4763–73. doi:10.4049/jimmunol.180.7.4763
79. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* (2002) **3**(10):944–50. doi:10.1038/ni833
80. Popi AF, Godoy LC, Xander P, Lopes JD, Mariano M. B-1 cells facilitate *Paracoccidioides brasiliensis* infection in mice via IL-10 secretion. *Microb Infect* (2008) **10**(7):817–24. doi:10.1016/j.micinf.2008.04.012
81. Popi AF, Zamboni DS, Mortara RA, Mariano M. Microbicidal property of B1 cell derived mononuclear phagocyte. *Immunobiology* (2009) **214**(8):664–73. doi:10.1016/j.imbio.2008.12.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 09 September 2016
doi: 10.3389/fimmu.2016.00324

B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production

*Nicole Baumgarth**

Department of Pathology, Microbiology and Immunology, Center for Comparative Medicine, University of California Davis, Davis, CA, USA

A small subset of B cells, termed B-1 cells, with developmental origins, phenotypes, and functions that are distinct from those of conventional B cells exist in mice. It contributes the vast majority of spontaneously produced "natural" IgM. Natural IgM is constitutively produced, even in the absence of microbiota, and fulfills many distinct functions in tissue homeostasis and host defense. B-1 cells also respond with IgM production to innate signals and pathogen exposure, while maintaining steady-state levels natural IgM. Thus, within the B-1 cell pool, cells of distinct and heterogeneous functionality must exist to facilitate these different functions. This review considers three factors that may contribute to this heterogeneity: first, developmental differences regarding the origins of the precursors, second, tissue-specific signals that may differentially affect B-1 cells in the tissue compartments, and finally responsiveness to self-antigens as well as innate and antigen-specific signals. All three are likely to shape the repertoire and responsiveness of B-1 cells to homeostatic- and antigen-induced signals and thus contribute to the functional heterogeneity among these innate-like B cells.

Keywords: natural IgM, B-1 cells, B cell development, immune regulation, innate-like lymphocytes

OPEN ACCESS

Edited by:
Nichol E. Holodick, The Feinstein Institute for Medical Research, USA

Reviewed by:
John Kearney, University of Alabama at Birmingham, USA
Heinz Kohler, University of Kentucky, USA

***Correspondence:**
Nicole Baumgarth
nbaumgarth@ucdavis.edu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *19 June 2016*
Accepted: *15 August 2016*
Published: *09 September 2016*

Citation:
Baumgarth N (2016) B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-Induced IgM Production. Front. Immunol. 7:324. doi: 10.3389/fimmu.2016.00324

INTRODUCTION

The B cell compartment of all jawed vertebrates contains populations of spontaneous "natural" Ig-secreting cells (1). These antibodies are broadly self-reactive and most are of the IgM isotype. The best-studied natural antibody-producing cell population is that of mice, in which a subset of CD5-expressing B cells, CD5 was thought of until then as a T cell-restricted surface receptor, was linked to natural IgM production (2). Further studies showed differences in B-1 cell development origins, tissue distribution, and responsiveness to antigens and mitogens compared with classical follicular B cells and thus identified them as distinct from the majority "conventional" B cell population [reviewed in Ref. (3, 4)]. Because these cells appear earlier in ontogeny than conventional B cells, they were termed "B-1 cells" and conventional B cells "B-2 cells"; CD5 expression is used to differentiate B-1 cells further into B-1a ($CD5^+$) and B-1b ($CD5^-$).

Numerous groups demonstrated that in addition to generating natural antibodies, B-1 cells also actively contribute to pathogen-induced immune responses (2). Explored have been responses to *Francisella* spp (5, 6), *Borrelia hermsii* (7–9), *Salmonella typhi* (10, 11), *Streptococcus pneumoniae* (12–14), and influenza virus (15–17). In each case, the response consisted of increased B-1 cell-derived IgM production, measured in regional lymph nodes, the spleen, and/or in serum. This raises important questions about the regulation of natural versus antigen-induced antibody production by

B-1 cells. Studies on influenza virus infection showed that despite an increased local production of B-1 cell-derived IgM, natural serum IgM levels remained unaffected (15), suggesting the presence of distinct subsets of B-1 cells that contribute systemic natural and enhanced infection-induced local IgM production, respectively.

At least two non-mutually exclusive models may explain these observations: a "division of labor" model, as proposed (14), in which distinct B-1 cell subsets exist, some responsible for natural antibody production. In the studies by Haas et al., B-1b cells responded to *S. pneumoniae* antigens by making antibodies, whereas B-1a cells constitutively produced natural IgM antibodies against other components of *S. pneumoniae*. However, given that in other infections (see below), B-1a cells were also shown to respond with increased antibody production, a simple division of labor between B-1a and B-1b seems unlikely. Another model is that the degree of self-antigen-mediated stimulation of the BCR and/or additional costimulatory signals may support a certain number of B-1 cells to differentiate to natural antibody-producing cells, while others remain quiescent, until stimulation by innate signals and/or antigen exposure activates these cells above a certain threshold required for their differentiation. Common to both models is the idea that the B-1 cell pool contains B-1 cells of different functional properties.

The literature supports the notion of a heterogeneous B-1 cell pool, but the causes for this heterogeneity are largely unknown and little explored. Below, we consider three factors that may modulate B-1 cell functions: (1) the multiple developmental origins of B-1 cells, (2) tissue-specific signals, and (3) differences in exposure to and responsiveness of B-1 cells to self- and foreign antigens. Determining the relative impact of these signals on the functionality of B-1 cells could clarify much of the biology of this cell population and of one of its important products: natural IgM.

MULTIPLE DEVELOPMENTAL ORIGINS CONTRIBUTE TO THE B-1 CELL POOL

Early studies demonstrated that B cell precursors exist in the splanchnopleura of the developing mouse embryo, which give rise only to B-1 but not B-2 cells, but the precursors were not identified (18). More recent reports supported these findings by showing that the earliest B-1 cells arise from extra-hematopoietic sources, the blood island cells of the yolk sac (19, 20), similar to the development of some macrophage populations (21). In 2006, Montecino-Rodriguez et al. identified B cell precursors in fetal liver and in small numbers also in the bone marrow of adult mice that give rise only to B-1 cells (22). These B-1 cell-restricted precursors (pre–pro-B) among otherwise "lineage negative cells" were differentiated from early conventional (B-2) cell precursors by their lack of CD45R (B220) and strong expression of CD19. While present also in the adult bone marrow, it was further reported that by 3–6 weeks after birth, B-1 cell precursors are no longer contributing significantly to the adult B-1 cell pool (23), consistent with a previous body of literature that showed B-1 cells to be a mainly fetal- and neonatal-developed population that is maintained by self-renewal rather than *de novo* development (24, 25).

It appears that the bone marrow precursors can be activated in situations of severe lymphopenia, however, as occurs following adoptive cell transfer of bone marrow into lethally irradiated recipients (26, 27). In that situation, the emerging B-1 cell populations are much more heavily skewed toward $CD5^-$ B-1b cell development. The reasons for this remain to be explored. Thus, existing data support the concept that the $CD5^+$ B-1a cell pool is largely, albeit not exclusively, fetal and neonatal derived (28). This conclusion was recently further underscored by the demonstration of a developmental switch between fetal and post-natal development, regulated by the transcription factor Lin28b that significantly affected B-1 cells (29, 30). The studies showed that the expression of Lin28b induces a regulatory network of transcriptional regulators that support the development of B-1a cells. In its absence, B-1a cell populations are greatly reduced, while forced overexpression of Lin28b in adult bone marrow precursors enhances B-1a cell output in adulthood (29, 30). In the latter case, BCR repertoire differences compared with B-1a cells generated from fetal precursors were noted (30), however, suggesting that other signals regulate development and/or selection of these cells.

The lack of sustained *de novo* B-1 cells development beginning from a few weeks after birth was first demonstrated by Lalor et al. (25). It can be exploited experimentally by transferring peritoneal cavity-derived B-1 cells into neonatal mice rendered B cell-deficient by allotype-specific anti-IgM antibody treatment (24, 31). Once recipient mice reach 6 weeks of age, discontinuation of antibody treatment will lead to the reemergence of bone marrow-derived B-2 cell populations, but only few B-1 cells. In that manner, one can generate chimeras in which B-1 cells and their Ig are marked by allotype, or lack or express certain genes only in one of the B cell compartments. Given that B-1 cells are maintained throughout life by self-renewal, i.e., continuous turnover, it will be important to explore the effects of aging on their functionality. Indeed, recent studies suggest alterations to these populations in the aging animals (32). Whether this affects primarily the production of natural IgM, antigen-induced responses of B-1 cells, or both will be an important future target for study.

Thus, the B-1 cell pool of adult mice is likely shaped by distinct waves of B-1 cells that develop from distinct precursors: the first wave of extra-hematopoietic yolk sac B-1 precursors that populate the fetal liver until about E15.5; the second wave of fetal liver precursors that presumably dominates the B-1 cell pool at birth; and the third set in the bone marrow that gives rise to B-1 cells developing during the first few weeks of life (33). All waves are expected to modulate the B-1 cell pool. An unanswered question is to what extent these distinct waves generate B-1 cells of different repertoires, tissue distribution, functionality, and/or lifespan.

Natural IgM Regulates B Cell Development

Recently, we demonstrated that mice unable to generate secreted (s)IgM contain few B-1 cells in the body cavities, while spleen and bone marrow B-1 cell populations appeared largely unchanged (34). B-2 cell development was also significantly affected. These studies were in apparent contrast to earlier reports that suggested the presence of increased B-1 cell frequencies in the peritoneal cavity of $sIgM^{-/-}$ mice (35, 36), which usually make about 60% of B cells at that site. The discrepancy is explained by our

findings that these mice harbor large numbers of anergic, $CD5^+$ conventional B cells in both spleen and peritoneal cavity, which due to their expression of CD5 were misidentified as B-1a cells. These anergic $CD5^+$ B cells are distinct, however, in that they are $CD19^{int}$, $B220^{hi}$, and $CD43^-$, in contrast to the $CD19^{hi}$, $B220^{lo}$, and $CD43^+$ B-1a cells (34).

Approximately 1.5–2% of B cells in the spleen of commonly used inbred mouse strains are B-1 cells (37). Interestingly, asplenic $Hox11^{-/-}$ mice were shown previously to have reduced numbers of peritoneal cavity B-1 cells (38) and splenectomy resulted in a reduction of already established peritoneal cavity B-1a cell pools over time (38, 39). Since the spleen is an important tissue source of natural IgM (40, 41) and the $Hox11^{-/-}$ mice showed reductions in serum IgM levels (38), it is unclear whether there is a precursor–offspring relationship between spleen and peritoneal cavity B-1 cells or whether the reductions in sIgM indirectly affected B-1 cell development/expansion.

Our findings, demonstrating alterations in B-2 cell development as early as the bone marrow pre-B cell stage when mice lack secreted IgM, are difficult to reconcile with previous suggestions that the B cell defects seen in the absence of natural IgM are due to lack of apoptotic cell clearance and other "housekeeping functions" attributed to IgM (36, 42). While this could explain the emergence of autoreactive, including anergic, B cells, it is hard to see how this would affect pre-B cell selection. Furthermore, mice that lack the Fc receptor for IgM (FcμR) have about twice the amount of natural serum IgM, yet they develop autoantibodies similar to $sIgM^{-/-}$ mice (43). Based on these and other findings, we suggest that natural IgM regulates B cell development by functions that are independent of its role in the removal of cell debris and autoantigens, but rather by yet to be discovered mechanisms that directly regulate B cell development.

Irrespective of the mechanisms, the findings of a dependency of normal B cell development on natural IgM indicates that the earliest waves of B-1 cells produces the natural IgM that allows normal B-1 and B-2 cell development to commence. The fact that peritoneal cavity B-1 cells, but not splenic B-1 cells, are affected by the lack of IgM further suggests that peritoneal cavity B-1 cells development is dependent on distinct signals and may occur later, after natural IgM production has been initiated in the murine fetus, as IgM, in contrast to IgG, does not effectively cross the placenta (44, 45). Indeed, while small numbers of B-1 cells, including IgM-secreting cells, are detectable before birth in the fetal liver and at birth in the mouse spleen, peritoneal cavity B-1 cells accumulate slowly and not until about 1–2 weeks after birth and the accumulation of B-1 cells in peritoneal and pleural cavity, but not the spleen, are dependent on secretion of the "follicular" homing chemokine CXCL13 (46).

Repertoire Development of Spleen and Body Cavity B-1 Cells

It is of note that the repertoire of B-1a cells in the peritoneal cavity is distinct from that of the spleen. This is exemplified by measuring frequencies of B-1a cells that bind to liposomes containing phosphotidyl choline (PtC) by FACS, many of which are encoded by IgHV11 (47). While the splenic compartment harbors only about 1–2% of PtC binders among B-1a cells, that frequency is approximately 10% in the peritoneal cavity (48). In mice lacking sIgM, frequencies of PtC binders in the peritoneal cavity and mRNA for IgHV11 are greatly diminished (34), suggesting that B-1a cells with that specificity emerge (or expand) later and then preferentially home to the body cavities.

A recent comprehensive RNA sequencing study on pooled RNA from FACS-purified B-1a and B-2 cells in spleen and peritoneal cavity at various ages of mice further supported previous findings of a B-1 cell B cell receptor (BCR) repertoire that is clearly distinct from that of the B-2 cell pool (49). The B-1 cell repertoire is enriched for self-reactivity, and there is a preponderance of Ig-regions that lack N-region insertions, again supporting the conclusion that B-1 cells are generated before birth, when the enzyme TdT is not yet expressed, and thus does not facilitate inclusions of N-regions during V-D-J recombination. However, both earlier single-cell PCR experiments (50, 51) and the more recent studies (32, 49) demonstrated that the repertoire of B-1a cells is broader, more diverse and not as devoid of N-region insertions as originally anticipated.

The recent B-1a cell repertoire studies support earlier conclusions, which had suggested that the repertoire of the B-1a cell pool is unaffected by foreign antigen exposure. Germ-free mice were shown to have a similar B-1a cell repertoire than that of mice held under SPF housing conditions (49). Similarly, germ-free mice have similar levels of natural IgM and numbers of natural IgM-producing cells in spleen and bone marrow than SPF mice (40). This is not, however, because natural IgM production or the B-1a cell pools are already fully developed at birth. Neonatal mice have very few IgM-secreting cells, few B-1 cells and their serum IgM levels increase dramatically during the first few weeks of life. This was shown already in the 1970s, when early studies demonstrated that antibody-producing cells specific for phosphoryl choline encoded by the T15 idiotype, later found to be expressed nearly exclusively by B-1a cells, did not appear in the spleen and bone marrow until about 1 week after birth and were absent from the fetal liver (52, 53). Frequencies of these antigen-specific cells, while varying in size between individual mice, were present at overall similar frequencies in germ-free and conventionally housed animals, and the pool size was independent of the genotype of the mothers. Similarly, Yang and colleagues noted that the repertoire of B-1a cells in fetal liver and at birth differed from that of B-1a cells in both peritoneal cavity and spleen at weaning and in adulthood (49). Thus, B-1a cells and natural IgM titers undergo dramatic expansions and changes during the first few weeks after birth. While this timing coincides with the first exposure to microbiota and other environmental antigens, the similarities in B-1a cell BCR repertoires between gnotobiotic and SPF-housed mice suggest that this repertoire and the production of natural IgM are regulated in ontogeny, at least in part, by age-specific factors, which could include the emergence of, or exposure to, other cell types, and/or self-antigens.

Modulating the Pool of Natural IgM-Secreting Cells

In apparent contrast to these findings, early studies demonstrated that B-1 cells can respond to α1–3 dextran (54). More recent studies by Kearney and colleagues have provided evidence that

injection of polysaccharides found on the facultative pathogen, *Aspergillus fumigatus*, as well as on house dust mites, permanently altered the natural IgM repertoire when given during a brief window of development just after birth [summarized in Ref. (55)]. While they could not detect significant changes in serum IgM levels, consistent with a body of work demonstrating the inability of newborns to respond to T-independent antigens, their data suggested instead that permanent changes occur affecting the repertoire of polysaccharide-specific B cells. They further demonstrated that this allowed mice to respond to subsequent antigen exposure more robustly with antibody production and a shift away from harmful allergic humoral responses to support immune protective responses (56, 57). These studies are of significance as they further underscore the crucial effects of IgM on the maintenance of tissue and immune homeostasis. They also provide a potential link to and mechanism in support of the "hygiene hypothesis," which predicts that exposure to pathogens, at an early age, may prime the immune system in such a way that development of allergic reactions are less likely (55).

Apart from the semantic argument of whether IgM antibodies induced in response to foreign antigen exposure/vaccination should still be considered "natural antibodies," the more important question is why such foreign antigen-induced changes do not seem to affect the BCR repertoire of B-1a cells. Potential explanations are that these antigen-stimulated cells do not arise from the stimulation of B-1 cells, or that following antigen exposure these cells change their phenotype such that they are no longer detectable as part of the "B-1a cell pool." It will be of interest to study the emerging antigen-specific B cells in greater detail.

TISSUE-SPECIFIC SIGNALS AS MODULATORS OF B-1 CELL FUNCTIONS

Adoptive transfer of adult-derived peritoneal cavity B-1 cells into newborn mice seeds all major B-1 cell niches in spleen, body cavities, and bone marrow, such that frequencies of B-1 cells and the antibody-producing cells, and natural serum IgM levels, are at normal levels compared with non-manipulated mice (24, 41, 54). This includes reconstitution of the recently described population of IgM plasma cells in the bone marrow [Ref. (58); Savage et al., under review[1]], demonstrating their B-1 cell origins. This is quite remarkable, given the functional differences of B-1 cells in peritoneal and pleural cavity versus the spleen, as well as their extensive gene expression differences. It is also in apparent contrast to the above data regarding the differential developmental requirements of spleen and body cavity B-1 cells.

It is possible that B-1 cell populations in the body cavities are heterogeneous, containing distinct B-1 cell subsets that will seed the spleen, bone marrow, or body cavities, respectively. Alternatively, B-1 cells are homogenous but have the plasticity to adapt to tissue-specific signals that induce the phenotypic and functional alterations between B-1 cells in these different locations. Overall, scientific evidence to date seems to support the latter. Weiss and colleagues attempted to address this question by adoptively transferring peritoneal cavity B-1 cells and then measuring expression of a handful of genes they had identified as being differentially expressed by spleen and peritoneal cavity B-1 cells (59). Their data showed that the gene expression profile of the donor cells was dependent on their tissue location and not their tissue of origin, thus supporting the idea of tissue-specific signals modulating the gene expression profile of the transferred B-1 cells. However, single-cell transfer or fate-mapping approaches would be necessary to formally rule out that this is due to cell selection rather than tissue-induced changes.

One long known tissue-induced difference between B-1 cells in body cavity and spleen is their differential expression of the β-2 integrin CD11b. While most peritoneal cavity B-1 cells express CD11b, the integrin appears to be lost rapidly after B-1 cell leave the body cavities and enter lymphoid tissues. The small number of $CD11b^-$ B-1 cells in the body cavities appears to identify recent arrivals to that site [reviewed in Ref. (4)]. While the tissue-specific signals that induce expression of CD11b on B-1 cells in the body cavities are unknown, we recently demonstrated one of its functions. Specifically, we showed that CD11b was required for effective homing of B-1a cells from body cavities to draining lymph nodes after infection (60). The lack of CD11b did not affect the emigration of B-1a cells from the body cavities. Instead, it was required for the enhanced accumulation of B-1a cells to the lymph nodes. Given that B-1a cells rapidly lose CD11b expression upon entering secondary lymphoid tissues, it appears that the interaction of CD11b with its ligand enhances the entrance of B-1a cells from the blood or lymphatic vessels into the lymph tissue. Thus, CD11b expression by B-1 cells is actively modulated by tissue-specific and/or inflammatory signals.

The abovementioned repertoire differences in BCR expression between B-1 cells in spleen and body cavity may also suggest the presence of tissue-specific factors that drive B-1 cell clonal expansion and/or selection. In the absence of obvious effects of the microbiota on these changes, tissue-restricted expression of autoantigens may account for the distinct repertoires of B-1 cells in different sites. However, this would then also suggest that the B-1 cell compartments of spleen, bone marrow, and body cavity do not usually interchange much. Yet, when we measured the migration of B-1 cells from the body cavity to other tissues, we found that labeled B-1 cells (labeled either with radioisotopes or fluorescent dyes) rapidly disappeared from these sites and then be found in the blood, indicating that there is continuous circulation of body cavity B-1 cells (60).

Another well explored difference between B-1 cells in body cavities and spleen/bone marrow is the fact that spontaneous "natural" IgM production is largely restricted to cells in spleen and bone marrow (41). An earlier study suggested that body cavity macrophages suppress B-1 cell antibody production *via* production of prostaglandin (61). Indeed, isolation of B-1 cells often leads to a larger production of IgM *in vitro*, than culturing cells in the context of the entire cell populations found in the cavities (41). That data fit well with the lack of spontaneous antibody production by B-1 cells in the body cavities and the fact that in response to stimulation, body cavity B-1 cells rapidly migrate to

[1]Savage, H.P., Yenson, V.M., and Baumgarth, N. (2016). Blimp-1 dependent and independent production of natural Ig by B-1 cells and B-1-derived plasma cells. submitted.

the environments of the spleen and lymph nodes, where they begin to secrete antibodies in response to innate and possibly antigen-specific stimulatory signals (5, 17, 60, 62–65).

Finally, early studies by Kroese and colleagues demonstrated that nearly half of the IgA-secreting plasma cells in the gut are B-1 cell derived (66, 67). Using allotype-chimeric mice similar to studies described by Kroese et al., we found that in the lung of young adult BALB/c mice about one-third of IgA-expressing cells to be B-1 cells (Baumgarth, unpublished)[2]. Based on *in vitro* and *in vivo* studies, it appears that B-1 cells like their B-2 counterparts require TGFβ-signaling for class-switch recombination to IgA, as TGFβ receptor-deficient mice are devoid of all IgA production (68). It is possible that activation of CD11b on peritoneal cavity B-1 cells drives their accumulation in the mesenteric lymph nodes in a manner similar to the activation described by us for B-1 cells from the pleural cavity and their migration to the mediastinal lymph nodes of the lung (60).

While many questions remain, overall there is strong evidence that the tissue environment contributes to the distinct phenotypes and functions of B-1 cells in each tissue compartment.

B-1 CELL RESPONSIVENESS TO SELF- AND FOREIGN ANTIGENS

B-1 Cell Responses to Foreign Antigens

The removal of tissue (body cavity)-specific inhibitory signals as a mechanism of regulation for B-1 cell antibody production is an attractive solution to the conundrum of B-1a cell response regulation, a cell that appears unable to respond to anti-IgM induced BCR cross-linking with proliferation or IgM production *in vitro* (69), yet seems to undergo at least limited clonal expansion in response to some antigen-specific signals *in vivo* (see above). However, this unlikely comprises the entire regulatory network controlling B-1 cell activation. Other signals that are known to induce B-1 cell activation include cytokines, specifically IL-5 and IL-10 (70). Lack of IL-5 or its receptor reduces B-1 cell numbers and natural IgM levels in mice (71, 72), including the levels of the classical T15 idiotype-expressing IgM, which binds oxidized low-density lipoproteins and pneumococcal polysaccharides (73). Overexpression of IL-5 *in vivo* (74, 75), exposure of B-1 cells to IL-5 *in vitro*, and injection of IL-5 into the peritoneal cavity *in vivo* induce secretion of IgM following the migration of B-1 cells to the spleen (70).

Consistent with the role of body cavity B-1 cells as surveyors of organ system health and rapid responders to an infection, injection of LPS (76), or bacteria (65) into the peritoneal cavity induces their rapid migration to lymph tissues, where they differentiate to antibody-producing cells. Activation of B-1 cells in the latter case was shown to be dependent on the innate signaling adaptor MyD88 (65) and thus appears mediated by innate rather than antigen-specific B-1 cell stimuli. Similarly, following respiratory tract infection of mice with influenza infection, we noted a rapid drop of B-1a, but not B-1b cells, in the pleural cavity and a concomitant accumulation of these cells in the regional lymph nodes of the respiratory tract (60). The enhanced infection-induced accumulation, but not the migration itself, was dependent on direct signaling *via* the type I IFNR by pleural cavity B-1a cells. Type I IFNR signaling was shown to activate the β-2 integrin CD11b to a high-affinity state, which can bind its ligands and thereby mediate the transfer of B-1 cells across the endothelium (60).

Importantly, in the latter case, the frequency of influenza-binding B-1 cells among those accumulating in the lymph nodes was not significantly different from those in the spleen before influenza infection (17). Also, B-1a cells that accumulated in the lymph nodes did not incorporate significant levels of BrDU, suggesting that these cells responded to influenza infection by relocation and IgM production in an antigen non-specific manner, rather than clonal expansion (17). Yet, following application of non-mitogenic LPS from *Francisella tularensis*, modest expansion of antigen-specific B-1a cells and even formation of memory B cells seemed to occur (5, 6), suggesting that the lack of B-1a cell expansion is not absolute and also depend on the type of antigen.

While responses to both influenza virus infection and non-mitogenic LPS from *F. tularensis* were shown to primarily activate B-1a cells (5), infections with *B. hermsii* (7), *S. pneumonia* (14), and *Salmonella* (10, 11) were shown to stimulate predominantly or exclusively B-1b. In response to infection with *Borrelia burgdorferi*, we found neither B-1a nor B-1b cells to respond in any significant fashion (Hastey et al., in preparation)[3]. What determines this differential responsiveness to antigens and pathogens is unexplored. Possible explanations include the BCR repertoire (50, 51, 77), i.e., differential antigen-specificity among each B cell subset, the site of infection/injection, the quality of the induced innate responses, or other factors. Addressing this question will be important to gain a more complete understanding of the signals that induce B-1 cell responses.

B-1 Cells and Self-Antigen Recognition

A final consideration for regulation of IgM production by B-1 cells is their responsiveness to self-antigens. Elegant studies by Hayakawa and colleagues demonstrated that the lack of self-antigen expression (in that case the T cell-expressed antigen "Thy-1") caused a failure to develop self-reactive B-1a cells to that specificity (78), usually a normal component of the B-1a cell repertoire of wild-type mice (79). This data provided the strongest evidence to date that the B-1a cell repertoire is selected for binding to self. Consistent with a positive selection event being necessary for the development of B-1a cells are data indicating that signals that enhance BCR-signaling usually cause increased B-1 cell pools, while mutations reducing BCR-signaling reduce such pools. Examples are deficiencies in the coreceptor CD19 or signaling molecule Btk, causing reduction of B-1a cell numbers, and deficiencies in coinhibitory molecules, such as CD72 increasing these pools [summarized in Ref. (3)]. Thus, positive selection and BCR-signaling appear to be critical elements of B-1a cell development. This is consistent with the resulting B-1 repertoire,

[2] Baumgarth, N. (2016). Unpublished observation.

[3] Hastey, C. J., Elsner, R. A., Olsen, K. J., Tunev, S. S., Escobar, E. D., Barthold, S. W., et al. (2016). Borrelia burgdorferi infection induced-IgM controls bacteremia but not bacterial dissemination or tissue burden. in preparation.

which is clearly skewed toward the recognition of self-molecules; particularly those expressed when tissues are altered, stressed, or senescent. Examples are oxidized low-density lipoproteins and antigens expressed by dead and dying cells. The binding of natural IgM to such antigens is thought to fulfill "housekeeping" functions by opsonizing these antigens for removal by phagocytic cells, such as macrophages, while avoiding or actively inhibiting inflammation (80). In certain disease states, however, the generation or accumulation of these antibodies correlates with disease. A prominent example is reperfusion injury, where the binding of natural IgM to tissues stressed or damaged by hypoxia following blood flow disruption is thought to activate the complement cascade and cause tissue injury (81–83).

The recently published comprehensive repertoire analysis conducted on B-1a cells confirmed and expanded previous studies, which together showed differences in the B-1a repertoire based on the age of the host (49). The study by Yang and colleagues reported quite dramatic alterations in the repertoire of spleen and peritoneal cavity B-1a cells, driven by the strong and unequal expansion of certain clones, right after weaning (3 weeks of age) (49). Since alterations in the microbiota were excluded as a potential cause, because similar changes were observed also in gnotobiotic mice, the data might suggest that alterations in, or exposure to certain self-antigens could be causing these repertoire shifts. However, other causes cannot be ruled out, in particular the potential influence of food antigens. The timing of these repertoire changes appears to occur later than the time window identified by Kearney and colleagues by which the B-1 repertoire to certain polysaccharides is amenable to change (55). The fact that B-1 cells have a phenotype similar to activated cells: they express CD43, lack expression of CD23, and have low expression of CD45R (B220) and IgD, could further support the idea that their arrival in the peritoneal cavity and clonal outgrowth is the result of encounter with self-antigens.

Natural IgM Production and B-1 Cell Repertoires Are Independently Regulated

It is important to distinguish between changes in the repertoire of the overall B-1 cell pool and the extent to which these changes affect the repertoire of the natural IgM-producing cells and thus the serum IgM specificities. Natural IgM-producing cells are distinct from peritoneal cavity B-1 cells and represent only a small subset of B-1 cells in the spleen, currently not distinguishable from non-secreting cells with surface markers. Although a small subset of $CD19^+$ $CD43^+$ B-1 cells expresses CD138 (84), these are not the major natural IgM producers in the spleen (Savage et al., under review)[1]. Therefore, it should not be assumed that the identified repertoire shifts within the B-1a cell pool necessarily affect the specificity of the secreted natural IgM. It will be important to identify and characterize the B-1 cell-derived natural IgM-producing cells in ontogeny and to compare their repertoire to that of the non-secreting B-1 cells.

The extent to which the secreted antibody repertoire is affected by age and/or antigen exposure is an important question, as this could have implication for immune defense and tissue homeostasis. Indeed, Holodick and colleagues recently provided evidence that IgG-depleted serum from very old mice lost its protective capacity for protection from *S. pneumoniae* infection compared with the same serum preparation from young mice (32). Thus, indicating that both B-1a cell repertoires and secreted IgM repertoires, or their effector functions, can shift with age. What underlies such shifts remains unresolved, given that much of the B-1 cell pool is maintained mainly through self-renewal rather than *de novo* development of B-1 cells, with bone marrow output although not completely abrogated, rather greatly constrained (23). Furthermore, although Patel and Kearney found that immunization during the first few days after birth can alter B-1 cell repertoires, they found no evidence for alterations in the natural IgM serum pool (57), again suggesting that B-1 cell repertoires and natural IgM production are independently regulated and differentially affected by innate and antigen-induced signals.

CONCLUSION

The heterogeneity of the B-1 cell precursors, including extra-hematopoietic origins, ontogenetic, and tissue-specific effects, that seem to act on these cells and the high degree of functional plasticity and ability to respond to self- and inflammatory signals generates a diverse set of B-1 cells. Some have effector functions such as IgM secretion or cytokine production. Others, particularly those in the body cavities, appear to be poised to function a natural memory compartment and rapid response system that responds to innate signals with rapid relocation and differentiation.

There are some striking similarities between B-1 cells and macrophages in terms of their developmental origins and effector functions. Not because of a shared ontogeny or phenotype, but rather because of their ontogenic and functional plasticity. Macrophages and B-1 cells both develop in waves from extra-hematopoietic and hematopoietic stem cell precursors (21). They both seed various tissues and differentiate based, at least in part, in response to tissue-specific signals (85). Whether some B-1 cells may also differentiate to become tissue restricted, such as microglia, alveolar macrophages, or other tissue-specific macrophages, remains to be studied. Both cell types also respond rapidly to innate signals with relocation and on-site differentiation. And finally, both cell types fulfill important "housekeeping functions," in order to maintain tissue homeostasis and to facilitate tissue repair (86). A better understanding of the plasticity and the heterogeneity among B-1 cells will be crucial to better understand the principles underlying their functions and responses.

AUTHOR CONTRIBUTION

NB conceived and wrote the manuscript.

ACKNOWLEDGMENTS

Work from our laboratory cited in this review article was supported by grants from the NIH/NIAID R01AI051354, R01AI085568, and U19AI109962. Stimulating discussions with current and previous members of the laboratory are gratefully acknowledged.

REFERENCES

1. Flajnik MF. Comparative analyses of immunoglobulin genes: surprises and portents. *Nat Rev Immunol* (2002) 2(9):688–98. doi:10.1038/nri889
2. Lalor PA. An evolutionarily-conserved role for murine Ly-1 B cells in protection against bacterial infections. *Autoimmunity* (1991) 10(1):71–6. doi:10.3109/08916939108997150
3. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* (2002) 20:253–300. doi:10.1146/annurev.immunol.20.100301.064833
4. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) 11(1):34–46. doi:10.1038/nri2901
5. Cole LE, Yang Y, Elkins KL, Fernandez ET, Qureshi N, Shlomchik MJ, et al. Antigen-specific B-1a antibodies induced by *Francisella tularensis* LPS provide long-term protection against *F. tularensis* LVS challenge. *Proc Natl Acad Sci U S A* (2009) 106(11):4343–8. doi:10.1073/pnas.0813411106
6. Yang Y, Ghosn EE, Cole LE, Obukhanych TV, Sadate-Ngatchou P, Vogel SN, et al. Antigen-specific antibody responses in B-1a and their relationship to natural immunity. *Proc Natl Acad Sci U S A* (2012) 109(14):5382–7. doi:10.1073/pnas.1121631109
7. Alugupalli KR, Gerstein RM, Chen J, Szomolanyi-Tsuda E, Woodland RT, Leong JM. The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *J Immunol* (2003) 170(7):3819–27. doi:10.4049/jimmunol.170.7.3819
8. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* (2004) 21(3):379–90. doi:10.1016/j.immuni.2004.06.019
9. Colombo MJ, Alugupalli KR. Complement factor H-binding protein, a putative virulence determinant of *Borrelia hermsii*, is an antigenic target for protective B1b lymphocytes. *J Immunol* (2008) 180(7):4858–64. doi:10.4049/jimmunol.180.7.4858
10. Gil-Cruz C, Bobat S, Marshall JL, Kingsley RA, Ross EA, Henderson IR, et al. The porin OmpD from nontyphoidal *Salmonella* is a key target for a protective B1b cell antibody response. *Proc Natl Acad Sci U S A* (2009) 106(24):9803–8. doi:10.1073/pnas.0812431106
11. Cunningham AF, Flores-Langarica A, Bobat S, Dominguez Medina CC, Cook CN, Ross EA, et al. B1b cells recognize protective antigens after natural infection and vaccination. *Front Immunol* (2014) 5:535. doi:10.3389/fimmu.2014.00535
12. Cosenza H, Kohler H. Specific suppression of the antibody response by antibodies to receptors. *Proc Natl Acad Sci U S A* (1972) 69(9):2701–5. doi:10.1073/pnas.69.9.2701
13. Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *J Exp Med* (1981) 153(3):694–705. doi:10.1084/jem.153.3.694
14. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity* (2005) 23(1):7–18. doi:10.1016/j.immuni.2005.04.011
15. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* (1999) 96(5):2250–5. doi:10.1073/pnas.96.5.2250
16. Baumgarth N, Chen J, Herman OC, Jager GC, Herzenberg LA. The role of B-1 and B-2 cells in immune protection from influenza virus infection. *Curr Top Microbiol Immunol* (2000) 252:163–9.
17. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med* (2008) 205(13):3053–64. doi:10.1084/jem.20080979
18. Godin IE, Garcia-Porrero JA, Coutinho A, Dieterlen-Lievre F, Marcos MA. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. *Nature* (1993) 364(6432):67–70. doi:10.1038/364067a0
19. Yoshimoto M, Montecino-Rodriguez E, Ferkowicz MJ, Porayette P, Shelley WC, Conway SJ, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci U S A* (2011) 108(4):1468–73. doi:10.1073/pnas.1015841108
20. Kobayashi M, Shelley WC, Seo W, Vemula S, Lin Y, Liu Y, et al. Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on Cbfbeta for their development. *Proc Natl Acad Sci U S A* (2014) 111(33):12151–6. doi:10.1073/pnas.1407370111
21. Perdiguero EG, Geissmann F. The development and maintenance of resident macrophages. *Nat Immunol* (2016) 17(1):2–8. doi:10.1038/ni.3341
22. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol* (2006) 7(3):293–301. doi:10.1038/ni1301
23. Barber CL, Montecino-Rodriguez E, Dorshkind K. Reduced production of B-1-specified common lymphoid progenitors results in diminished potential of adult marrow to generate B-1 cells. *Proc Natl Acad Sci U S A* (2011) 108(33):13700–4. doi:10.1073/pnas.1107172108
24. Lalor PA, Herzenberg LA, Adams S, Stall AM. Feedback regulation of murine Ly-1 B cell development. *Eur J Immunol* (1989) 19(3):507–13. doi:10.1002/eji.1830190315
25. Lalor PA, Stall AM, Adams S, Herzenberg LA. Permanent alteration of the murine Ly-1 B repertoire due to selective depletion of Ly-1 B cells in neonatal animals. *Eur J Immunol* (1989) 19(3):501–6. doi:10.1002/eji.1830190314
26. Duber S, Hafner M, Krey M, Lienenklaus S, Roy B, Hobeika E, et al. Induction of B-cell development in adult mice reveals the ability of bone marrow to produce B-1a cells. *Blood* (2009) 114(24):4960–7. doi:10.1182/blood-2009-04-218156
27. Holodick NE, Repetny K, Zhong X, Rothstein TL. Adult BM generates CD5+ B1 cells containing abundant N-region additions. *Eur J Immunol* (2009) 39(9):2383–94. doi:10.1002/eji.200838920
28. Hardy RR, Hayakawa K. Perspectives on fetal derived CD5+ B1 B cells. *Eur J Immunol* (2015) 45(11):2978–84. doi:10.1002/eji.201445146
29. Yuan J, Nguyen CK, Liu X, Kanellopoulou C, Muljo SA. Lin28b reprograms adult bone marrow hematopoietic progenitors to mediate fetal-like lymphopoiesis. *Science* (2012) 335(6073):1195–200. doi:10.1126/science.1216557
30. Zhou Y, Li YS, Bandi SR, Tang L, Shinton SA, Hayakawa K, et al. Lin28b promotes fetal B lymphopoiesis through the transcription factor Arid3a. *J Exp Med* (2015) 212(4):569–80. doi:10.1084/jem.20141510
31. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* (2000) 192(2):271–80. doi:10.1084/jem.192.2.271
32. Holodick NE, Vizconde T, Hopkins TJ, Rothstein TL. Age-related decline in natural IgM function: diversification and selection of the B-1a cell pool with age. *J Immunol* (2016) 196(10):4348–57. doi:10.4049/jimmunol.1600073
33. Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity* (2012) 36(1):13–21. doi:10.1016/j.immuni.2011.11.017
34. Nguyen TT, Elsner RA, Baumgarth N. Natural IgM prevents autoimmunity by enforcing B cell central tolerance induction. *J Immunol* (2015) 194(4):1489–502. doi:10.4049/jimmunol.1401880
35. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* (1998) 160(10):4776–87.
36. Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A* (1998) 95:10089–1093. doi:10.1073/pnas.95.17.10089
37. Dexter CM, Corley RB. Expression of the Fc gamma receptor on Ly-1+ B lymphocytes. *Eur J Immunol* (1987) 17(6):867–71. doi:10.1002/eji.1830170621
38. Wardemann H, Boehm T, Dear N, Carsetti R. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J Exp Med* (2002) 195(6):771–80. doi:10.1084/jem.20011140
39. Kretschmer K, Stopkowicz J, Scheffer S, Greten TF, Weiss S. Maintenance of peritoneal B-1a lymphocytes in the absence of the spleen. *J Immunol* (2004) 173(1):197–204. doi:10.4049/jimmunol.173.1.197
40. Hooijkaas H, Benner R, Pleasants JR, Wostmann BS. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. *Eur J Immunol* (1984) 14(12):1127–30. doi:10.1002/eji.1830141212
41. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol* (2012) 42(1):120–9. doi:10.1002/eji.201141890

42. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci U S A* (2000) 97(3):1184–9. doi:10.1073/pnas.97.3.1184
43. Ouchida R, Mori H, Hase K, Takatsu H, Kurosaki T, Tokuhisa T, et al. Critical role of the IgM Fc receptor in IgM homeostasis, B-cell survival, and humoral immune responses. *Proc Natl Acad Sci U S A* (2012) 109(40):E2699–706. doi:10.1073/pnas.1210706109
44. Avrech OM, Samra Z, Lazarovich Z, Caspi E, Jacobovich A, Sompolinsky D. Efficacy of the placental barrier for immunoglobulins: correlations between maternal, paternal and fetal immunoglobulin levels. *Int Arch Allergy Immunol* (1994) 103(2):160–5. doi:10.1159/000236622
45. Ben-Hur H, Gurevich P, Elhayany A, Avinoach I, Schneider DF, Zusman I. Transport of maternal immunoglobulins through the human placental barrier in normal pregnancy and during inflammation. *Int J Mol Med* (2005) 16(3):401–7. doi:10.3892/ijmm.16.3.401
46. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* (2002) 16(1):67–76. doi:10.1016/S1074-7613(01)00257-6
47. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J Immunol* (1989) 142(10):3643–51.
48. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G. Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J Exp Med* (1988) 168(2):687–98. doi:10.1084/jem.168.2.687
49. Yang Y, Wang C, Yang Q, Kantor AB, Chu H, Ghosn EE, et al. Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *Elife* (2015) 4:e09083. doi:10.7554/eLife.09083
50. Kantor AB. V-gene usage and N-region insertions in B-1a, B-1b and conventional B cells. *Semin Immunol* (1996) 8(1):29–35. doi:10.1006/smim.1996.0005
51. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* (1997) 158(3):1175–86.
52. Sigal NH, Pickard AR, Metcalf ES, Gearhart PJ, Klinman NR. Expression of phosphorylcholine-specific B cells during murine development. *J Exp Med* (1977) 146(4):933–48. doi:10.1084/jem.146.4.933
53. Fung J, Kohler H. Late clonal selection and expansion of the TEPC-15 germ-line specificity. *J Exp Med* (1980) 152(5):1262–73. doi:10.1084/jem.152.5.1262
54. Forster I, Rajewsky K. Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic, newborn mice. *Eur J Immunol* (1987) 17(4):521–8. doi:10.1002/eji.1830170414
55. Kearney JF, Patel P, Stefanov EK, King RG. Natural antibody repertoires: development and functional role in inhibiting allergic airway disease. *Annu Rev Immunol* (2015) 33:475–504. doi:10.1146/annurev-immunol-032713-120140
56. Kin NW, Stefanov EK, Dizon BL, Kearney JF. Antibodies generated against conserved antigens expressed by bacteria and allergen-bearing fungi suppress airway disease. *J Immunol* (2012) 189(5):2246–56. doi:10.4049/jimmunol.1200702
57. Patel PS, Kearney JF. Neonatal exposure to pneumococcal phosphorylcholine modulates the development of house dust mite allergy during adult life. *J Immunol* (2015) 194(12):5838–50. doi:10.4049/jimmunol.1500251
58. Reynolds AE, Kuraoka M, Kelsoe G. Natural IgM is produced by CD5- plasma cells that occupy a distinct survival niche in bone marrow. *J Immunol* (2015) 194(1):231–42. doi:10.4049/jimmunol.1401203
59. Stoermann B, Kretschmer K, Duber S, Weiss S. B-1a cells are imprinted by the microenvironment in spleen and peritoneum. *Eur J Immunol* (2007) 37(6):1613–20. doi:10.1002/eji.200636640
60. Waffarn EE, Hastey CJ, Dixit N, Soo Choi Y, Cherry S, Kalinke U, et al. Infection-induced type I interferons activate CD11b on B-1 cells for subsequent lymph node accumulation. *Nat Commun* (2015) 6:8991. doi:10.1038/ncomms9991
61. Chace JH, Fleming AL, Gordon JA, Perandones CE, Cowdery JS. Regulation of differentiation of peritoneal B-1a (CD5+) B cells. Activated peritoneal macrophages release prostaglandin E2, which inhibits IgM secretion by peritoneal B-1a cells. *J Immunol* (1995) 154(11):5630–6.
62. Ohdan H, Swenson KG, Kruger Gray HS, Yang YG, Xu Y, Thall AD, et al. Mac-1-negative B-1b phenotype of natural antibody-producing cells, including those responding to Gal alpha 1,3Gal epitopes in alpha 1,3-galactosyltransferase-deficient mice. *J Immunol* (2000) 165(10):5518–29. doi:10.4049/jimmunol.165.10.5518
63. Watanabe N, Ikuta K, Fagarasan S, Yazumi S, Chiba T, Honjo T. Migration and differentiation of autoreactive B-1 cells induced by activated gamma/delta T cells in antierythrocyte immunoglobulin transgenic mice. *J Exp Med* (2000) 192(11):1577–86. doi:10.1084/jem.192.11.1577
64. Kawahara T, Ohdan H, Zhao G, Yang YG, Sykes M. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. *J Immunol* (2003) 171(10):5406–14. doi:10.4049/jimmunol.171.10.5406
65. Ha SA, Tsuji M, Suzuki K, Meek B, Yasuda N, Kaisho T, et al. Regulation of B1 cell migration by signals through toll-like receptors. *J Exp Med* (2006) 203(11):2541–50. doi:10.1084/jem.20061041
66. Kroese FG, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg LA. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *Int Immunol* (1989) 1(1):75–84. doi:10.1093/intimm/1.1.75
67. Kroese FG, de Waard R, Bos NA. B-1 cells and their reactivity with the murine intestinal microflora. *Semin Immunol* (1996) 8(1):11–8. doi:10.1006/smim.1996.0003
68. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity* (2000) 13(4):443–51. doi:10.1016/S1074-7613(00)00044-3
69. Morris DL, Rothstein TL. Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes. *J Exp Med* (1993) 177(3):857–61. doi:10.1084/jem.177.3.857
70. Nisitani S, Tsubata T, Murakami M, Honjo T. Administration of interleukin-5 or -10 activates peritoneal B-1 cells and induces autoimmune hemolytic anemia in anti-erythrocyte autoantibody-transgenic mice. *Eur J Immunol* (1995) 25(11):3047–52. doi:10.1002/eji.1830251110
71. Yoshida T, Ikuta K, Sugaya H, Maki K, Takagi M, Kanazawa H, et al. Defective B-1 cell development and impaired immunity against *Angiostrongylus cantonensis* in IL-5R alpha-deficient mice. *Immunity* (1996) 4(5):483–94. doi:10.1016/S1074-7613(00)80414-8
72. Moon BG, Takaki S, Miyake K, Takatsu K. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. *J Immunol* (2004) 172(10):6020–9. doi:10.4049/jimmunol.172.10.6020
73. Chen Y, Duan Y, Kang Y, Yang X, Jiang M, Zhang L, et al. Activation of liver X receptor induces macrophage interleukin-5 expression. *J Biol Chem* (2012) 287(52):43340–50. doi:10.1074/jbc.M112.403394
74. Vaux DL, Lalor PA, Cory S, Johnson GR. In vivo expression of interleukin 5 induces an eosinophilia and expanded Ly-1B lineage populations. *Int Immunol* (1990) 2(10):965–71. doi:10.1093/intimm/2.10.965
75. Tominaga A, Takaki S, Koyama N, Katoh S, Matsumoto R, Migita M, et al. Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production. *J Exp Med* (1991) 173(2):429–37. doi:10.1084/jem.173.2.429
76. Murakami M, Tsubata T, Shinkura R, Nisitani S, Okamoto M, Yoshioka H, et al. Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. *J Exp Med* (1994) 180(1):111–21. doi:10.1084/jem.180.1.111
77. Tornberg UC, Holmberg D. B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. *EMBO J* (1995) 14(8):1680–9.
78. Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, et al. Positive selection of natural autoreactive B cells. *Science* (1999) 285(5424):113–6. doi:10.1126/science.285.5424.113
79. Hayakawa K, Carmack CE, Hyman R, Hardy RR. Natural autoantibodies to thymocytes: origin, VH genes, fine specificities, and the role of Thy-1 glycoprotein. *J Exp Med* (1990) 172(3):869–78. doi:10.1084/jem.172.3.869
80. Vas J, Gronwall C, Silverman GJ. Fundamental roles of the innate-like repertoire of natural antibodies in immune homeostasis. *Front Immunol* (2013) 4:4. doi:10.3389/fimmu.2013.00004

81. Rahyab AS, Alam A, Kapoor A, Zhang M. Natural antibody – biochemistry and functions. *Glob J Biochem* (2011) 2(4):283–8.
82. van der Pol P, Roos A, Berger SP, Daha MR, van Kooten C. Natural IgM antibodies are involved in the activation of complement by hypoxic human tubular cells. *Am J Physiol Renal Physiol* (2011) 300(4):F932–40. doi:10.1152/ajprenal.00509.2010
83. Elvington A, Atkinson C, Kulik L, Zhu H, Yu J, Kindy MS, et al. Pathogenic natural antibodies propagate cerebral injury following ischemic stroke in mice. *J Immunol* (2012) 188(3):1460–8. doi:10.4049/jimmunol.1102132
84. Holodick NE, Vizconde T, Rothstein TL. Splenic B-1a cells expressing CD138 spontaneously secrete large amounts of immunoglobulin in naive mice. *Front Immunol* (2014) 5:129. doi:10.3389/fimmu.2014.00129
85. Okabe Y, Medzhitov R. Tissue biology perspective on macrophages. *Nat Immunol* (2016) 17(1):9–17. doi:10.1038/ni.3320
86. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* (2005) 5(12):953–64. doi:10.1038/nri1733

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 20 June 2017
doi: 10.3389/fimmu.2017.00672

CD25$^+$ B-1a Cells Express *Aicda*

Hiroaki Kaku[1†‡], Nichol E. Holodick[1‡], Joseph R. Tumang[2†] and Thomas L. Rothstein[1,3,4†]*

[1] Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, Manhasset, NY, United States, [2] Compass Therapeutics LLC, Cambridge, MA, United States, [3] Department of Medicine, The Hofstra Northwell School of Medicine, Manhasset, NY, United States, [4] Department of Molecular Medicine, The Hofstra Northwell School of Medicine, Manhasset, NY, United States

OPEN ACCESS

Edited by:
Harry W. Schroeder, University of Alabama at Birmingham, United States

Reviewed by:
Paulo Vieira, Institut Pasteur de Paris, France
Paolo Casali, The University of Texas Health Science Center San Antonio, United States

***Correspondence:**
Hiroaki Kaku
hiroaki.kaku@med.wmich.edu

†Present address:
Hiroaki Kaku, Nichol E. Holodick and Thomas L. Rothstein, Center for Immunobiology, Western Michigan University Homer Stryker MD School of Medicine, Kalamazoo, MI, United States; Joseph R. Tumang, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, United States

‡These authors have contributed equally to this work.

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: 16 September 2016
Accepted: 23 May 2017
Published: 20 June 2017

Citation:
Kaku H, Holodick NE, Tumang JR and Rothstein TL (2017) CD25$^+$ B-1a Cells Express Aicda. Front. Immunol. 8:672. doi: 10.3389/fimmu.2017.00672

B-1a cells are innate-like B-lymphocytes producing natural antibodies. Activation-induced cytidine deaminase (AID), a product of the *Aicda* gene, plays a central role in class-switch recombination and somatic hypermutation in B cells. Although a role for *Aicda* in B-1a cells has been suggested on the basis of experiments with knock out (KO) mice, whether B-1a cells express *Aicda*, and if so, which B-1a cell subpopulation expresses *Aicda*, remains unknown. Here, we demonstrate that B-1 cells express *Aicda*, but at a level below that expressed by germinal center (GC) B cells. We previously reported that B-1a cells can be subdivided based on CD25 expression. We show here that B-1a cell *Aicda* expression is concentrated in the CD25$^+$ B-1a cell subpopulation. These results suggest the possibility that previous studies of memory B cells identified on the basis of *Aicda* expression may have inadvertently included an unknown number of CD25$^+$ B-1a cells. Although B-1a cells develop normally in the absence of *Aicda*, a competitive reconstitution assay reveals enhanced vigor for AID KO B-1a cell bone marrow (BM) progenitors, as compared with wild-type BM B-1 cell progenitors. These results suggest that AID inhibits the development of B-1a cells from BM B-1 cell progenitors in a competitive environment.

Keywords: AID, B-1a cells, CD25, B-1 cell subset, peritoneal cavity

INTRODUCTION

B-1 cells are innate-like B-lymphocytes that spontaneously and constitutively produce natural antibodies, which provide immediate protection against infection and rapid removal of dying cell debris (1, 2). Mouse B-1 cells are distinguished from B-2 cells both by phenotype and by function (3). Phenotypically, B-1 cells are characterized as IgM^{hi}, IgD^{lo}, B220^{lo}, CD23$^-$, and CD43$^+$ (and CD11b$^+$ in the peritoneal cavity) (3). B-1 cells are either CD5$^+$ (B-1a) or CD5$^-$ (B-1b). Recent studies have shown B-1 cells can be further subdivided based on the expression of CD25 (4), CD73 (5), PD-L2 (CD273) (6–9), or ENPP1 (PC1) (10), suggesting different roles for each subpopulation.

Activation-induced cytidine deaminase (AID) plays a central role in class-switch recombination and somatic hypermutation (11). AID is expressed abundantly in germinal center (GC) B cells (12) and at a low level in immature B cells (13–15). It was recently reported that B-1 cells accumulate immunoglobulin somatic hypermutation and increase class switching from 1 week of age up to 6 months of age, and these changes are diminished in the absence of AID (16). Nonetheless, AID expression in B-1 cells has not been documented and is yet to be directly addressed, raising the possibility that B-1 cell changes in AID KO mice may represent indirect effects.

We previously found that expression of CD25 on B-1a cells is activation dependent and these CD25$^+$ B-1a cells express leukemia inhibitory factor receptor as well as increased levels of activated STAT3 as compared to CD25$^-$ B-1a cells (4). We explored the possibility that B-1 cells express AID

and found that AID is expressed in B-1a cells and that this expression is concentrated in the activated, CD25+ B-1a cell pool.

MATERIALS AND METHODS

Mice

Male BALB/c-ByJ and C57BL/6 mice were obtained from The Jackson Laboratory at 6–8 weeks of age. CB17-SCID or CB17 mice of 6–8 weeks of age were obtained from Taconic. AID KO mice on a BALB/c background were obtained from Dr. Michel Nussenzweig with Dr. Tasuku Honjo's permission. All mice were used for experimentation at 8–14 weeks of age. All studies were approved by the Institutional Animal Care and Use Committee at the Feinstein Institute for Medical Research. Mice were cared for and handled in accordance with the National Institutes of Health and institutional guidelines.

Cell Purification and Flow Cytometry

Peritoneal washout cells and splenocytes were obtained from 8- to 14-week-old wild-type (WT) or AID knock out (KO) mice and were stained with fluorescence-labeled antibodies to B220, CD5, CD25, CD23, and GL-7 and with peanut agglutinin (PNA). B-cell populations (peritoneal B-1a cells: $B220^{lo}$/$CD5^+$, peritoneal CD25+ B-1a cells: $B220^{lo}$/$CD5^+CD25^+$, peritoneal CD25− B-1a cells: $B220^{lo}$/$CD5^+CD25^-$, splenic B2 cells: $B220^{hi}CD5^-CD23^+$, or GC B cells: $B220^+$/GL-7^+/PNA^{high}) were isolated using the Influx cell sorter (BD Biosciences). Post-sort, reanalysis of the B-cell populations showed them to be ≥98% pure. Cells were blocked with rat anti-mouse CD16/CD32 antibody (clone 2.4G2), stained with immunofluorescent antibodies, and then analyzed on a FACSCalibur flow cytometer (BD Biosciences) with appropriate gating. Images were constructed with FlowJo 6.0 software (Tree Star). PE-conjugated rat anti-mouse CD25 (clone PC61) was obtained from BD Pharmingen. The following antibodies were obtained from Biolegend: perCP-Cy5.5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2); Alexa 647-conjugated rat anti-mouse CD5 (clone 53-7.3); and Alexa 647-conjugated rat anti-mouse GL-7. FITC-PNA was obtained from Sigma. CD23-PE-Cy7 (clone 2G8) was obtained from Abcam.

Gene Expression

Gene expression was assayed by real-time PCR as previously described (17). Briefly, RNA was prepared from B cells using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. cDNA was prepared using avian myeloblastosis virus reverse transcriptase (Bio-Rad). Gene expression was then measured by real-time PCR using iTaq SYBR Green (Bio-Rad) and normalized with β_2-microglobulin. The following primer sets were used: β_2-microglobulin (F-CCCGCCTCACATTGAAATCC/R-GCGTATGTATCAGTCTCAGTGG); AID (AGAAAGTCACG CTGGAGACC/CTCCTCTTCACCACGTAGCA). Gene expression was also measured by real-time PCR using TaqMan chemistry. Primer and probe sets were obtained from Applied Biosystems for Aicda (Mm01184115_m1) and β-actin, which was used for normalization.

Adoptive Transfer

Bone marrow (BM) was obtained from 2-month-old BALB/c-ByJ (IgM^a) mice and 2-month-old CB17 mice (IgM^b). BM B-1a cell progenitors (lineage negative, $CD19^+B220^{lo/-}AA4.1^+$) were sort-purified using the Influx cell sorter (BD Biosciences), washed twice in 1× PBS, resuspended in 1× PBS, and then injected (i.v.) into recipient CB17-SCID mice at 0.6×10^6 cells per mouse in 0.2 ml. Recipient mice were not irradiated prior to transfer. Serum samples, spleens, and peritoneal washout cells were collected from euthanized CB17-SCID recipients 6 weeks post transfer.

Statistics

Comparisons were conducted between WT and AID KO mice using Graphpad Prism 6.0 with two-tailed tests as indicated in the figure legends.

RESULTS

B-1a Cells Express *Aicda* and Gene Expression Is Restricted to the CD25+ B-1a Cell Subset

The expression level of *Aicda* was evaluated in sort-purified peritoneal B-1a cells, peritoneal CD25+ B-1a cells (4), peritoneal CD25− B-1a cells, splenic B2 cells, and GC B cells from unmanipulated mice. The sorting strategy for isolating these populations is shown in **Figure 1A**. GC B cells displayed a high level of *Aicda* expression, which is consistent with previous reports (12), in contrast to splenic B-2 cells that expressed very little *Aicda*. We found that peritoneal B-1a cells expressed more *Aicda* than that by splenic B-2 cells, but less than that by GC B cells (**Figure 1B**). We then examined CD25+ B-1a cells in comparison to CD25− B-1a cells and found that CD25+ B-1a cells demonstrated a higher level of *Aicda* expression than did CD25− B-1a cells, total B-1a cells, and splenic B-2 cells, although this was still less than the level expressed by GC B cells. These results were confirmed using Taqman primers and probe (Figure S1 in Supplementary Material). Peritoneal CD25+ B-1a cells from C57BL/6 mice were also found to express *Aicda* in greater amounts than that by CD25− B-1a cells (Figure S2 in Supplementary Material). The mean level of *Aicda* expression in BALB/c CD25+ B-1a cells was 18-fold more than that of splenic B-2 cells but 40-fold less than that of GC B cells. Thus, B-1a cells, especially CD25+ B-1a cells, express *Aicda*.

The Number of CD25+ B-1a Cells Is Unchanged in AID KO

Mice lacking the AID gene on the BALB/c background were assessed for numbers of total peritoneal cells, total peritoneal lymphocytes, B-1a cells, CD25+ B-1a cells, and CD25− B-1a cells. There was no significant difference in the total number of peritoneal lymphocytes in AID KO mice ($4.3 \times 10^6 \pm 0.71$) compared to that in WT mice ($3.0 \times 10^6 \pm 0.17$) (**Figure 2A**), although the total number of cells in the peritoneal cavities of AID KO mice was greater than the number in WT mice, presumably due to

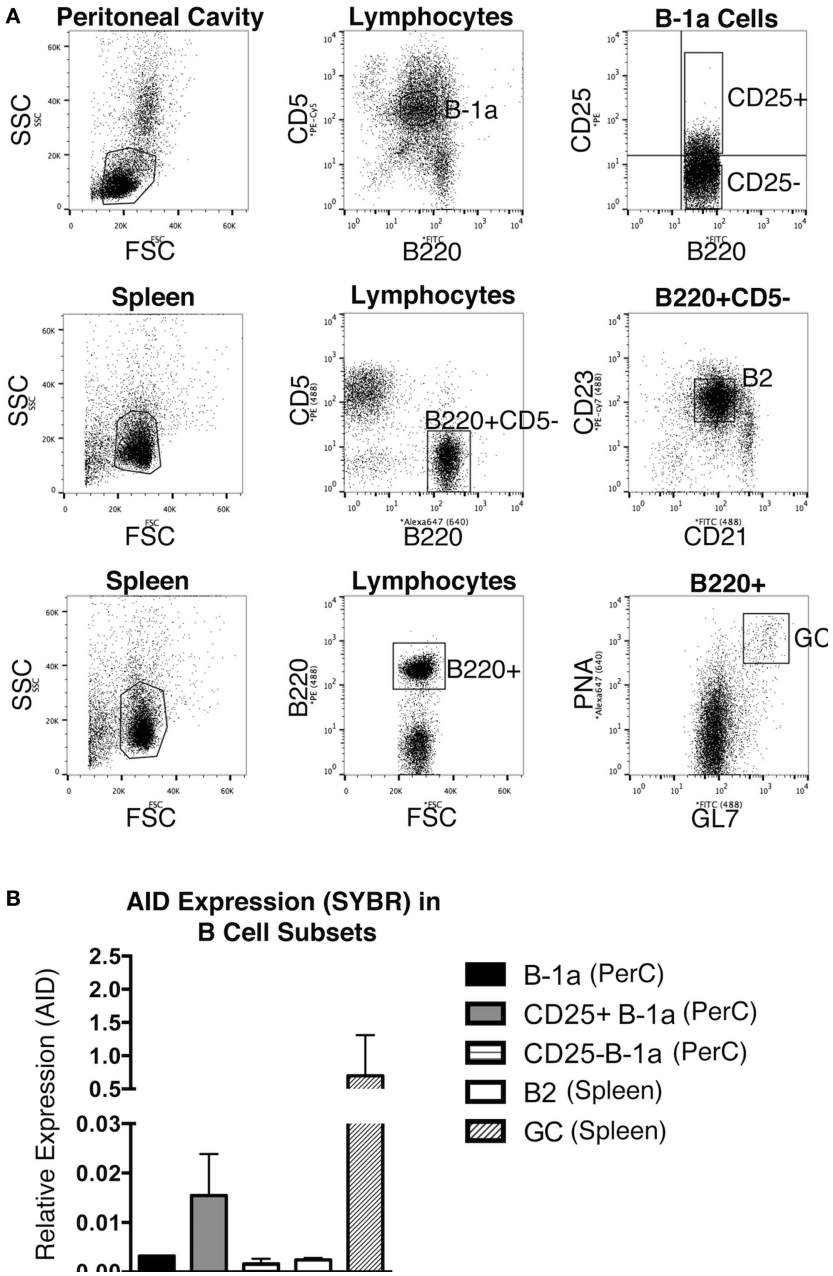


FIGURE 1 | *Aicda* gene expression in B cells. Peritoneal washout cells and spleen cells were obtained from 3-month-old BALB/c-ByJ mice, immunofluorescently stained, and sorted for peritoneal B-1a ($B220^{lo}CD5^{+}$), $CD25^{+}$ B-1a ($B220^{lo}CD5^{+}CD25^{+}$), $CD25^{-}$ B-1a ($B220^{lo}CD5^{+}CD25^{-}$), splenic B2 ($B220^{+}CD5^{-}CD23^{+}$), and germinal center (GC, $B220^{+}$/GL-7^{+}/PNA^{high}) cells. The sorting strategy for these populations is shown in **(A)**. RNA was prepared from each sort-purified B cell subset and reverse transcribed. **(A)** The level of *Acida* relative to β_2-microglobulin was determined by real-time PCR (SYBR Green) with the primers described in Section "Materials and Methods." The means of three independent experiments are shown in **(B)**, along with lines indicating SEMs.

differences in a non-lymphoid population, such as myeloid cells. Next, the total numbers of B-1a, $CD25^{+}$ B-1a, and $CD25^{-}$ B-1a cells were assessed in WT and AID KO mice. The results demonstrated that there is no significant difference in the total numbers of peritoneal B-1a, $CD25^{+}$ B-1a, or $CD25^{-}$ B-1a cells from AID KO mice compared to those in WT controls (**Figure 2B**). Thus, AID does not appear to be required for the development of early appearing $CD25^{+}$ or $CD25^{-}$ B-1a cells.

AID Impairs BM B-1a Cell Development

It has been previously shown that *Aicda* deficiency impairs B-cell development (13); however, it is unknown whether this effect extends to B-1a cell development. To directly test the extent to which *Aicda* affects B-1a cell development, we set up a mixed chimera system. **Figure 3A** illustrates the experimental design, which involved adoptive transfer of B-1 cell-specific progenitors ($Lin^{-}B220^{lo/-}CD19^{+}AA4.1^{+}$) obtained from the BM of AID KO

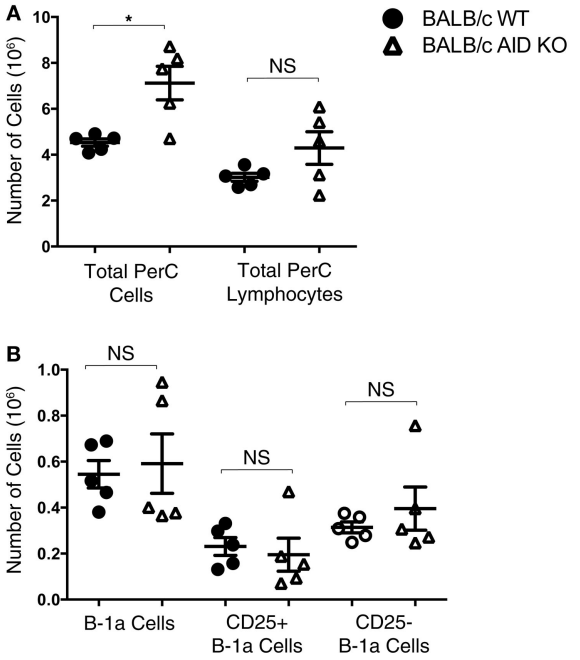


FIGURE 2 | Number of B-1a cells in activation-induced cytidine deaminase (AID) knock out (KO) mice. Peritoneal washout cells were obtained from 3-month-old wild-type (WT) and AID KO mice on the BALB/c-ByJ background. **(A)** The total number of peritoneal cells and the total number of lymphocytes (based on the lymphocyte gate) are shown. **(B)** Peritoneal washout cells were stained with anti-B220-pCP-Cy5.5, anti-CD5-Alexa 647, anti-CD25-PE, and CD23-PE-Cy7. Based on staining for B-1a ($B220^{lo}CD5^{+}$), $CD25^{+}$ B-1a ($B220^{lo}CD5^{+}CD25^{+}$), and $CD25^{-}$ B-1a ($B220^{lo}CD5^{+}CD25^{-}$), the absolute number of each subset is shown. Results are shown as means of five independent mice, along with lines indicating the SEMs.

mice and WT mice. Three groups of chimera mice were set up: (1) SCID mice were injected with 600,000 B-1-specific progenitors from the BM of BALB/c AID KO mice plus 600,000 B-1-specific progenitors from the BM of CB17 WT mice; (2) SCID mice were injected with 600,000 B-1-specific progenitors from the BM of BALB/c AID KO mice; and (3) SCID mice were injected with 600,000 B-1-specific progenitors from the BM of CB17 WT mice. Allotypic differences between BALB/c-ByJ (IgM^{a}) and CB17 (IgM^{b}) mice were used to assess the individual contributions of WT (IgM^{b})- and AID KO (IgM^{a})-derived B-1a cells to the B-1a cell pool.

We first examined the percent of total lymphocytes in the peritoneal washouts that were either IgM^{a+} or IgM^{b+}. Interestingly, in the chimera mice receiving B-1 cell progenitors from both AID KO and WT BM, there were more AID KO-derived (IgM^{a+}) B cells than WT-derived (IgM^{b+}) B cells ($p = 0.01$) (**Figure 3A**). On the other hand, there was no difference in the percent of IgM^{a+} or IgM^{b+} lymphocytes that were peritoneal B-1a cells (**Figure 3B**) in mice receiving B-1 cell progenitors from both AID KO and WT BM. Thus, the total number of peritoneal B-1a cells derived from WT BM B-1 cell progenitors was significantly lower than those derived from AID KO BM B-1 cell progenitors ($p = 0.01$) (**Figure 3C**). As a control, BM B-1 cell progenitors from WT mice or AID KO mice were transferred alone into SCID recipients to detect any differences in reconstitution when the progenitors from these two sources (BALB/c or CD17 mice) were transferred alone. The results of the single transfers demonstrated no significant differences in the overall reconstitution of WT or AID KO mouse B-1 cell progenitors when transferred individually (**Figures 3A–C**). Together, these results demonstrate that *Aicda* inhibits the development of B-1a cells from BM B-1 cell progenitors in a competitive environment.

DISCUSSION

We found that B-1a cells, particularly $CD25^{+}$ B-1a cells, express *Aicda*. This *Aicda* expression occurs in the absence of intentional stimulation and without participation in GCs. Our results support the findings of Herzenberg and colleagues regarding the loss of somatic mutation and isotype switching from B-1 cell immunoglobulin in the absence of AID (16). Thus, we have extended previously reported functional results by directly demonstrating that WT B-1a cells express *Aicda*, and we have shown that *Aicda* expression is concentrated within the $CD25^{+}$ B-1a cell subpopulation.

Recent studies have utilized AID reporter constructs to identify memory B cells that developed in GCs (18–20). Our results inject a note of caution regarding the interpretation of these kinds of experiments by showing that some mature B cells, specifically $CD25^{+}$ B-1a cells, express *Aicda* and thus could register as reporter-positive despite not having resided in a GC. Further complicating this issue is the recent evidence that some B-1 cells may themselves be memory B cells (21). It is clear from the work reported here and by others that further study will be needed to tease out the extent to which *Aicda* expression marks naïve B-1a as well as memory B-2 cells and/or marks memory B cells regardless of whether they are B-1a or B-2.

We previously found that about 20% of peritoneal B-1a cells express CD25, a component of the high-affinity IL-2 receptor, and $CD25^{+}$ B-1a cells express increased levels of activated signaling intermediates (4). However, these B-1a cells lack expression of CD122 and are not responsive to IL-2 (4). In some systems, AID expression appears to impact viability. Immature murine B cells express AID and AID-deficient immature B cells are more resistant to apoptosis than immature B cells that express AID (15). Moreover, AID-deficient GC B cells are more resistant to apoptosis (22). These data suggest a role for AID in regulating cell viability. Along these lines, we found more B-1 cells in the peritoneal cavities of SCID mice after reconstitution with AID KO B-1 progenitor cells, and this was accentuated in BM chimeras wherein competition exists between WT and KO B-1 cell progenitors. Thus, AID is likely involved in the development and/or viability of B-1a cells.

The phenotype of mouse-like human B-1 cells has recently been redefined (23) from its previous focus on CD5 (24), an unreliable marker for B-1 cells in *Homo sapiens* and other species (25–27). It has been reported that AID-deficient hyper IgM syndrome patients are prone to develop autoimmune or inflammatory diseases such as diabetes mellitus, polyarthritis, autoimmune hepatitis, hemolytic anemia, and immune thrombocytopenia

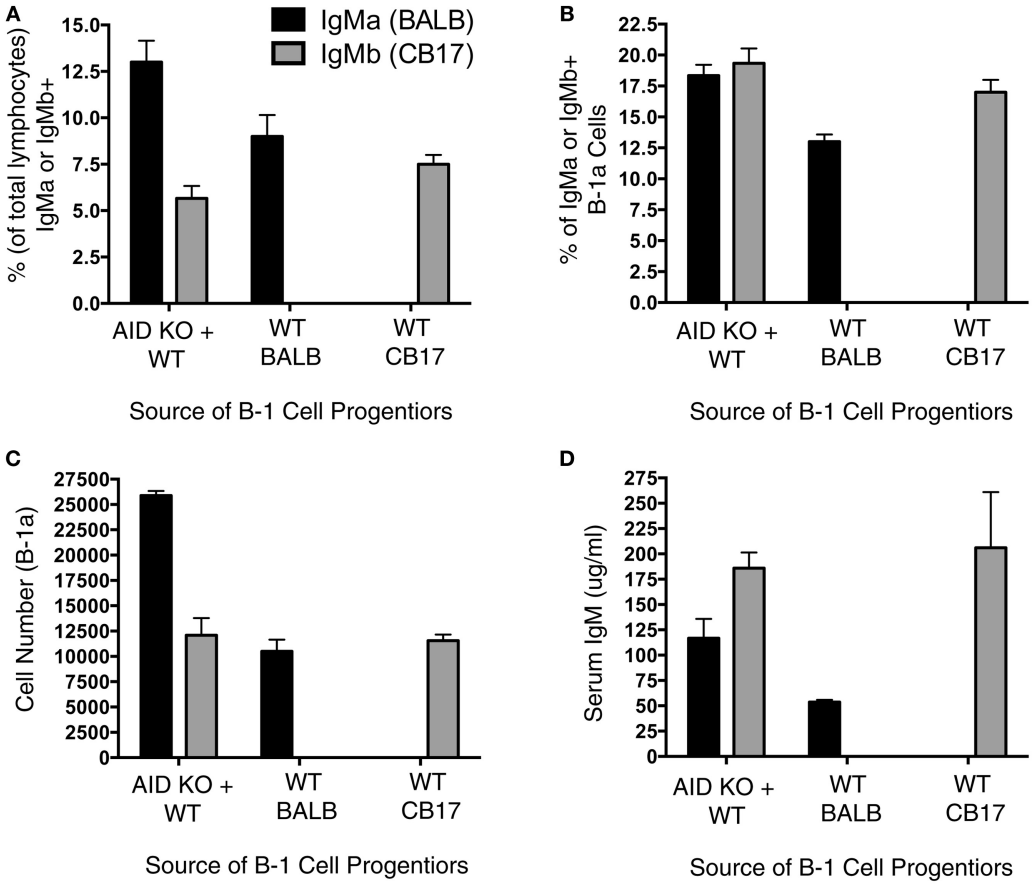


FIGURE 3 | *Aicda* impairs B-1a cell development. Allotype mixed chimeras were set up by injecting (i.v.): (1) 600,000 B-1 cell-specific progenitors ($Lin^-B220^{lo/-}CD19^+AA4.1^+$) obtained from activation-induced cytidine deaminase (AID) knock out (KO)-BALB/c bone marrow (BM) (IgM^a) along with 600,000 B-1 cell-specific progenitors from wild-type (WT)-CB17 BM (IgM^b); (2) 600,000 B-1 cell-specific progenitors obtained from WT-BALB/c BM (IgM^a); or (3) 600,000 B-1 cell-specific progenitors from WT-CB17 BM (IgM^b) into CB17-SCID recipients. Six weeks after the transfer, peritoneal cells were collected for the flow analysis. **(A)** The percent of live lymphocytes positive for IgM^a (black bars) or IgM^b (gray bars) in the collected washout cells was assessed. **(B)** The percent of live lymphocytes that phenotyped as IgM^a (black bars) or IgM^b (gray bars) B-1a cells in the collected washout cells was assessed. **(C)** The total number of peritoneal B-1a cells derived from BALB/c (IgM^a, black bars) or CB17 (IgM^b, gray bars) mice was assessed. **(D)** Serum IgM from BALB/c (IgM^a, black bars) or CB17 (IgM^b, grey bars) mice was assessed by ELISA

(28, 29). Our results suggest that B-1 cells should be considered as the pathogenesis of AID-deficient autoimmunity is probed.

AUTHOR CONTRIBUTIONS

HK, NH, and JT designed and performed the research, analyzed and interpreted data, and wrote the manuscript; TR interpreted data and wrote the manuscript.

FUNDING

This work was supported by Public Health Service grant AI029690 awarded to TR by the National Institutes of Health.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00672/full#supplementary-material.

FIGURE S1 | *Aicda* gene expression in B cells by Taqman assay. Peritoneal washout cells and spleen cells were obtained from 3-month-old BALB/c-ByJ mice, immunofluorescently stained, and sorted for peritoneal B-1a ($B220^{lo}CD5^+$), $CD25^+$ B-1a ($B220^{lo}CD5^+CD25^+$), $CD25^-$ B-1a ($B220^{lo}CD5^+CD25^-$), splenic B2 ($B220^+CD5^-CD23^+$), and GC ($B220^+$/GL-7^+/PNA^{high}) cells, as shown in **Figure 1**. RNA was prepared from each sort-purified B cell subset and reverse transcribed. The level of *Aicda* relative to actin was determined by real-time PCR (Taqman) with the primers described in Section "Materials and Methods." The means of three independent experiments are shown, along with lines indicating SEMs.

FIGURE S2 | *Aicda* gene expression in C57BL/6 B cells. Peritoneal washout cells and spleen cells were obtained from 3-month-old C57BL/6J mice, immunofluorescently stained, and sorted for peritoneal B-1a ($B220^{lo}CD5^+$), $CD25^+$ B-1a ($B220^{lo}CD5^+CD25^+$), $CD25^-$ B-1a ($B220^{lo}CD5^+CD25^-$), splenic B2 ($B220^+CD5^-CD23^+$), and GC ($B220^+$/GL-7^+/PNA^{high}) cells, as shown in **Figure 1**. RNA was prepared from each sort-purified B cell subset and reverse transcribed. The level of *Acida* relative to β_2-microglobulin was determined by real-time PCR (SYBR Green) with the primers described in Section "Materials and Methods." The means of three independent experiments are shown in **(A)**, along with lines indicating SEMs. The level of *Aicda* relative to actin was determined by real-time PCR (Taqman) with the primers described in Section "Materials and Methods." The means of three independent experiments are shown in **(B)**, along with lines indicating SEMs.

REFERENCES

1. Rothstein TL, Griffin DO, Holodick NE, Quach TD, Kaku H. Human B-1 cells take the stage. *Ann N Y Acad Sci* (2013) 1285:97–114. doi:10.1111/nyas.12137
2. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci U S A* (2000) 97(3):1184–9. doi:10.1073/pnas.97.3.1184
3. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) 11(1):34–46. doi:10.1038/nri2901
4. Tumang JR, Holodick NE, Vizconde TC, Kaku H, Frances R, Rothstein TL. A CD25(−) positive population of activated B1 cells expresses LIFR and responds to LIF. *Front Immunol* (2011) 2:6. doi:10.3389/fimmu.2011.00006
5. Kaku H, Cheng KF, Al-Abed Y, Rothstein TL. A novel mechanism of B cell-mediated immune suppression through CD73 expression and adenosine production. *J Immunol* (2014) 193(12):5904–13. doi:10.4049/jimmunol.1400336
6. Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. *Eur J Immunol* (2007) 37(9):2405–10. doi:10.1002/eji.200737461
7. Zhong X, Lau S, Bai C, Degauque N, Holodick NE, Steven SJ, et al. A novel subpopulation of B-1 cells is enriched with autoreactivity in normal and lupus-prone mice. *Arthritis Rheum* (2009) 60(12):3734–43. doi:10.1002/art.25015
8. Kaku H, Rothstein TL. Octamer binding protein 2 (Oct2) regulates PD-L2 gene expression in B-1 cells through lineage-specific activity of a unique, intronic promoter. *Genes Immun* (2010) 11(1):55–66. doi:10.1038/gene.2009.68
9. Zhong X, Rothstein TL. L2pB1: a new player in autoimmunity. *Mol Immunol* (2011) 48(11):1292–300. doi:10.1016/j.molimm.2010.12.006
10. Wang H, Shin DM, Abbasi S, Jain S, Kovalchuk AL, Beaty N, et al. Expression of plasma cell alloantigen 1 defines layered development of B-1a B-cell subsets with distinct innate-like functions. *Proc Natl Acad Sci U S A* (2012) 109(49):20077–82. doi:10.1073/pnas.1212428109
11. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* (2000) 102(5):553–63. doi:10.1016/S0092-8674(00)00078-7
12. Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, et al. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* (1999) 274(26):18470–6. doi:10.1074/jbc.274.26.18470
13. Kuraoka M, Holl TM, Liao D, Womble M, Cain DW, Reynolds AE, et al. Activation-induced cytidine deaminase mediates central tolerance in B cells. *Proc Natl Acad Sci U S A* (2011) 108(28):11560–5. doi:10.1073/pnas.1102571108
14. Kuraoka M, McWilliams L, Kelsoe G. AID expression during B-cell development: searching for answers. *Immunol Res* (2011) 49(1–3):3–13. doi:10.1007/s12026-010-8185-7
15. Kuraoka M, Kelsoe G. A novel role for activation-induced cytidine deaminase: central B-cell tolerance. *Cell Cycle* (2011) 10(20):3423–4. doi:10.4161/cc.10.20.17693
16. Yang Y, Wang C, Yang Q, Kantor AB, Chu H, Ghosn EE, et al. Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *Elife* (2015) 4:e09083. doi:10.7554/eLife.09083
17. Tumang JR, Frances R, Yeo SG, Rothstein TL. Cutting edge: spontaneously Ig-secreting B-1 cells violate the accepted paradigm for expression of differentiation-associated transcription factors. *J Immunol* (2005) 174(6):3173–7. doi:10.4049/jimmunol.174.6.3173
18. Crouch EE, Li Z, Takizawa M, Fichtner-Feigl S, Gourzi P, Montano C, et al. Regulation of AID expression in the immune response. *J Exp Med* (2007) 204(5):1145–56. doi:10.1084/jem.20061952
19. Mullins CD, Su MY, Hucthagowder V, Chu L, Lu L, Kulkarni S, et al. Germinal center B-cells resist transformation by Kras independently of tumor suppressor Arf. *PLoS One* (2013) 8(6):e67941. doi:10.1371/journal.pone.0067941
20. Healy JA, Nugent A, Rempel RE, Moffitt AB, Davis NS, Jiang X, et al. GNA13 loss in germinal center B cells leads to impaired apoptosis and promotes lymphoma in vivo. *Blood* (2016) 127(22):2723–31. doi:10.1182/blood-2015-07-659938
21. Yang Y, Ghosn EE, Cole LE, Obukhanych TV, Sadate-Ngatchou P, Vogel SN, et al. Antigen-specific memory in B-1a and its relationship to natural immunity. *Proc Natl Acad Sci U S A* (2012) 109(14):5388–93. doi:10.1073/pnas.1121627109
22. Boulianne B, Rojas OL, Haddad D, Zaheen A, Kapelnikov A, Nguyen T, et al. AID and caspase 8 shape the germinal center response through apoptosis. *J Immunol* (2013) 191(12):5840–7. doi:10.4049/jimmunol.1301776
23. Quach TD, Rodriguez-Zhurbenko N, Hopkins TJ, Guo X, Hernandez AM, Li W, et al. Distinctions among circulating antibody-secreting cell populations, including B-1 cells, in human adult peripheral blood. *J Immunol* (2016) 196(3):1060–9. doi:10.4049/jimmunol.1501843
24. Casali P, Notkins AL. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5+ B lymphocytes. *Annu Rev Immunol* (1989) 7:513–35. doi:10.1146/annurev.iy.07.040189.002501
25. Freedman AS, Freeman G, Whitman J, Segil J, Daley J, Levine H, et al. Expression and regulation of CD5 on in vitro activated human B cells. *Eur J Immunol* (1989) 19(5):849–55. doi:10.1002/eji.1830190511
26. Raman C, Knight KL. CD5+ B cells predominate in peripheral tissues of rabbit. *J Immunol* (1992) 149(12):3858–64.
27. Guelpa-Fonlupt V, Tonnelle C, Blaise D, Fougereau M, Fumoux F. Discrete early pro-B and pre-B stages in normal human bone marrow as defined by surface pseudo-light chain expression. *Eur J Immunol* (1994) 24(1):257–64. doi:10.1002/eji.1830240140
28. Jesus AA, Duarte AJ, Oliveira JB. Autoimmunity in hyper-IgM syndrome. *J Clin Immunol* (2008) 28(Suppl 1):S62–6. doi:10.1007/s10875-008-9171-x
29. Meyers G, Ng YS, Bannock JM, Lavoie A, Walter JE, Notarangelo LD, et al. Activation-induced cytidine deaminase (AID) is required for B-cell tolerance in humans. *Proc Natl Acad Sci U S A* (2011) 108(28):11554–9. doi:10.1073/pnas.1102600108

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 15 March 2016
doi: 10.3389/fimmu.2016.00099

Emerging Functions of Natural IgM and Its Fc Receptor FCMR in Immune Homeostasis

Hongsheng Wang[1], John E. Coligan[2] and Herbert C. Morse III[1]**

[1] Virology and Cellular Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA, [2] Receptor Cell Biology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA

Most natural IgM antibodies are encoded by germline Ig sequences and are produced in large quantities by both mice and humans in the absence of intentional immunization. Natural IgM are reactive with many conserved epitopes, including those shared by microorganisms and autoantigens. As a result, these antibodies play important roles in clearing intruding pathogens, as well as apoptotic/necrotic cells and otherwise damaged tissues. While natural IgM binds to target structures with low affinity due to a lack of significant selection by somatic hypermutation, its pentameric structure with 10 antigen-binding sites enables these antibodies to bind multivalent target antigens with high avidity. Opsonization of antigen complexed with IgM is mediated by cell surface Fc receptors. While the existence of Fc alpha/mu receptor has been known for some time, only recently has the Fc receptor specific for IgM (FCMR) been identified. In this review, we focus on our current understandings of how natural IgM and FCMR regulate the immune system and maintain homeostasis under physiological and pathological conditions.

Keywords: natural IgM, FCMR, autoimmunity, homeostasis, inflammation

OPEN ACCESS

Edited by:
Ana María Hernández, Center of Molecular Immunology, Cuba

Reviewed by:
Laurence Morel, University of Florida, USA
Yoelys Cruz Leal, University of Havana, Cuba

****Correspondence:***
Hongsheng Wang
wanghongs@niaid.nih.gov;
Herbert C. Morse III
hmorse@niaid.nih.gov

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *08 January 2016*
Accepted: *29 February 2016*
Published: *15 March 2016*

Citation:
Wang H, Coligan JE and Morse HC III (2016) Emerging Functions of Natural IgM and Its Fc Receptor FCMR in Immune Homeostasis. Front. Immunol. 7:99. doi: 10.3389/fimmu.2016.00099

INTRODUCTION

Studies of IgG antibodies produced in response to foreign antigens have provided a wealth of information about the mechanisms involved in generating a seemingly limitless array of antigen-binding specificities by diversification of their antigen-binding domains through somatic recombination and mutation. In addition, a large number of investigations have shown that the effector functions of these antibodies are mediated through the interactions of their Fc domains with a series of isotype-restricted receptors expressed on a variety of hematopoietic cell types.

An Fc receptor specific for IgM, now termed FCMR (1), was defined only 8 years ago. Importantly, and in contrast to most secreted IgG antibodies, secreted IgM (sIgM) can be subdivided into natural and immune IgM. Natural IgM is found at equivalent levels in sera of normal and germ-free mice, and it is thought that exposure to natural antigens is responsible for its production. In addition, natural IgM is often polyreactive, whereas immune IgM is selected for antigen-specificity and is usually produced following exposure to pathogens. Because it is impossible to separate the effects of natural IgM from immune IgM when we evaluate the consequences of interactions between FCMR and an IgM molecule in non-immunized individuals; in this review, serum sIgM is taken to be synonymous to natural IgM.

Over the last several years, advances in understanding the various functions of sIgM and its interactions with FCMR have been accelerated by the generation of knockout mice that lack sIgM or FCMR. The purpose of this review is to describe new aspects of the nature and functions of the sIgM–FCMR axis.

NATURAL IgM, AN OVERVIEW

sIgM exists in all vertebrate species including fishes, amphibians, reptiles, birds, and mammals (2). Both mice and humans have large quantities of circulating sIgM (300–800 μg/ml for mice and 400–2300 μg/ml for humans). The serum levels of sIgM are maintained similarly in mice that are raised in pathogen-free, germ-free, or chemically defined antigen-free environments indicating that endogenous stimuli are responsible for its production (3, 4). The cellular origin of sIgM has been postulated to be predominantly B-1 cells found in the peritoneal cavity (5). However, this view has recently been revised by evidence that B-1 cells residing in the spleen and bone marrow are significant producers of sIgM (6). Other cell types including marginal zone B cells have also been implicated in the production of natural IgM (7). B-1 cells, particularly the $CD5^+$ B-1a subset, belong to a stable population maintained by self-renewal independent of gut microbiomes (8, 9). B-1a cells, especially the $PC1^{lo}$ subset (10), constantly migrate out of the peritoneum with some migrating to the spleen where they undergo activation and secret sIgM. In addition, the bone marrow is found to host a small number of $CD5^-$ plasma cells originating from the peritoneum that likely contribute to long-term sIgM production (11). A thorough discussion of natural antibody-producing cells can be found in a recent elegant review (12).

Polyreactivity and autoreactivity are two prominent features found within the sIgM population. Prior studies with monoclonal natural antibodies including IgM and IgG isotypes demonstrated that a single natural IgM or IgG is capable of binding more than three apparently structurally unrelated antigens (13–15). Based on immunoadsorption experiments or immunoblotting using a panel of self-antigens, it has been estimated that 5–100% of normal mouse or human sera is autoreactive [Reviewed in Ref. (16)]. These properties of sIgM have been attributed to the germline configuration of their V region structures, characterized by enrichment of positively charged amino acids, especially arginine (17, 18). Also, compared to induced IgM antibodies, the V regions of sIgM have relatively higher frequencies of tyrosine and serine residues, which bear side-chain hydroxyl groups, allowing sIgM to bind various epitopes via ionic and hydrogen bonding (17, 18). These interactions, however, are usually of low affinity (19). Nevertheless, the polymeric binding between pentameric sIgM and a target antigen reaches a functional affinity (avidity) much higher than the intrinsic affinity (20, 21). By using the surface plasmon resonance technique to determine binding avidities, Diaw et al. analyzed five monoclonal sIgM against dissimilar autoantigens including cytoskeletal antigens and DNA. The kinetic binding constants of all five sIgM were indistinguishable from those observed for immune antibodies (22).

The biological functions of sIgM have been described to include removal of apoptotic cells, protection from infection, and tissue homeostasis (16, 23). The third function is attributed to sIgM-mediated clearance of tissue-breakdown molecules and binding to cell surface molecules on B and T cells to inhibit cell division and/or activation, thereby minimizing inflammation (16). This immunomodulatory role of sIgM has been confirmed by studies with two independently genetically engineered mouse strains lacking serum IgM ($S\mu^{-/-}$) (24, 25). These mice exhibit abnormal B cell development, impaired antibody responses, and enhanced production of autoantibodies (see below).

THE IgM Fc RECEPTOR, FCMR

Originally identified and termed as TOSO [encoded by Fas apoptosis inhibitory molecule 3 (FAIM3)] in 1998 (26), FCMR was recently rediscovered and characterized as an IgM-specific Fc receptor (27, 28). A consensus nomenclature for this molecule has recently been proposed as FCMR (1). FCMR is a transmembrane protein with a predicted molecular weight of ~41 KDa, but with heavily O-linked glycosylation in the extracellular domain, a mature molecule can reach ~60 KDa (26, 27, 29). The extracellular domain contains a single Ig-like domain with binding activity to the IgM Fc region, while the intracellular domain contains tyrosine residues that serve as phosphorylation sites to initiate/mediate signaling cascades. A thorough review of the molecular features of FCMR has recently been published (30).

To date, most of our understanding of the functions of FCMR comes from studies of FCMR-deficient mice. Natural mutations of FCMR in humans have not been reported. There are three independently generated *Fcmr* gene knockout strains have been reported (31–34) and two of them were characterized in detail (31, 32, 34) (**Table 1**). Clear differences exist among these mice, possibly due to the nature of gene targeting strategies, differing involvement of 129/Sv ES cells, extent of backcrossing to the B6 background, and husbandry environment. Readers are reminded of a recent report that revealed an astonishing "side effect" of passenger mutations of the 129 line that persists even after extensive backcrossing (35). This finding could explain why distinct knockout strains for the same gene often yield discrepant functional results.

EXPRESSION OF FCMR

The expression of FCMR in different cell types has been investigated at both the mRNA and protein levels by a number of investigators without complete agreement. The ImmGen database, a microarray-based public resource of mouse transcript expression (www.immgen.org), indicates a broad expression pattern of *Fcmr* among all lymphoid and myeloid cells tested, with relative expression levels ranging from ~50 in T cells, NK, DCs, myeloid, and stromal cells to ~5000 in B cells. Our quantitative PCR analyses of sorted populations revealed a similar pattern of expression, also with B cells expressing the highest levels (31). Northern blot analysis of human tissues also revealed a broad expression pattern of FCMR in lymphoid and non-lymphoid tissues (26, 28).

Fc receptor specific for IgM protein expression has been assessed in a variety of cell types using several monoclonal antibodies. Kubagawa and colleagues reported that FCMR

TABLE 1 | Phenotypes of *Fcmr*$^{-/-}$ mice.

Reports by		Choi et al. (31)	Ouchida et al. (34)	Honjo et al. (32)	Nguyen et al. (33)
Strain		Fcmr^{tm1Mak}	Fcmr^{tm1Ohno}		Fcmr$^{tm1.2Khl}$
Genetic manipulation		Deleted exons 2–8. Involving 129/Sv ES cells and backcrossing with C57BL/6 mice. The Neo gene cassette was not deleted after recombination	Deleted exons 2–4. Involving 129/Sv ES cells and backcrossing with C57BL/6 mice. The Neo gene cassette was deleted after recombination		Deleted exons 4–7. The Neo gene cassette was deleted after recombination. Pure B6 background
B cells	BM	Small pre-B↓ Immature B↓	Not changed	Not changed	NR
	Spleen	FOB↓, MZB unchanged	FOB unchanged, MZB↓, transitional B↑	MZB↓, B-1↑	B cells↓
	PerC	B-1a↑, B-2↓	Not changed	Not changed	NR
T cells Basal Ig levels		Not changed IgG1↓, No change for other classes	Not changed IgM↑, no change for total IgG	Not changed IgM↑, IgG3↑, no change for other classes	Not changed NR
TI responses		Enhanced	Reduced	Phosphorylcholine-specific responses are enhanced	NR
TD responses		Enhanced	Reduced	Reduced	NR
Responses to infectious pathogens		NR	NR	IgM and IgG3 responses to low doses of *Streptococcus pneumoniae* are enhanced	NR
Spontaneous autoantibody production		Anti-ds-DNA and ANAs ↑	Anti-DNA, -rheumatoid factor, and ANAs ↑	Anti-DNA, -chromatin, and ANAs ↑	NR

NR, not reported.

expression was restricted to human B, T, and NK cells, and mouse B cells (27, 32). The lack of expression of mouse FCMR by non-B cells was confirmed by Ohno and colleagues (28, 34). However, Lang et al. using a different monoclonal antibody reported expression of FCMR on myeloid cells (36). On the other hand, Honjo et al. could not detect expression of exon 2 mRNA of *Fcmr* and FCMR protein in granulocytes with their monoclonal antibodies (37). Analysis of FCMR expression is complicated by the fact that it undergoes internalization after binding IgM (29). Freshly isolated tonsillar B and T cells are negative for FCMR on the cell surface; however, these cells become positive for FCMR after a brief culture with IgM-negative medium *in vitro* (27). Therefore, detection of FCMR at the cell membrane becomes problematic and ambiguous depending on the method used for study. In addition, future studies are warranted to determine whether the various anti-FCMR monoclonal antibodies recognize the same or alternative forms of FCMR expressed in different tissues.

It should be recognized that FCMR, while specific for IgM, is not the only cell surface Fc receptor capable of binding IgM. The Fcα/μ receptor (FCA/MR), encoded by the *FCA/MR* gene in humans, is an unusual Fc receptor in that it binds to two different antibody isotypes, IgA, and IgM (38). The receptor is broadly expressed in humans and mice, but with significant differences in expression patterns between the two species, particularly on hematopoietic cells (39). Both IgA and IgM cross-compete for binding to the mouse receptor suggesting a common site of interaction. Pentameric IgM does not have to contain J chain to bind the receptor (40).

A second receptor with dual specificity for IgA and IgM is the polymeric immunoglobulin receptor, PIGR. This receptor only binds polymeric IgA and IgM associated with the J chain at high affinities (41). In contrast to FCA/MR and FCMR, PIGR is expressed only on epithelial cells (42).

SIGNALING POTENTIAL OF FCMR

The intracellular domain of FCMR contains several tyrosine residues but lacks a commonly present immunoreceptor tyrosine-based activation motif (ITAM) and/or the immunoreceptor tyrosine-based inhibition motif (ITIM) (27). However, the FCMR cytoplasmic tail does contain an Asp–X_5–Asp–Tyr^{401}–Ile–Asn sequence that matches the recently identified immunoglobulin tail tyrosine (ITT) phosphorylation motif Glu/Asp–X_{6-7}–Asp–Tyr–X–Asn present in membrane IgG (mIgG) and mIgE (43). This consensus motif is found to amplify BCR signals in class-switched memory B cells by recruiting the adaptor Grb2, thereby allowing switched memory B cells to respond more quickly and vigorously than primary B cells to secondary exposures to antigens (43, 44). Therefore, the ITT motif of FCMR could serve as a molecular platform to interact with and influence the BCR signaling pathway. In fact, cross linking FCMR with either anti-FCMR monoclonal antibodies or preformed IgM immune complexes induced phosphorylation of tyrosine and serine residues of FCMR in B cells (27) and phosphorylation of PLC-γ and Erk1/2 in NK cells (45). Our analyses also showed that FCMR positively modulates tonic BCR signaling. The basal levels of phospho-Syk in pre-B, MZ, and B-1a cells were lower in *Fcmr*$^{-/-}$ mice than WT controls (31). Ligation of FCMR alone did not affect cellular proliferation and survival (34). However, BCR-induced proliferation and survival were reduced in the absence of FCMR (31, 34).

Moreover, ligation of FCMR had no effect on LPS or anti-CD40 antibody-induced proliferation and survival (31, 34). These results argue that FCMR more closely approximates the activity of BCR than TLR or CD40 on the surface of B cells. Therefore, it is conceivable that FCMR plays a modulatory role in BCR signaling that supports survival.

THE sIgM–FCMR AXIS IN REGULATION OF EARLY B CELL DEVELOPMENT

Early stages of B cell development are characterized by ordered gene expression consisting of H and L chain gene rearrangements in pro-B and pre-B cells, respectively. The expression of productive μH chains leads to assembly and expression of pre-BCRs on the surface of pre-B cells. In conjunction with the IL-7 signaling, pre-BCRs trigger clonal expansion for approximately six divisions (46) and upregulate expression of IRF4, which promotes pre-B cell dissociation from stromal cells, a critical step leading to differentiation of small pre-B cells and L chain gene rearrangement (47).

The expression levels of FCMR in early B cells gradually increases starting from pro-B to pre-B and to immature B cells (ImmGen database), similar to our assessment by qPCR (31). In the absence of FCMR, as noted in the Fcmr^{tm1Mak} strain but not the Fcmr^{tm1Ohno} strain (**Table 1**), the development of pre-B and immature B cells was significantly reduced (31). Remarkably, Sμ$^{-/-}$ mice lacking sIgM exhibited similar deficiencies in pre-B and immature B cells (31, 48). Therefore, the sIgM–FCMR axis may represent a positive feedback loop promoting development and maturation of early B cells. Indeed, irradiated normal recipient mice receiving WT hematopoietic stem cells (HSCs) and sIgM-containing sera generated significantly more pre-B cells than recipients of WT HSCs and sIgM-deficient sera (31). This observation suggests that sIgM could enhance generation of pre-B and immature B cells in a FCMR-dependent fashion (31).

THE sIgM–FCMR AXIS IN REGULATION OF LATE STAGES OF B CELL DEVELOPMENT

Fc receptor specific for IgM deficiency in mice was associated with altered distributions of peripheral B cells. The Fcmr^{tm1Mak} strain exhibited reduced numbers of splenic follicular B cells, but the numbers of MZ B cells remained unchanged (31). In the peritoneum, the frequencies of B-1a cells were increased while B-2 cells in this strain were decreased (31). In contrast, the Fcmr^{tm1Ohno} strain exhibited reduced numbers of MZ B cells, increased transitional B cells and B-1 cells in the spleen, and no change in peritoneal B cells (32, 34) (**Table 1**). The differences in distribution of B cells between the two strains are also associated with differences between the strains in basal levels of serum immunoglobulins in naive mice (**Table 1**) (31, 32, 34, 48).

Our parallel assessments of Sμ$^{-/-}$ and Fcmr^{tm1Mak} mice revealed strikingly similar changes in peripheral B cells: reduced numbers of FO but increased numbers of MZ B cells in the spleen, and increased numbers of B-1a cells but reduced B-2 cells in the peritoneum (31). A second Sμ$^{-/-}$ strain also exhibited expansion of MZ and B-1a cells and reduction in FO B cells (25). These data agree with the conclusion that the sIgM–FCMR axis may promote FO B cell development. Regarding B-1 cells, Nguyen et al. recently reported that peritoneal CD5^{+} B cells in Sμ$^{-/-}$ mice were not "regular" B-1a cells but exhibited characteristics of "anergic" B cells (48). They further showed that the lack of sIgM in Sμ$^{-/-}$ mice not only altered B cell numbers (reduced FO and expanded MZ B cells), confirming our analyses (31), but also fundamentally altered the B cell repertoire (the usage of V_H genes) and selection (48). More importantly, administration of sIgM into Sμ$^{-/-}$ mice reversed the MZ/FO B cell ratio and mostly restored normal B cell development (48, 49). These results strongly suggest that the sIgM–FCMR axis does indeed modulate differentiation of peripheral B cells.

THE sIgM–FCMR AXIS IN REGULATION OF T-INDEPENDENT IMMUNE RESPONSES

Immunization of the Fcmr^{tm1Mak} strain with NP-LPS stimulated increased levels of plasma cell development and secretion of IgM antibodies (31). This data can be explained by increased numbers of peritoneal B-1a cells in *Fcmr*$^{-/-}$ mice as B-1a cells are the primary responders to NP-LPS challenge in this setting (31). Immunization of Fcmr^{tm1Mak} mice with NP-FICOLL, which predominantly activates MZ B cells, resulted in moderate increases in plasma cells without a significant increase in sIgM, which seems to correlate with relatively normal numbers of MZ B cells in this strain (31). In contrast, studies of the Fcmr^{tm1Ohno} strain yielded opposing results, i.e., reduced production of sIgM and IgG following NP-FICOLL immunization (34) (**Table 1**). This discrepancy could be due to the fact that the MZ B cell compartment is reduced in the Fcmr^{tm1Ohno} strain but remains unchanged (if not slightly expanded) in the Fcmr^{tm1Mak} strain. By using a complicated antigen, *Streptococcus pneumoniae* (R36A), Honjo et al. showed increased IgM and IgG3 responses to the carbohydrate phosphorylcholine (PC), epitope of the pathogen, in Fcmr^{tm1Ohno} mice (32).

Previous studies in Sμ$^{-/-}$ mice revealed enhanced production of IgG2a antibodies following immunization with NP-FICOLL (50). Similar findings were reported in another Sμ$^{-/-}$ strain (25). Taken together, these results suggest that the sIgM–FCMR pathway may negatively regulate TI immune responses likely through modulating the sensitivity of responding B cells.

THE sIgM–FCMR AXIS IN REGULATION OF T-DEPENDENT IMMUNE RESPONSES

Studies of TD immune responses in Fcmr^{tm1Ohno} and Fcmr^{tm1Mak} mice have also yielded conflicting results. Our analyses in the Fcmr^{tm1Mak} strain revealed a moderate increase in primary IgM but not of IgG2b responses following immunization with NP-KLH/alum (31). During a recall immune response, FCMR-deficient mice produced significantly more germinal centers and plasma cells but only exhibited subtle differences in overall serum

antibody levels (31). In contrast, following a similar immunization protocol, Ouchida et al. and Honjo et al. reported that the Fcmr^{tm1Ohno} mice developed generally decreased TD immune responses (32, 34) (**Table 1**). Given the fact that the antibody responses elicited in both strains were relatively moderate, we believe that the role of FCMR in TD immune responses may be limited.

Previous studies of Sμ$^{-/-}$ mice indicated that sIgM is required to elicit optimal TD immune responses because in the absence of sIgM, Sμ$^{-/-}$ mice produced significantly lower levels of IgG1 anti-NP antibodies, and antibody affinity maturation was also delayed (25). However, injection of sIgM into Sμ$^{-/-}$ mice before immunization increased NP-specific IgG1 responses (25). These results suggest that sIgM could augment TD humoral responses, but whether this was mediated by sIgM-mediated antigen processing/presentation, or FCMR-mediated signaling that renders B cells hyperresponsive to activation signals, or both, remains to be determined.

THE sIgM–FCMR AXIS SUPPRESSES DEVELOPMENT OF AUTOIMMUNITY

Spontaneous production of increased levels of autoreactive antibodies, including anti-DNA and anti-nuclear antibodies, was detected in both FCMR-deficient strains (**Table 1**). Surprisingly, the existence of these autoantibodies in FCMR-deficient mice was insufficient to induce pathology, e.g., glomerular damage common to lupus-like diseases. Introducing *Fcmr* deficiency onto the autoimmune-prone B6.MRL Fas$^{lpr/lpr}$ background accelerated development of autoreactive antibodies but still had no effect on severity of renal pathology and function or overall survival (51). These results suggest that FCMR acts to inhibit activation of non-pathogenic autoreactive B cells.

Absence of sIgM in Sμ$^{-/-}$ mice was also associated with accelerated development of IgG autoantibodies (48, 52, 53). Similar to FCMR-deficient mice, both Sμ$^{-/-}$ strains spontaneously generated IgG anti-DNA autoantibodies (52, 53). Immune complex deposition in the kidneys was observed in a small proportion of mice (53). When Sμ$^{-/-}$ mice were bred to MRL$^{lpr/lpr}$ mice, the progeny exhibited accelerated development of IgG autoantibodies and autoimmune disease (52). Moreover, adoptive transfer of Sμ$^{-/-}$ BM cells into recipient mice containing normal levels of sIgM abrogated anti-nuclear antibody development (48). Thus, sIgM and FCMR may negatively regulate autoimmunity through the sIgM–FCMR pathway not withstanding that sIgM may also modulate autoimmune responses through FCMR-independent mechanisms, such as complement fixation, immune complex uptake, and antigen presentation (23).

In addition to the functions of FCMR in the above lupus model, Lang et al. studied the roles of FCMR in an experimental autoimmune encephalomyelitis (EAE) model and demonstrated that Fcmr^{tm1Mak} mice were resistant to EAE (54). While changes in expression of IL-17 in T cells were not observed in this report (54), a more recent study using single cell RNA-sequencing in isolated Th17 cells identified a strong correlation between expression of FCMR and the Th17 cytokine signature (55). Indeed, FCMR-deficient T cells fail to produce IL-17A upon stimulation (55). IL-17A is a critical driving cytokine for EAE (56). Thus, FCMR may promote pathogenesis of Th17-mediated diseases.

DOES FCMR PLAY A ROLE IN CONTROLLING INFECTION?

While sIgM has long been postulated to play an important role as first line in defense against infectious agents (2), there are also indications that FCMR may play a role during inflammatory responses to infection. Honjo et al. immunized Fcmr^{tm1Ohno} mice with a live attenuated strain of *S. pneumoniae* (R36A) to examine antigen-specific immune responses against the bacterial PC and protein determinants (32). While the FCMR-deficient mice successfully elicited anti-PC antibody responses, they failed to generate anti-protein antibody responses (32). This result suggests that FCMR has discrete roles in B cells (possibly coupled with specificity of the BCR) in responding to protein and non-protein determinants of live pathogens.

Lang et al. performed wide range analyses of the Fcmr^{tm1Mak} strain infected with *Listeria* (36). FCMR-deficient mice were resistant to LPS-induced septic shock and failed to control Listeria infection. This phenotype is associated with decreased systemic production of IFN-γ, IL-12, and IL-6 in FCMR-deficient mice (36). While most of the changes in this model were attributed to absence of FCMR in phagocytes, including monocytes, macrophages, and granulocytes (36), the expression of FCMR by these myeloid cells was questioned by Honjo et al. who failed to detect expression of exon 2 of *Fcmr* by PCR and protein by FACS in granulocytes (37). More recently, Lang et al. have reported that FCMR is required for development and function of inflammatory dendritic cells (iDCs) (57). FCMR mutant mice were deficient for iDCs in the liver and thereby failed to recruit and activate CD8$^+$ T cells to clear lymphocytic choriomeningitis virus (57). Because the expression levels of FCMR transcripts in granulocytes and DCs are about 100-fold lower than in B cells (ImmGen database) and it remains unknown whether infection could upregulate expression of FCMR in granulocytes and DCs, future studies are warranted to determine the molecular mechanisms of FCMR action in non-B cells in response to infection.

CONCLUDING REMARKS

It is becoming broadly accepted that sIgM has protective functions in defense against infection and is important for maintaining tissue homeostasis. Earlier studies have revealed that sIgM binds to infectious agents, as well as altered self-antigens, and that sIgM-antigen complexes activate the complement cascade and initiate inflammatory responses. The recent identification of FCMR has provided new insights into the mechanisms of sIgM function *in vivo*. While evidence of the beneficial effects of sIgM on survival have dominated the literature, it is worth noting that sIgM can also play pathogenic roles under certain circumstances. For example, natural IgM is critical for development of inflammation and damage in a model of ischemia–reperfusion

injury (58–61). In this case, sIgM binds to damaged tissue and activates the complement cascade causing massive inflammatory responses. Blocking sIgM binding to modified self-antigens with an anti-annexin IV single-chain antibody (scFv) significantly reduced graft inflammation and injury (58). Recently, Panzer et al. reported that injection of sIgM into B-cell-deficient μMT mice induced albuminuria in a complement-induced glomerular disease model (62). These observations raise a note of caution regarding postulated application of intravenous IgM (IVIgM) (63), a homolog of intravenous IgG (IVIg), to the treatment of autoimmune and inflammatory diseases. More recently, Brenner et al. reported that a FCMR-Fc fusion protein comprising the extracellular domain of FCMR exhibited therapeutic effects by inhibiting MOG-induced neuroinflammatory responses in an EAE model (54). An anti-FCMR monoclonal antibody has also been shown to be beneficial in experimental malaria infection (64). Future studies are warranted to determine whether blockade of FCMR could be a therapeutic approach to treat autoimmune and inflammatory diseases.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Programs of the NIH, National Institute of Allergy and Infectious Diseases (NIAID) (HW, JC, and HM).

REFERENCES

1. Kubagawa H, Carroll MC, Jacob CO, Lang KS, Lee KH, Mak T, et al. Nomenclature of Toso, Fas apoptosis inhibitory molecule 3, and IgM FcR. *J Immunol* (2015) **194**:4055–7. doi:10.4049/jimmunol.1500222
2. Boyden SV. Natural antibodies and the immune response. *Adv Immunol* (1966) **5**:1–28. doi:10.1016/S0065-2776(08)60271-0
3. Coutinho A, Kazatchkine MD, Avrameas S. Natural autoantibodies. *Curr Opin Immunol* (1995) **7**:812–8. doi:10.1016/0952-7915(95)80053-0
4. Pereira P, Forni L, Larsson EL, Cooper M, Heusser C, Coutinho A. Autonomous activation of B and T cells in antigen-free mice. *Eur J Immunol* (1986) **16**:685–8. doi:10.1002/eji.1830160616
5. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* (1999) **96**:2250–5. doi:10.1073/pnas.96.5.2250
6. Choi YS, Dieter JA, Rothaeusler K, Luo Z, Baumgarth N. B-1 cells in the bone marrow are a significant source of natural IgM. *Eur J Immunol* (2012) **42**:120–9. doi:10.1002/eji.201141890
7. Ichikawa D, Asano M, Shinton SA, Brill-Dashoff J, Formica AM, Velcich A, et al. Natural anti-intestinal goblet cell autoantibody production from marginal zone B cells. *J Immunol* (2015) **194**:606–14. doi:10.4049/jimmunol.1402383
8. Ha SA, Tsuji M, Suzuki K, Meek B, Yasuda N, Kaisho T, et al. Regulation of B1 cell migration by signals through Toll-like receptors. *J Exp Med* (2006) **203**:2541–50. doi:10.1084/jem.20061041
9. Hardy RR. B-1 B cells: development, selection, natural autoantibody and leukemia. *Curr Opin Immunol* (2006) **18**:547–55. doi:10.1016/j.coi.2006.07.010
10. Wang H, Shin DM, Abbasi S, Jain S, Kovalchuk AL, Beaty N, et al. Expression of plasma cell alloantigen 1 defines layered development of B-1a B-cell subsets with distinct innate-like functions. *Proc Natl Acad Sci U S A* (2012) **109**:20077–82. doi:10.1073/pnas.1212428109
11. Reynolds AE, Kuraoka M, Kelsoe G. Natural IgM is produced by CD5-plasma cells that occupy a distinct survival niche in bone marrow. *J Immunol* (2015) **194**:231–42. doi:10.4049/jimmunol.1401203
12. Savage HP, Baumgarth N. Characteristics of natural antibody-secreting cells. *Ann N Y Acad Sci* (2015) **1362**:132–42. doi:10.1111/nyas.12799
13. Dighiero G, Lymberi P, Mazie JC, Rouyre S, Butler-Browne GS, Whalen RG, et al. Murine hybridomas secreting natural monoclonal antibodies reacting with self antigens. *J Immunol* (1983) **131**:2267–72.
14. Prabhakar BS, Saegusa J, Onodera T, Notkins AL. Lymphocytes capable of making monoclonal autoantibodies that react with multiple organs are a common feature of the normal B cell repertoire. *J Immunol* (1984) **133**:2815–7.
15. Seigneurin JM, Guilbert B, Bourgeat MJ, Avrameas S. Polyspecific natural antibodies and autoantibodies secreted by human lymphocytes immortalized with Epstein-Barr virus. *Blood* (1988) **71**:581–5.
16. Avrameas S, Ternynck T. Natural autoantibodies: the other side of the immune system. *Res Immunol* (1995) **146**:235–48. doi:10.1016/0923-2494(96)80259-8
17. Baccala R, Quang TV, Gilbert M, Ternynck T, Avrameas S. Two murine natural polyreactive autoantibodies are encoded by nonmutated germ-line genes. *Proc Natl Acad Sci U S A* (1989) **86**:4624–8. doi:10.1073/pnas.86.12.4624
18. Conger JD, Sage HJ, Corley RB. Correlation of antibody multireactivity with variable region primary structure among murine anti-erythrocyte autoantibodies. *Eur J Immunol* (1992) **22**:783–90. doi:10.1002/eji.1830220323
19. Nakamura M, Burastero SE, Ueki Y, Larrick JW, Notkins AL, Casali P. Probing the normal and autoimmune B cell repertoire with Epstein-Barr virus. Frequency of B cells producing monoreactive high affinity autoantibodies in patients with Hashimoto's disease and systemic lupus erythematosus. *J Immunol* (1988) **141**:4165–72.
20. Adib-Conquy M, Avrameas S, Ternynck T. Monoclonal IgG and IgM autoantibodies obtained after polyclonal activation, show reactivities similar to those of polyclonal natural autoantibodies. *Mol Immunol* (1993) **30**:119–27. doi:10.1016/0161-5890(93)90083-N
21. Ternynck T, Avrameas S. Murine natural monoclonal autoantibodies: a study of their polyspecificities and their affinities. *Immunol Rev* (1986) **94**:99–112. doi:10.1111/j.1600-065X.1986.tb01166.x
22. Diaw L, Magnac C, Pritsch O, Buckle M, Alzari PM, Dighiero G. Structural and affinity studies of IgM polyreactive natural autoantibodies. *J Immunol* (1997) **158**:968–76.
23. Gronwall C, Silverman GJ. Natural IgM: beneficial autoantibodies for the control of inflammatory and autoimmune disease. *J Clin Immunol* (2014) **34**(Suppl 1):S12–21. doi:10.1007/s10875-014-0025-4
24. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med* (1998) **188**:2381–6. doi:10.1084/jem.188.12.2381
25. Ehrenstein MR, O'Keefe TL, Davies SL, Neuberger MS. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A* (1998) **95**:10089–93. doi:10.1073/pnas.95.17.10089
26. Hitoshi Y, Lorens J, Kitada SI, Fisher J, LaBarge M, Ring HZ, et al. Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. *Immunity* (1998) **8**:461–71. doi:10.1016/S1074-7613(00)80551-8
27. Kubagawa H, Oka S, Kubagawa Y, Torii I, Takayama E, Kang DW, et al. Identity of the elusive IgM Fc receptor (FcmuR) in humans. *J Exp Med* (2009) **206**:2779–93. doi:10.1084/jem.20091107
28. Shima H, Takatsu H, Fukuda S, Ohmae M, Hase K, Kubagawa H, et al. Identification of TOSO/FAIM3 as an Fc receptor for IgM. *Int Immunol* (2010) **22**:149–56. doi:10.1093/intimm/dxp121
29. Vire B, David A, Wiestner A. TOSO, the Fcmicro receptor, is highly expressed on chronic lymphocytic leukemia B cells, internalizes upon IgM binding, shuttles to the lysosome, and is downregulated in response to TLR activation. *J Immunol* (2011) **187**:4040–50. doi:10.4049/jimmunol.1100532
30. Kubagawa H, Kubagawa Y, Jones D, Nasti TH, Walter MR, Honjo K. The old but new IgM Fc receptor (FcmuR). *Curr Top Microbiol Immunol* (2014) **382**:3–28. doi:10.1007/978-3-319-07911-0_1

31. Choi SC, Wang H, Tian L, Murakami Y, Shin DM, Borrego F, et al. Mouse IgM Fc receptor, FCMR, promotes B cell development and modulates antigen-driven immune responses. *J Immunol* (2013) **190**:987–96. doi:10.4049/jimmunol.1202227
32. Honjo K, Kubagawa Y, Jones DM, Dizon B, Zhu Z, Ohno H, et al. Altered Ig levels and antibody responses in mice deficient for the Fc receptor for IgM (FcmuR). *Proc Natl Acad Sci U S A* (2012) **109**:15882–7. doi:10.1073/pnas.1206567109
33. Nguyen XH, Lang PA, Lang KS, Adam D, Fattakhova G, Foger N, et al. Toso regulates the balance between apoptotic and nonapoptotic death receptor signaling by facilitating RIP1 ubiquitination. *Blood* (2011) **118**:598–608. doi:10.1182/blood-2010-10-313643
34. Ouchida R, Mori H, Hase K, Takatsu H, Kurosaki T, Tokuhisa T, et al. Critical role of the IgM Fc receptor in IgM homeostasis, B-cell survival, and humoral immune responses. *Proc Natl Acad Sci U S A* (2012) **109**:E2699–706. doi:10.1073/pnas.1210706109
35. Vanden Berghe T, Hulpiau P, Martens L, Vandenbroucke RE, Van Wonterghem E, Perry SW, et al. Passenger mutations confound interpretation of all genetically modified congenic mice. *Immunity* (2015) **43**:200–9. doi:10.1016/j.immuni.2015.06.011
36. Lang KS, Lang PA, Meryk A, Pandyra AA, Boucher LM, Pozdeev VI, et al. Involvement of Toso in activation of monocytes, macrophages, and granulocytes. *Proc Natl Acad Sci U S A* (2013) **110**:2593–8. doi:10.1073/pnas.1222264110
37. Honjo K, Kubagawa Y, Kubagawa H. Is Toso/IgM Fc receptor (FcmuR) expressed by innate immune cells? *Proc Natl Acad Sci U S A* (2013) **110**:E2540–1. doi:10.1073/pnas.1304904110
38. Shibuya A, Sakamoto N, Shimizu Y, Shibuya K, Osawa M, Hiroyama T, et al. Fc alpha/mu receptor mediates endocytosis of IgM-coated microbes. *Nat Immunol* (2000) **1**:441–6. doi:10.1038/80886
39. Kikuno K, Kang DW, Tahara K, Torii I, Kubagawa HM, Ho KJ, et al. Unusual biochemical features and follicular dendritic cell expression of human Fcalpha/mu receptor. *Eur J Immunol* (2007) **37**:3540–50. doi:10.1002/eji.200737655
40. Yoo EM, Trinh KR, Lim H, Wims LA, Morrison SL. Characterization of IgA and IgM binding and internalization by surface-expressed human Fcalpha/mu receptor. *Mol Immunol* (2011) **48**:1818–26. doi:10.1016/j.molimm.2011.05.011
41. Johansen FE, Braathen R, Brandtzaeg P. The J chain is essential for polymeric Ig receptor-mediated epithelial transport of IgA. *J Immunol* (2001) **167**:5185–92. doi:10.4049/jimmunol.167.9.5185
42. Mostov KE, Kraehenbuhl JP, Blobel G. Receptor-mediated transcellular transport of immunoglobulin: synthesis of secretory component as multiple and larger transmembrane forms. *Proc Natl Acad Sci U S A* (1980) **77**:7257–61. doi:10.1073/pnas.77.12.7257
43. Engels N, Konig LM, Heemann C, Lutz J, Tsubata T, Griep S, et al. Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched B cells. *Nat Immunol* (2009) **10**:1018–25. doi:10.1038/ni.1764
44. Engels N, Wienands J. The signaling tool box for tyrosine-based costimulation of lymphocytes. *Curr Opin Immunol* (2011) **23**:324–9. doi:10.1016/j.coi.2011.01.005
45. Murakami Y, Narayanan S, Su S, Childs R, Krzewski K, Borrego F, et al. Toso, a functional IgM receptor, is regulated by IL-2 in T and NK cells. *J Immunol* (2012) **189**:587–97. doi:10.4049/jimmunol.1200840
46. Rolink AG, Winkler T, Melchers F, Andersson J. Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J Exp Med* (2000) **191**:23–32. doi:10.1084/jem.191.1.23
47. Johnson K, Hashimshony T, Sawai CM, Pongubala JM, Skok JA, Aifantis I, et al. Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling. *Immunity* (2008) **28**:335–45. doi:10.1016/j.immuni.2007.12.019
48. Nguyen TT, Elsner RA, Baumgarth N. Natural IgM prevents autoimmunity by enforcing B cell central tolerance induction. *J Immunol* (2015) **194**:1489–502. doi:10.4049/jimmunol.1401880
49. Baker N, Ehrenstein MR. Cutting edge: selection of B lymphocyte subsets is regulated by natural IgM. *J Immunol* (2002) **169**:6686–90. doi:10.4049/jimmunol.169.12.6686
50. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* (1998) **160**:4776–87.
51. Honjo K, Kubagawa Y, Suzuki Y, Takagi M, Ohno H, Bucy RP, et al. Enhanced auto-antibody production and Mott cell formation in FcmuR-deficient autoimmune mice. *Int Immunol* (2014) **26**:659–72. doi:10.1093/intimm/dxu070
52. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci U S A* (2000) **97**:1184–9. doi:10.1073/pnas.97.3.1184
53. Ehrenstein MR, Cook HT, Neuberger MS. Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. *J Exp Med* (2000) **191**:1253–8. doi:10.1084/jem.191.7.1253
54. Brenner D, Brustle A, Lin GH, Lang PA, Duncan GS, Knobbe-Thomsen CB, et al. Toso controls encephalitogenic immune responses by dendritic cells and regulatory T cells. *Proc Natl Acad Sci U S A* (2014) **111**:1060–5. doi:10.1073/pnas.1323166111
55. Gaublomme JT, Yosef N, Lee Y, Gertner RS, Yang LV, Wu C, et al. Single-cell genomics unveils critical regulators of Th17 cell pathogenicity. *Cell* (2015) **163**:1400–12. doi:10.1016/j.cell.2015.11.009
56. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, et al. Regulation of inflammatory responses by IL-17F. *J Exp Med* (2008) **205**:1063–75. doi:10.1084/jem.20071978
57. Lang PA, Meryk A, Pandyra AA, Brenner D, Brustle A, Xu HC, et al. Toso regulates differentiation and activation of inflammatory dendritic cells during persistence-prone virus infection. *Cell Death Differ* (2015) **22**:164–73. doi:10.1038/cdd.2014.138
58. Atkinson C, Qiao F, Yang X, Zhu P, Reaves N, Kulik L, et al. Targeting pathogenic postischemic self-recognition by natural IgM to protect against post-transplantation cardiac reperfusion injury. *Circulation* (2015) **131**:1171–80. doi:10.1161/CIRCULATIONAHA.114.010482
59. Haas MS, Alicot EM, Schuerpf F, Chiu I, Li J, Moore FD, et al. Blockade of self-reactive IgM significantly reduces injury in a murine model of acute myocardial infarction. *Cardiovasc Res* (2010) **87**:618–27. doi:10.1093/cvr/cvq141
60. Kulik L, Fleming SD, Moratz C, Reuter JW, Novikov A, Chen K, et al. Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury. *J Immunol* (2009) **182**:5363–73. doi:10.4049/jimmunol.0803980
61. Weiser MR, Williams JP, Moore FD Jr, Kobzik L, Ma M, Hechtman HB, et al. Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement. *J Exp Med* (1996) **183**:2343–8. doi:10.1084/jem.183.5.2343
62. Panzer SE, Laskowski J, Renner B, Kulik L, Ljubanovic D, Huber KM, et al. IgM exacerbates glomerular disease progression in complement-induced glomerulopathy. *Kidney Int* (2015) **88**:528–37. doi:10.1038/ki.2015.120
63. Kaveri SV, Silverman GJ, Bayry J. Natural IgM in immune equilibrium and harnessing their therapeutic potential. *J Immunol* (2012) **188**:939–45. doi:10.4049/jimmunol.1102107
64. Lapke N, Tartz S, Lee KH, Jacobs T. The application of anti-Toso antibody enhances CD8(+) T cell responses in experimental malaria vaccination and disease. *Vaccine* (2015) **33**:6763–70. doi:10.1016/j.vaccine.2015.10.065

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 March 2016
doi: 10.3389/fimmu.2016.00108

Expansion of B-1a Cells with Germline Heavy Chain Sequence in Lupus Mice

Nichol E. Holodick[1], Leilani Zeumer[2], Thomas L. Rothstein[1,3,4] and Laurence Morel[2]**

[1] Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, Manhasset, NY, USA, [2] Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL, USA, [3] Department of Medicine, The Hofstra Northwell School of Medicine, Manhasset, NY, USA, [4] Department of Molecular Medicine, The Hofstra Northwell School of Medicine, Manhasset, NY, USA

B6.*Sle1.Sle2.Sle3* (B6.TC) lupus-prone mice carrying the NZB allele of *Cdkn2c,* encoding for the cyclin-dependent kinase inhibitor $P18^{INK4}$, accumulate B-1a cells due to a higher rate of proliferative self-renewal. However, it is unclear whether this affects primarily early-appearing B-1a cells of fetal origin or later-appearing B-1a cells that emerge from bone marrow. B-1a cells are the major source of natural autoantibodies, and it has been shown that their protective nature is associated with a germline-like sequence, which is characterized by few N-nucleotide insertions and a repertoire skewed toward rearrangements predominated during fetal life, V_H11 and V_H12. To determine the nature of B-1a cells expanded in B6.TC mice, we amplified immunoglobulin genes by PCR from single cells in mice. Sequencing showed a significantly higher proportion of B-1a cell antibodies that display fewer N-additions in B6.TC mice than in B6 control mice. Following this lower number of N-insertions within the CDR-H3 region, the B6.TC B-1a cells display shorter CDR-H3 length than B6 B-1a cells. The absence of N-additions is a surrogate for fetal origin, as TdT expression starts after birth in mice. Therefore, our results suggest that the B-1a cell population is not only expanded in autoimmune B6.TC mice but also qualitatively different with the majority of cells from fetal origin. Accordingly, our sequencing results also demonstrated the overuse of V_H11 and V_H12 in autoimmune B6.TC mice as compared to B6 controls. These results suggest that the development of lupus autoantibodies in these mice is coupled with skewing of the B-1a cell repertoire and possible retention of protective natural antibodies.

Keywords: B cells, B-1 cells, autoimmunity, lupus erythematosus, systemic, repertoire analysis, mouse model, natural antibodies

OPEN ACCESS

Edited by:
George C. Tsokos, Harvard Medical School, USA

Reviewed by:
Subbarao Bondada, University of Kentucky, USA
Yoshiteru Sasaki, Kyoto University, Japan

****Correspondence:***
Nichol E. Holodick
nholodick@iclould.com;
Laurence Morel
morel@ufl.edu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *29 January 2016*
Accepted: *10 March 2016*
Published: *24 March 2016*

Citation:
Holodick NE, Zeumer L, Rothstein TL and Morel L (2016) Expansion of B-1a Cells with Germline Heavy Chain Sequence in Lupus Mice. Front. Immunol. 7:108. doi: 10.3389/fimmu.2016.00108

INTRODUCTION

Murine B-1a cells are a unique B-lymphocyte lineage characterized by phenotypic, functional, and ontologic characteristics (1, 2). B-1a cells are defined by surface marker expression of $IgM^{hi}IgD^{lo}CD45R^{lo}CD5^{+}CD43^{+}CD19^{hi}$ and are found in the peritoneal cavity, spleen, and bone marrow (3, 4). Functionally, B-1a cells exhibit unique signaling characteristics (4–6), are potent antigen-presenting

cells (7), and spontaneously produce 80–90% of natural serum IgM in mice (8). Natural IgM is non-immune, low-affinity immunoglobulin (Ig) that is both polyreactive and autoreactive. It functions in infection, atherosclerosis, B cell homeostasis, inflammation, and autoimmunity [reviewed in Ref. (9)]. Minimal N-region addition contributes to the germline-like nature of natural IgM. Furthermore, natural IgM manifests biased variable heavy chain (VH) gene usage in favor of V_H11 and V_H12, which are specific for phosphatidylcholine (PtC), a major component of cell membrane phospholipids (3, 10–13). This unique germline structure of natural IgM is established during the early fetal and neonatal development of B-1a cells (8).

The polyreactive nature of natural IgM provides initial defense against both bacterial and viral pathogens, which affords the organism protection during the period preceding generation of high-affinity antigen-specific antibodies produced by germinal center B-2 cells (3, 4, 14–17). The autoreactive quality of natural IgM has been shown to aid in the elimination of excess autoantigens through the removal of apoptotic cells and noxious molecular debris, thereby maintaining homeostasis and preventing inflammation (9, 18). These autoreactive natural antibodies are often directed against cell membrane components, such as PtC and phosphorylcholine (PC), which is the polar head group of PtC and is a major microbial cell wall determinant (19). Interestingly, such components are closely related to those also present on pathogens, which suggest that the natural autoreactive repertoire also react with common pathogens (20).

While this cross-reactivity of natural autoreactive antibodies with pathogens is beneficial, it highlights the importance of regulating B-1a cell expansion. Accumulation of B-1a cells has been shown in the NZM2410 (NZB × NZW F_1 hybrid) mouse model of systemic lupus erythematosus (SLE) (21, 22). However, the role of B-1a cells in lupus is still unclear (23). Some studies have demonstrated a role for B-1a cells *via* production of IL-10 (22), increase in antigen presentation (22), or with overexpression of osteopontin, resulting in expansion of B-1a cells and increased anti-dsDNA antibody production (24). In other models, however, B-1a cells do not contribute to disease (25, 26).

The expansion of B-1a cells in the NZM2410 model was traced to the *Sle2c1* lupus susceptibility locus, which contains *Ckdn2c* (27, 28). The *Ckdn2c* gene encodes for $p18^{INK4c}$, which is a cyclin-dependent kinase inhibitor that controls progression through G1 of the cell cycle (28). These studies demonstrated that the expansion of B-1a cells is intrinsic. B6.*Sle2c1* B-1a cells showed increased proliferation at rest, as well as increased resistance to cell death. In addition, B-1a cell reconstitution from fetal liver, adult bone marrow, and adult spleen was higher in B6.*Sle2c1* than in control C57/BL6 (B6) mice following lethal irradiation (21). Furthermore, comparison of $p18^{-/-}$ mice with B6.*Sle2c1* mice demonstrated that both produced autoantibodies; however, the amount produced by $p18^{-/-}$ mice was greater. This demonstrates that the control of the B-1a cell population depends on the amount of p18. B6.*Sle2c1* mouse B cells have fourfold less *Ckdn2c* than normal mice, whereas $p18^{-/-}$ mice completely lack *Ckdn2c* (28). Together, these results demonstrate an important role for p18 in B-1a cell numbers, which in turn affects the production of autoantibodies and development of autoimmunity. However, the origin of B-1a cell expansion in B6.TC, B6.Slec1, and $p18^{-/-}$ mice could be due to an increase in proliferation of early-appearing fetal-derived B-1a cells or heightened production of later-appearing bone marrow-derived B-1a cells. As the repertoires of early- and later-appearing B-1a cells differ, these two possibilities can be distinguished. Herein, we investigated whether significant changes to the natural IgM repertoire occur in triple congenic B6.*Sle1.Sle2.Sle3* (B6.TC) lupus-prone mice. These mice carry the *Sle2c1* locus that drives B-1a cell expansion and present clinical autoimmune pathology that has been described for the NZM2410 pathology (29). B6.TC mice carry the NZM2410 susceptibility loci on a B6 genetic background (>95%) that includes both heavy and light immunoglobulin chains, which allow to directly compare the lupus-prone B6.TC mice to the control B6 mice. Specifically, we found that the expansion of B-1a cells in B6.TC mice is associated with repertoire skewing toward V_H11 and V_H12 usage.

MATERIALS AND METHODS

Mice

B6.NZM-*Sle1*$^{NZM2410/Aeg}$*Sle2*$^{NZM2410/Aeg}$*Sle3*$^{NZM2410/Aeg}$/LmoJ (B6.TC) congenic mice have been previously described (29). B6.TC lupus-prone and C57BL6/J (B6) control mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

Single-Cell Sequencing and Analysis

Peritoneal washout cells were obtained from 8-week-old wild-type B6 mice and 8-week-old B6.TC mice (two each). The cells were stained with fluorescence-labeled antibodies to CD45R/B220 (clone RA3-6B2), CD5 (clone 53-7.3), and CD23 (clone B3B4) (BD Biosciences). B-1a cells ($CD45R^{lo}/CD5^{+}/CD23^{-}$) were then purified using an Influx cell sorter (BD Biosciences). Post-sort reanalysis of B cell populations showed them to be ≥98% pure. Peritoneal B-1a cells were sorted into a 96-well plate containing 20 µl of lysis buffer per well (dH_2O, RNase Out, 5× SuperScript III Buffer, DTT, IgePAL, and Carrier RNA). Reverse transcription was performed (42°C – 10 min, 25°C – 10 min, 50°C – 60 min, 94°C – 5 min, hold at 4°C) after addition of random hexamers, dNTP mix, and SuperScript III reverse transcriptase. Semi-nested PCR was performed using the cDNA diluted 1:1. Using Qiagen's HotStart Taq Plus, 2.5 µl of cDNA was used for the first PCR reaction with previously described primers (30). The product from this first PCR reaction was then diluted 1:100 and 2 µl of the diluted product was added to the second PCR reaction. The products were purified and then sequenced (Genewiz) using the forward primer. Sequences were then analyzed using an online sequence analysis tool for VDJ sequences (IMGT, the international ImMunoGeneTics information system).

Statistics

Comparisons were conducted between the pooled B6.TC and B6 sequences and the two strains using Graphpad Prism 6.0 with two-tailed tests, as indicated in the figure legends.

RESULTS

Lupus-Prone Triple Congenic Mice Display an Increase in Duplicate Sequences

The B-1a cell repertoire of B6.TC mice was compared to control B6 mice. Repertoire analysis was performed by PCR amplification of the V_HDJ_H region from individual peritoneal B-1a cells, which were previously shown to accumulate in B6.TC mice (21, 28). Interestingly, the B-1a cells from B6.TC mice had a significantly larger number of IgM sequences with identical V_H, D_H, and J_H segments as well as identical CDR3 regions than B6 mice (**Table 1**, B6.TC: 108 out of 146 total sequences; B6: 50 out of 105 total sequences; $p = 0.0335$, Mann–Whitney test). As stated in previously published work (30), it cannot be determined whether these sequences containing identical V_H, D_H, J_H, and CDR3 regions result from a single clonal expansion or from analysis of independent cells with identical rearrangements. Therefore, we will refer to such sequences as duplicate sequences instead of clones. Furthermore, V_H usage within the duplicate sequences differed significantly between B6 and B6.TC mice. As shown in **Table 1**, the duplicate sequences with the highest frequency in B6 mice utilized V_H1–55 (58%), whereas the most frequent duplicate sequences in B6.TC mice utilized V_H11 and V_H12 (43 and 46%, respectively). While both V_H11 and V_H12 utilization (20 and 8%, respectively) was observed in B6 mice, this percentage of duplicate sequences was significantly less than that seen in B6.TC mice (**Figure 1**). These results suggest that the accumulation of peritoneal B-1a cells seen in B6.TC mice might be influenced by autoantigen since there is an expansion of B-1a cells utilizing V_H11 and V_H12, which are specific for PtC, a major component of cell membrane phospholipids.

V_H–D_H–J_H Usage Shows Differences between B6 and B6.TC Mouse Repertoires

For analysis of V_H, D_H, and J_H usage, we evaluated the repertoire in two ways. First, we analyzed only sequences with unique CDR-H3 regions by removing all duplicate sequences. In the second method, we analyzed all sequences, which included the duplicate sequences.

When analyzing only sequences with unique CDR-H3 regions, we found overall similarity in D_H and J_H usage with only one major significant difference in V_H usage. Among V_H gene segments, V_H1 was expressed significantly less frequently by B6.TC B-1a cells (26%) as compared to B6 B-1a cells (48%) ($p = 0.0034$, chi-square test) (**Figure 2A**).

We found numerous differences in V_H–D_H–J_H usage when analyzing all sequences, including the duplicate sequences (**Figure 2B**). V_H1 and V_H5 were expressed significantly less frequently by B6.TC B-1a cells (10 and 3%, respectively), as compared to B6 B-1a cells (55 and 9%, respectively) ($p < 0.0001$ and $p = 0.0397$, chi-square test). Conversely, V_H11 and V_H12 were utilized significantly more frequently by B6.TC B-1a cells (35 and 34%, respectively), as compared to B6 B-1a cells (11 and 10%, respectively) ($p < 0.0001$ and $p < 0.0001$, chi-square test). Among D_H gene segments, no difference was observed for the unique sequences between strains (**Figure 3A**). When all sequences were compared, DFL16.1 was expressed less frequently, and DSP was expressed more frequently by B6.TC B-1a

TABLE 1 | Duplicate sequences.

Sample	CDR3 AA sequence	VH	DH	JH	Number of duplicate sequences	Percent of duplicates
B6	AGDSHGYWYFDV	IGHV12-3*01	IGHD1-1*02	IGHJ1*03	2	
	AGDVTGYWYFDV	IGHV12-3*01	IGHD4-1*01	IGHJ1*02	2	
	ARFYYYGSSYAMDY	IGHV1-55*01	IGHD1-1*01	IGHJ4*01	7	
	ARRDYGSSYWYFDV	IGHV1-55*01	IGHD1-1*01	IGHJ1*02	22	
	ARHYYGSSYYFDY	IGHV5-6*01	IGHD1-1*01	IGHJ2*01	4	
	MRYGNYWYFDV	IGHV11-2*01	IGHD2-1*01	IGHJ1*03	7	
	MRYSNYWYFDV	IGHV11-2*01	IGHD2-5*01	IGHJ1*03	3	
	TREDYYYYGSSYYAMDY	IGHV5-9-1*02	IGHD1-1*01	IGHJ4*01	3	
					50	48
B6.TC	AGDNDGYWYFDV	IGHV12-3*01	IGHD2-3*01	IGHJ1*03	3	
	AGDNDGYYGFAY	IGHV12-3*01	IGHD2-3*01	IGHJ3*01	2	
	AGDYDGYWYFDV	IGHV12-3*01	IGHD2-3*01	IGHJ1*03	37	
	AGDYYGYWYFDV	IGHV12-3*01	IGHD1-1*02	IGHJ1*03	4	
	ARDYYGSSHYFDY	IGHV1-82*01	IGHD1-1*01	IGHJ2*01	2	
	ARELIYYGNYGYFDV	IGHV1-72*01	IGHD2-1*01	IGHJ1*03	2	
	ARPYYSNYYAMDY	IGHV2-9-1*01	IGHD2-5*01	IGHJ4*01	2	
	ARYYYGSSYAMDY	IGHV7-3*01	IGHD1-1*01	IGHJ4*01	2	
	MRYGNYWYFDV	IGHV11-2*01	IGHD2-1*01	IGHJ1*03	39	
	MRYGSSYWYFDV	IGHV11-2*01	IGHD1-1*01	IGHJ1*03	3	
	MRYSNYWYFDV	IGHV11-2*01	IGHD2-5*01	IGHJ1*03	8	
	TRTSGYFDY	IGHV6-6*01	IGHD1-3*01	IGHJ2*01	2	
	VRHYGSSYFDY	IGHV10-1*01	IGHD1-1*01	IGHJ2*01	2	
					108	74

Peritoneal B-1a cells were single-cell sorted from 8-week-old C57BL/6 mice (B6) and B6.Sle1.Sle2.Sle3 (triple congenic, B6.TC) lupus-prone mice. IgM was amplified and sequenced as detailed in the Section "Materials and Methods." Sequencing analysis revealed a number of sequences with identical CDR-H3 regions, which we refer to as duplicate sequences.

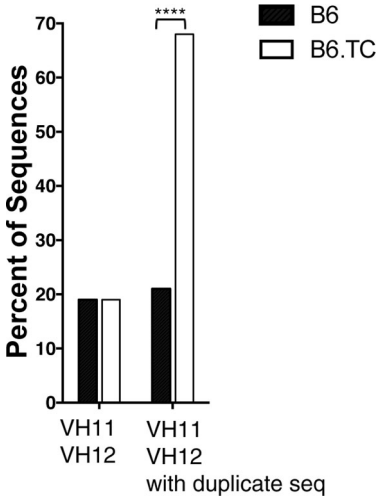


FIGURE 1 | Percent of V_H11 and V_H12 representation. Peritoneal B-1a cells were single-cell sorted from 8-week-old B6 control mice and B6.TC lupus-prone mice. IgM was amplified and sequenced as detailed in the Section "Materials and Methods." The percent of sequences that utilized V_H11 and V_H12 are shown for all unique sequences (left, B6 $n = 62$; B6.TC $n = 47$), and all sequences obtained, including the duplicates (right, B6 $n = 105$; B6.TC $n = 146$). Statistical analysis was performed using chi-square analysis (2 × 2 using the VH of interest and all others as the two categories), VH11/VH12 with duplicate sequences ****$p < 0.0001$.

cells (17 and 74%, respectively) as compared to B6 B-1a cells (54 and 32%, respectively) ($p < 0.0001$ and $p < 0.0001$, chi-square test) (**Figure 3B**). As for J_H segments, no difference was observed between strains among J_H gene segments in unique sequences (**Figure 4A**). However, when all sequences were compared, J_H1 was expressed more frequently (74%) ($p < 0.0001$), and J_H2 and J_H4 were expressed less frequently (11 and 8%, respectively) ($p = 0.0293$ and $p < 0.0001$, respectively) by B6.TC B-1a cells as compared to B6 B-1a cells (45, 21, and 28%, respectively) (**Figure 4B**). Thus, distinct V_H, D_H, and J_H gene segment usage separated B6.TC from B6 peritoneal B-1a cells across all sequences.

The B-1a Cell Repertoire Is Less Diverse in B6.TC Mice as Compared to B6 Mice

N-region addition provides diversity to the CDR-H3 region of antibodies *via* random insertion of nucleotides at the V–D and D–J junctions by the enzyme TdT. It is well-documented that peritoneal B-1a cells have limited N-addition due to the lack of TdT expression during fetal development (31). We analyzed N-addition at the D–J and V–D junctions and determined CDR3 length. No significant differences were found when analyzing sequences with only unique CDR-H3 regions (**Table 2**). In contrast, analysis of all sequences, including the duplicates, demonstrated significant differences between B-1a cells from B6.TC and B6 mice. We found that the number of N-additions at the D–J or V–D junctions of B6.TC B-1a cells was significantly less than B6 B-1a cells ($p < 0.0001$ and $p = 0.0120$, respectively) (**Table 2**). B6.TC B-1a cells were also found to contain significantly fewer N-additions when analyzing the sum of the two junctions as compared to B6 B-1a cells ($p < 0.0001$). We further examined CDR-H3 length and found, consistent with the differences in N-addition between B6.TC and B6 B-1a cells, the average CDR-H3 lengths differed significantly when analyzing all sequences ($p = 0.0044$), but did not differ when analyzing only sequences with unique CDR-H3 regions (**Table 2**). These results demonstrate that the B6.TC B-1a cell population expresses immunoglobulin that is less diverse due to fewer N-region additions as compared to the B6 peritoneal B-1a cell population.

We then focused the analysis of N-addition on sequences utilizing V_H11 and V_H12, which are overrepresented in the B6.TC B-1a cells when considering all sequences, including the duplicate sequences. Interestingly, the number of B6.TC B-1a cell sequences lacking N-additions at both junctions in cells utilizing V_H11 and V_H12 (98 and 100%, respectively) was significantly different than B6 B-1a cell sequences (92 and 10%, respectively) ($p = 0.0381$ and $p < 0.0001$, respectively, by chi-square test), particularly with respect to V_H12, although the number of B6 V_H11/V_H12 sequences was small ($n = 12/n = 10$). These results are summarized in **Figure 5**.

CDR-H3s Are More Charged in B6.TC than B6 B-1a Cells

Autoreactive antibodies, and in particular anti-dsDNA antibodies, are often enriched for charged amino acids in their CDR-H3 loop region (32–34). This region normally contains neutral, hydrophilic amino acids, which is due partly to usage of certain D_H and J_H sequences and use of the D reading frame I (32, 35). Amino acid changes within the loop region are also affected by N-region insertions (32). Upon evaluation of B6.TC B-1a cell CDR-H3 charge, we found that the average charge was increased in B6.TC B-1a cells (−0.213) over B6 B-1a cells (−0.142); however, this difference did not reach significance in the analysis of unique sequences (**Figure 6A**). When analyzing all sequences (including the duplicates), again the average charge of the CDR-H3 loop region was increased in B6.TC B-1a cells (−0.298) over B6 B-1a cells (−0.201) ($p = 0.0015$) (**Figure 6B**). Furthermore, comparing the average charge of the CDR-H3 loop region of B-1a cells utilizing V_H11 and V_H12 also demonstrated a greater charge in B6.TC B-1a cells (−0.362) than B6 B-1a cells (−0.290) ($p = 0.0197$) (**Figure 6C**). These results correlate with the differences observed in N-region addition between B6.TC and B6 B-1a cells utilizing V_H11 and V_H12.

DISCUSSION

Primary repertoire analysis of B-1a cells from 8-week-old B6.*Sle1.Sle2.Sle3* (B6.TC) lupus-prone mice demonstrated a large number of sequences that express identical CDR-H3 regions as compared to B-1a cells from healthy 8-week-old C57BL/6 (B6). This analysis demonstrates a significant increase in identical V_H, D_H, J_H usage in B6.TC mice. Although it is not possible to determine whether the duplicate sequences observed herein result from a single clonal expansion or from analysis of multiple cells with identical rearrangements, it has been well-documented over the years that B-1 cells have a limited repertoire (11, 14, 36–38), can undergo clonal expansion (39–42), and are self-replenishing (8). Therefore, these

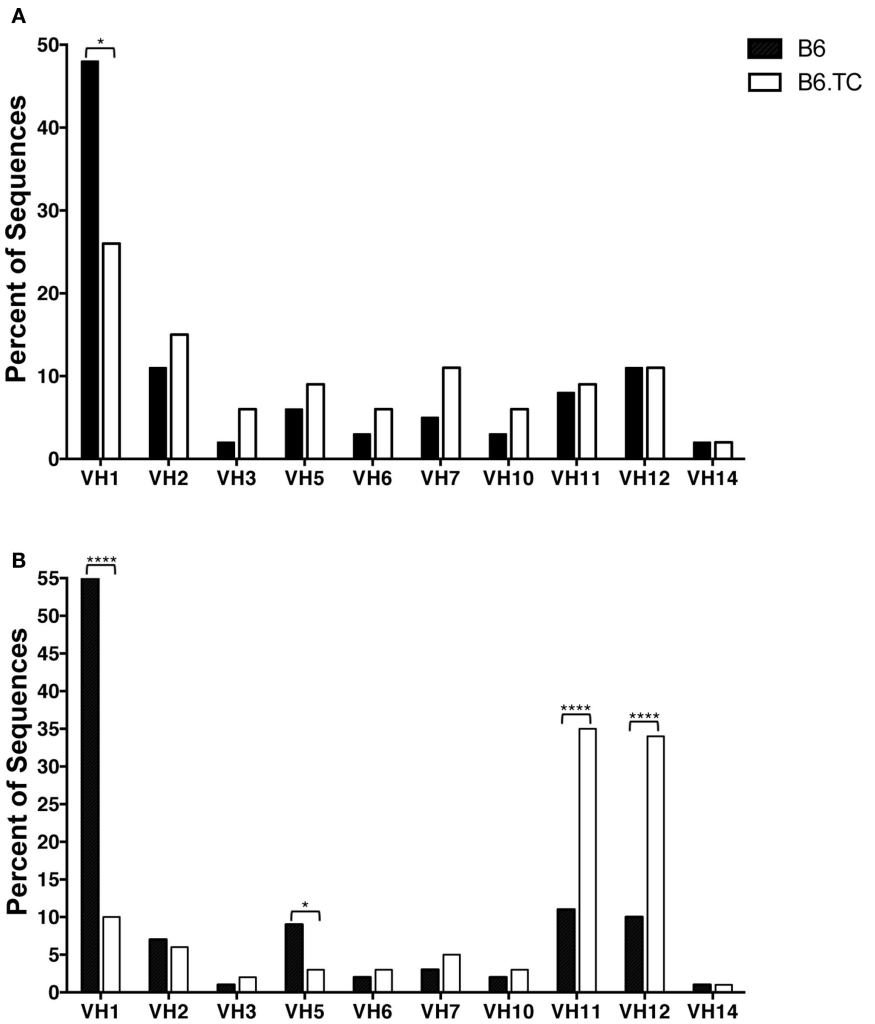


FIGURE 2 | V_H analysis of IgM from C57BL/6 and triple congenic peritoneal B-1a cells. Immunoglobulins were amplified by PCR from single-cell sorted peritoneal B-1a cells obtained from B6 and B6.TC mice and evaluated for the variable (V) segment heavy chain usage. The percent of cells (sequences) expressing the V segment usage is displayed. Chi-squared test was used to determine significance. **(A)** Analysis of sequences with only unique CDR-H3 regions (B6, *n* = 62; B6.TC *n* = 47). **(B)** Analysis of all sequences obtained, including the duplicate sequences (B6, *n* = 105; B6.TC *n* = 146).

duplicate sequences are most likely due to expansion of single B-1a cells. Further analysis, including the duplicate sequences, reveals that the B6.TC B-1a cell repertoire displays early fetal/neonatal-like characteristics, which consists of an increase in use of J_H1 [**Figure 4B**; Ref. (43)], few N-additions at both the V–D and D–J junctions, and a shorter average CDR-H3 length (**Table 2**). In addition, the B6.TC repertoire overused V_H11 and V_H12 as compared to B6 (**Figures 1** and **2**). Interestingly, V_H11 and V_H12 rearrangements are utilized almost exclusively by B-1a cells and target the cell membrane component PtC (19). Studies have shown V_H11 in particular is a V_H gene utilized during fetal development but not during adult development (44, 45). More recently, Yang et al. have shown overuse of V_H11 in the normal healthy peritoneal B-1a cell pool (38). Our results demonstrate the most common CDR3 in peritoneal B-1a cells from our normal healthy 2-month old B6 mice is ARRDYGSSYWYFDV (V_H1-55, D_H1-1, J_H1). Examining Yang et al's most common CDR3 in peritoneal B-1a cells from their normal healthy 2-month old B6 mice, it is ARFYYYGSSYAMDY, (V_H1-55, D_H1-1, J_H4), which does not share the exact same CDR3 as ours but does share the same V_H and D_H region. Our second most common CDR3 sequences (two are tied for second place) are identical to Yang et al's first and second most common CDR3 sequences ARFYYYGSSYAMDY and MRYGNYWYFDV (V_H11-2, D2-8, J_H1), respectively. The rank order of the sequences we identified is very similar to that of Yang et al. with only minor differences. Together, these results indicate that the B-1a cell repertoire in B6.TC mice reflects fetal rearrangements to a much greater extent than the B6 B-1a cell repertoire.

The mechanism for this selection toward fetal rearrangements in B6.TC mice is unknown; however, it can be speculated that the *Sle2c1* lupus susceptibility locus, which contains *Ckdn2c* and results in less p18 expression, could lead to a difference in

expansion of B-1a cells with different BCR signaling requirements. It is possible the V_H11 and V_H12 specificities require a different level of BCR signaling, which the reduced level of p18 might provide, thereby allowing for increased proliferation of V_H11 and V_H12 expressing B-1a cells. In this view, *Ckdn2c* does not encode B-1a cell expansion on its own but does so in collaboration with BCR signals of requisite intensity. Our data suggest that such signals might be provided by self-antigen binding of PtC-specific immunoglobulins in preference to other elements of the B-1a cell repertoire.

Various studies have shown that B-1a cell-derived natural IgM provides protection against not only infection but also autoimmunity (9, 18, 46). The role of IgM in protection against autoimmunity was recently demonstrated in a mouse model lacking secretory IgM. This study demonstrated that natural IgM is required to control the accumulation of autoantibodies *via* its ability to regulate B cell development and selection (46). In mice lacking secretory IgM, the number of peritoneal B-1a cells was

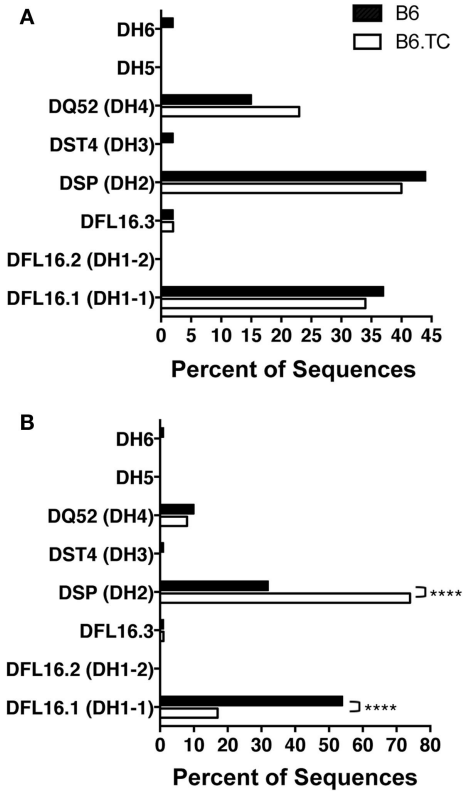


FIGURE 3 | D_H analysis of IgM from C57BL/6 and triple congenic mouse peritoneal B-1a cells. Immunoglobulins were amplified by PCR from single-cell sorted peritoneal B-1a cells obtained from B6 and B6.TC mice and evaluated for the diversity (D) segment heavy chain usage. The percent of cells (sequences) expressing the D segment usage is displayed. Chi-squared test was used to determine significance. **(A)** Analysis of sequences with only unique CDR-H3 regions (B6, $n = 62$; B6.TC $n = 47$). **(B)** Analysis of all sequences obtained, including the duplicate sequences (B6, $n = 105$; B6.TC $n = 146$).

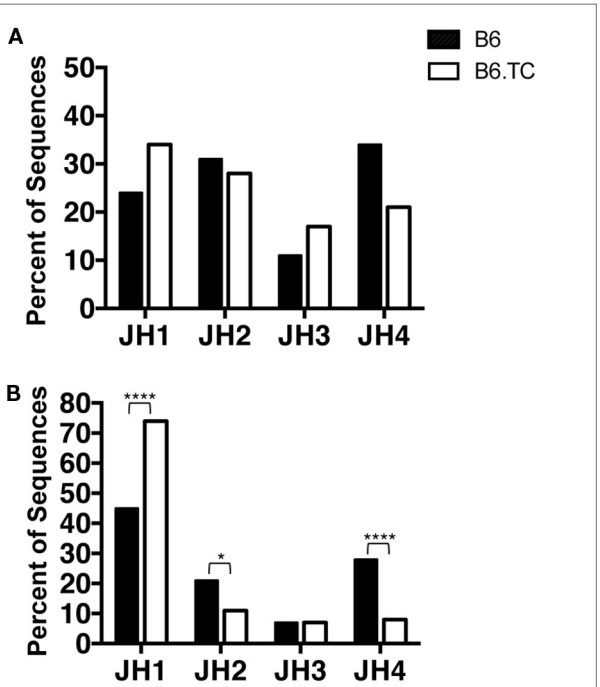


FIGURE 4 | J_H analysis of IgM from C57BL/6 and triple congenic mouse peritoneal B-1a cells. Immunoglobulins were amplified by PCR from single-cell sorted peritoneal B-1a cells obtained from B6 and B6.TC mice and evaluated for the junction (J) segment heavy chain usage. The percent of cells (sequences) expressing the J segment usage is displayed. Chi-squared test was used to determine significance. **(A)** Analysis of sequences with only unique CDR-H3 regions (B6, $n = 62$; B6.TC $n = 47$). **(B)** Analysis of all sequences obtained, including the duplicate sequences (B6, $n = 105$; B6.TC $n = 146$).

TABLE 2 | N-region addition and CDR3 length analysis of IgM from C57BL/6 and triple congenic mouse peritoneal B-1a cells.

	CDR-H3 length	V–D	D–J	Sum
B6	11.9 (±0.295)	1.74 (±0.265)	0.89 (±0.203)	2.63 (±0.338)
B6.TC	12.0 (±0.680)	1.57 (±0.322)	2.34 (±1.81)	3.92 (±1.80)
With duplicate sequences	**CDR-H3 length**	**V–D**	**D–J**	**Sum**
B6	12.5 (±0.208)	2.07 (±0.215)	0.533 (±0.127)	2.60 (±0.244)
B6.TC	11.7 (±0.224)	0.63 (±0.129)	0.329 (±0.148)	0.959 (±0.204)

Immunoglobulins were amplified by PCR from single-cell sorted peritoneal B-1a cells obtained from B6 and B6.TC mice and evaluated for N-region additions and CDR3 lengths. The average number of N-additions at each junction or sum of the two junctions is displayed (± SEM).

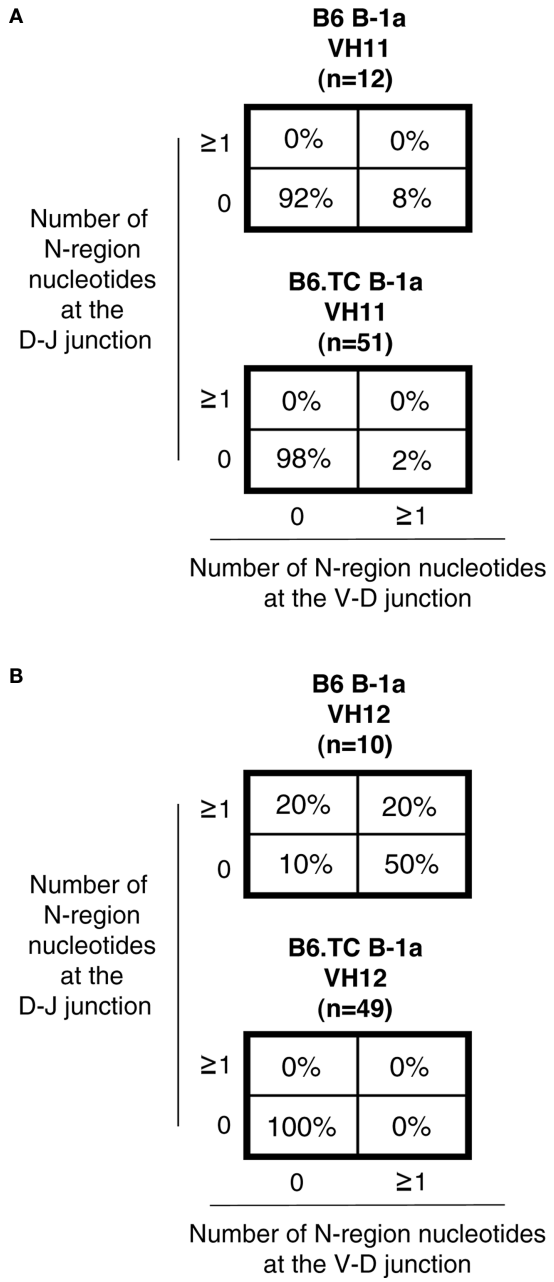


FIGURE 5 | N-region addition analysis of IgM from C57BL/6 and triple congenic mouse peritoneal B-1a cells utilizing V_H11 and V_H12. Immunoglobulins were amplified by PCR from single-cell sorted peritoneal B-1a cells obtained from B6 and B6.TC mice and evaluated for N-region additions. The percent of sequences with 0 N-additions at both junctions, one or more N-additions at both junctions, 0 N-additions at V–D and one or more at D–J junctions, or 0 N-additions at D–J and one or more at V–D junctions is shown. **(A)** N-addition analysis of B-1a cells utilizing V_H11 (B6 $n = 12$; B6.TC $n = 51$). **(B)** N-addition analysis of B-1a cells utilizing V_H12 (B6 $n = 10$; B6.TC $n = 49$).

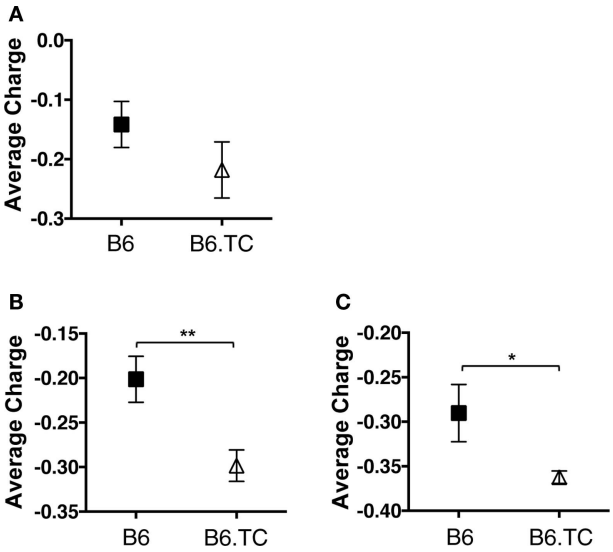


FIGURE 6 | Average charge of CDR-H3 loop region of IgM from C57BL/6 and triple congenic mouse peritoneal B-1a cells. Immunoglobulins were amplified by PCR from single-cell sorted peritoneal B-1a cells obtained from B6 and B6.TC mice. IgM was amplified and sequenced as detailed in the Section "Materials and Methods." The average charge of the CDR-H3 loop region of IgM is shown. **(A)** Analysis of sequences with only unique CDR-H3 regions (B6, $n = 62$; B6.TC $n = 47$). **(B)** Analysis of all sequences obtained, including the duplicate sequences (B6, $n = 105$; B6.TC $n = 146$). **(C)** Analysis of all VH11 and VH12 sequences, including the duplicate sequences (B6, $n = 22$; B6.TC $n = 100$).

significantly decreased and of the B-1a cells present, there was little V_H11 expression, which correlated with a lack of PtC-binding B-1a cells (46). These findings would seem to be at odds with previous studies demonstrating that mice with the *Sle2c1* lupus susceptibility locus have an expansion of peritoneal B-1a cells and yet they develop autoimmunity (28). Furthermore, results presented herein reveal that the B-1a cell repertoire in B6.TC mice is significantly more skewed toward V_H11 and V_H12 than control B6 mice (**Figures 1** and **2**). Together, these studies raise the question as to why the B6.TC mice are not protected against autoimmunity if they have an expansion of B-1a cells producing protective natural IgM. As demonstrated by Nguyen et al., selection of the B cell repertoire is affected by the presence of IgM (46). Herein, we demonstrate the expanded B-1a cells in mice carrying the *Sle2c1* lupus susceptibility locus are skewed toward a different specificity than control B6 mice. Together, these studies suggest that altering the pool of natural IgM disrupts the balance of antibodies, which enable selection of a healthy non-autoreactive repertoire. In other words, alteration of the natural IgM repertoire in the TC.B6 mice could then lead to selection of an autoreactive repertoire instead of a non-autoreactive repertoire, despite natural IgM being present. Furthermore, the role of B-1a cells in autoimmunity may not be limited to the antibodies they produce. B-1a cells have been shown to be potent antigen-presenting cells, which could also contribute to autoimmunity (7, 47, 48). In addition, B-1a cells have immunoregulatory functions through the secretion of IL-10 (49), ability to produce adenosine (50, 51), and ability to class switch in sites of inflammation (52).

The greater expansion/overuse of V_H11 and V_H12 might not help in the regulation of autoimmunity; however, it might afford increased protection from sepsis. It has been shown that the mice

lacking secretory IgM are more susceptible to sepsis induced by cecal ligation and puncture (53). Interestingly, these mice could be rescued by injection of anti-PtC antibody, but not anti-PC antibody (53). Future studies could provide insight into whether the B6.TC lupus-prone mice might be more protected against bacterial sepsis. Such resistance to bacteria has been shown for mice with the *Sle3* lupus susceptibility locus (54); however, a role for B cells in such resistance has yet to be investigated.

The results presented herein demonstrate that the available B-1a cell repertoire present in 8-week-old B6.TC lupus-prone mice is more characteristic of an early fetal/neonatal B cell repertoire than that of B-1a cells from healthy aged-matched B6 mice. Thus, B6.TC-enhanced B-1a cell expansion is established early on and affects developing B-1a cells in a BCR-specific manner. Nonetheless, further analysis is required to determine the mechanism of B-1a cell expansion, and in particular, whether certain BCR specificities have a growth advantage in B6.TC mice. Overall, our results together with previous studies suggest the development of natural IgM that is protective against both bacterial infections and autoimmunity might require a balance of repertoire specificities. Previously published studies suggest this balance is greatly influenced by the IgM repertoire present (46). Further repertoire analyses of healthy and autoimmune models will help uncover factors that might affect this balance of protection against both infection and autoimmunity.

ETHICS STATEMENT

Experiments using animals were conducted under approved Institutional Animal Care and Use Committee (IACUC) at the University of Florida.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, LM, NH, and TR; investigation, NH, LZ, and LM; writing – original draft, NH; writing – review and editing, LM, TR, and NH; funding acquisition, resources, and supervision, LM and TR.

FUNDING

This work was supported by Public Health Service grants AI029690 (TR) and AI058150 (LM) awarded by the National Institutes of Health.

REFERENCES

1. Herzenberg LA, Tung JW. B cell lineages: documented at last! *Nat Immunol* (2006) **7**:225–6. doi:10.1038/ni0306-225
2. Rothstein TL, Griffin DO, Holodick NE, Quach TD, Kaku H. Human B-1 cells take the stage. *Ann N Y Acad Sci* (2013) **1285**:97–114. doi:10.1111/nyas.12137
3. Hardy RR, Hayakawa K. B cell development pathways. *Annu Rev Immunol* (2001) **19**:595–621. doi:10.1146/annurev.immunol.19.1.595
4. Rothstein TL. Cutting edge commentary: two B-1 or not to be one. *J Immunol* (2002) **168**:4257–61. doi:10.4049/jimmunol.168.9.4257
5. Wong SC, Chew WK, Tan JE, Melendez AJ, Francis F, Lam KP. Peritoneal CD5+ B-1 cells have signaling properties similar to tolerant B cells. *J Biol Chem* (2002) **277**:30707–15. doi:10.1074/jbc.M202460200
6. Holodick NE, Tumang JR, Rothstein TL. Continual signaling is responsible for constitutive ERK phosphorylation in B-1a cells. *Mol Immunol* (2009) **46**:3029–36. doi:10.1016/j.molimm.2009.06.011
7. Zhong X, Gao W, Degauque N, Bai C, Lu Y, Kenny J, et al. Reciprocal generation of Th1/Th17 and T(reg) cells by B1 and B2 B cells. *Eur J Immunol* (2007) **37**:2400–4. doi:10.1002/eji.200737296
8. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) **11**:34–46. doi:10.1038/nri2901
9. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* (2010) **10**:778–86. doi:10.1038/nri2849
10. Feeney AJ. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J Exp Med* (1990) **172**:1377–90. doi:10.1084/jem.172.5.1377
11. Pennell CA, Arnold LW, Haughton G, Clarke SH. Restricted Ig variable region gene expression among Ly-1+ B cell lymphomas. *J Immunol* (1988) **141**:2788–96.
12. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J Immunol* (1989) **142**:3643–51.
13. Wang H, Clarke SH. Positive selection focuses the VH12 B-cell repertoire towards a single B1 specificity with survival function. *Immunol Rev* (2004) **197**:51–9. doi:10.1111/j.0105-2896.2004.0098.x
14. Su SD, Ward MM, Apicella MA, Ward RE. The primary B cell response to the O/core region of bacterial lipopolysaccharide is restricted to the Ly-1 lineage. *J Immunol* (1991) **146**:327–31.
15. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* (1999) **286**:2156–9. doi:10.1126/science.286.5447.2156
16. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* (2000) **192**:271–80. doi:10.1084/jem.192.2.271
17. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity* (2005) **23**:7–18. doi:10.1016/j.immuni.2005.04.011
18. Gronwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. *Front Immunol* (2012) **3**:66. doi:10.3389/fimmu.2012.00066
19. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G. Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J Exp Med* (1988) **168**:687–98. doi:10.1084/jem.168.2.687
20. Baumgarth N, Tung JW, Herzenberg LA. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin Immunopathol* (2005) **26**:347–62. doi:10.1007/s00281-004-0182-2
21. Xu Z, Butfiloski EJ, Sobel ES, Morel L. Mechanisms of peritoneal B-1a cells accumulation induced by murine lupus susceptibility locus Sle2. *J Immunol* (2004) **173**:6050–8. doi:10.4049/jimmunol.173.10.6050
22. Mohan C, Morel L, Yang P, Wakeland EK. Accumulation of splenic B1a cells with potent antigen-presenting capability in NZM2410 lupus-prone mice. *Arthritis Rheum* (1998) **41**:1652–62. doi:10.1002/1529-0131(199809)41:9<1652::AID-ART17>3.0.CO;2-W
23. Xu Z, Morel L. Contribution of B-1a cells to systemic lupus erythematosus in the NZM2410 mouse model. *Ann N Y Acad Sci* (2015) **1362**:215–23. doi:10.1111/nyas.12607
24. Iizuka J, Katagiri Y, Tada N, Murakami M, Ikeda T, Sato M, et al. Introduction of an osteopontin gene confers the increase in B1 cell population and the production of anti-DNA autoantibodies. *Lab Invest* (1998) **78**:1523–33.
25. Reap EA, Sobel ES, Cohen PL, Eisenberg RA. Conventional B cells, not B-1 cells, are responsible for producing autoantibodies in lpr mice. *J Exp Med* (1993) **177**:69–78. doi:10.1084/jem.177.1.69
26. Atencio S, Amano H, Izui S, Kotzin BL. Separation of the New Zealand Black genetic contribution to lupus from New Zealand Black determined expansions

of marginal zone B and B1a cells. *J Immunol* (2004) **172**:4159–66. doi:10.4049/jimmunol.172.7.4159
27. Xu Z, Potula HH, Vallurupalli A, Perry D, Baker H, Croker BP, et al. Cyclin-dependent kinase inhibitor Cdkn2c regulates B cell homeostasis and function in the NZM2410-derived murine lupus susceptibility locus Sle2c1. *J Immunol* (2011) **186**:6673–82. doi:10.4049/jimmunol.1002544
28. Potula HH, Xu Z, Zeumer L, Sang A, Croker BP, Morel L. Cyclin-dependent kinase inhibitor Cdkn2c deficiency promotes B1a cell expansion and autoimmunity in a mouse model of lupus. *J Immunol* (2012) **189**:2931–40. doi:10.4049/jimmunol.1200556
29. Morel L, Croker BP, Blenman KR, Mohan C, Huang G, Gilkeson G, et al. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *Proc Natl Acad Sci U S A* (2000) **97**:6670–5. doi:10.1073/pnas.97.12.6670
30. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* (1997) **158**:1175–86.
31. Gregoire KE, Goldschneider I, Barton RW, Bollum FJ. Ontogeny of terminal deoxynucleotidyl transferase-positive cells in lymphohemopoietic tissues of rat and mouse. *J Immunol* (1979) **123**:1347–52.
32. Zemlin M, Ippolito GC, Zemlin C, Link J, Monestier M, Schroeder HW Jr. Adult lupus-prone MRL/MpJ2+ mice express a primary antibody repertoire that differs in CDR-H3 length distribution and hydrophobicity from that expressed in the C3H parental strain. *Mol Immunol* (2005) **42**:789–98. doi:10.1016/j.molimm.2004.07.049
33. Radic MZ, Mackle J, Erikson J, Mol C, Anderson WF, Weigert M. Residues that mediate DNA binding of autoimmune antibodies. *J Immunol* (1993) **150**:4966–77.
34. Kirkham PM, Schroeder HW Jr. Antibody structure and the evolution of immunoglobulin V gene segments. *Semin Immunol* (1994) **6**:347–60. doi:10.1006/smim.1994.1045
35. Gu H, Kitamura D, Rajewsky K. B cell development regulated by gene rearrangement: arrest of maturation by membrane-bound D mu protein and selection of DH element reading frames. *Cell* (1991) **65**:47–54. doi:10.1016/0092-8674(91)90406-O
36. Forster I, Rajewsky K. Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic, newborn mice. *Eur J Immunol* (1987) **17**:521–8. doi:10.1002/eji.1830170414
37. Hayakawa K, Carmack CE, Hyman R, Hardy RR. Natural autoantibodies to thymocytes: origin, VH genes, fine specificities, and the role of Thy-1 glycoprotein. *J Exp Med* (1990) **172**:869–78. doi:10.1084/jem.172.3.869
38. Yang Y, Wang C, Yang Q, Kantor AB, Chu H, Ghosn EE, et al. Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *Elife* (2015) **4**:e09083. doi:10.7554/eLife.09083
39. Forster I, Gu H, Rajewsky K. Germline antibody V regions as determinants of clonal persistence and malignant growth in the B cell compartment. *EMBO J* (1988) **7**:3693–703.
40. Tarlinton D, Stall AM, Herzenberg LA. Repetitive usage of immunoglobulin VH and D gene segments in CD5+ Ly-1 B clones of (NZB x NZW)F1 mice. *EMBO J* (1988) **7**:3705–10.
41. Tornberg UC, Holmberg D. B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. *EMBO J* (1995) **14**:1680–9.
42. Stall AM, Farinas MC, Tarlinton DM, Lalor PA, Herzenberg LA, Strober S, et al. Ly-1 B-cell clones similar to human chronic lymphocytic leukemias routinely develop in older normal mice and young autoimmune (New Zealand Black-related) animals. *Proc Natl Acad Sci U S A* (1988) **85**:7312–6. doi:10.1073/pnas.85.19.7312
43. Gu H, Forster I, Rajewsky K. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. *EMBO J* (1990) **9**:2133–40.
44. Wasserman R, Li YS, Shinton SA, Carmack CE, Manser T, Wiest DL, et al. A novel mechanism for B cell repertoire maturation based on response by B cell precursors to pre-B receptor assembly. *J Exp Med* (1998) **187**:259–64. doi:10.1084/jem.187.2.259
45. Hardy RR. Development of VH11+ B cells: a model for selection of B cells producing natural autoantibodies. *Curr Dir Autoimmun* (2003) **6**:196–211. doi:10.1159/000066862
46. Nguyen TT, Elsner RA, Baumgarth N. Natural IgM prevents autoimmunity by enforcing B cell central tolerance induction. *J Immunol* (2015) **194**:1489–502. doi:10.4049/jimmunol.1401880
47. Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. *Eur J Immunol* (2007) **37**:2405–10. doi:10.1002/eji.200737461
48. Zhong X, Lau S, Bai C, Degauque N, Holodick NE, Steven SJ, et al. A novel subpopulation of B-1 cells is enriched with autoreactivity in normal and lupus-prone mice. *Arthritis Rheum* (2009) **60**:3734–43. doi:10.1002/art.25015
49. O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* (1992) **22**:711–7. doi:10.1002/eji.1830220314
50. Kaku H, Cheng KF, Al-Abed Y, Rothstein TL. A novel mechanism of B cell-mediated immune suppression through CD73 expression and adenosine production. *J Immunol* (2014) **193**:5904–13. doi:10.4049/jimmunol.1400336
51. Wang H, Shin DM, Abbasi S, Jain S, Kovalchuk AL, Beaty N, et al. Expression of plasma cell alloantigen 1 defines layered development of B-1a B-cell subsets with distinct innate-like functions. *Proc Natl Acad Sci U S A* (2012) **109**:20077–82. doi:10.1073/pnas.1212428109
52. Enghard P, Humrich JY, Chu VT, Grussie E, Hiepe F, Burmester GR, et al. Class switching and consecutive loss of dsDNA-reactive B1a B cells from the peritoneal cavity during murine lupus development. *Eur J Immunol* (2010) **40**:1809–18. doi:10.1002/eji.200940050
53. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med* (1998) **188**:2381–6. doi:10.1084/jem.188.12.2381
54. Mehrad B, Park SJ, Akangire G, Standiford TJ, Wu T, Zhu J, et al. The lupus-susceptibility locus, Sle3, mediates enhanced resistance to bacterial infections. *J Immunol* (2006) **176**:3233–9. doi:10.4049/jimmunol.176.5.3233

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 06 June 2016
doi: 10.3389/fimmu.2016.00198

Role of Natural Autoantibodies and Natural IgM Anti-Leucocyte Autoantibodies in Health and Disease

*Peter Isaac Lobo**

Department of Internal Medicine, Division of Nephrology, Center of Immunology, Inflammation and Regenerative Medicine, University of Virginia Health Center, Charlottesville, VA, USA

We review how polyreactive natural IgM autoantibodies (IgM-NAA) protect the host from invading micro-organisms and host neo-antigens that are constantly being produced by oxidation mechanisms and cell apoptosis. Second, we discuss how IgM-NAA and IgM anti-leukocyte antibodies (IgM-ALA) inhibits autoimmune inflammation by anti-idiotypic mechanisms, enhancing removal of apoptotic cells, masking neo-antigens, and regulating the function of dendritic cells (DC) and effector cells. Third, we review how natural IgM prevents autoimmune disorders arising from pathogenic IgG autoantibodies, triggered by genetic mechanisms (e.g., SLE) or micro-organisms, as well as by autoreactive B and T cells that have escaped tolerance mechanisms. Studies in IgM knockout mice have clearly demonstrated that regulatory B and T cells require IgM to effectively regulate inflammation mediated by innate, adaptive, and autoimmune mechanisms. It is, therefore, not surprising why the host positively selects such autoreactive B1 cells that generate IgM-NAA, which are also evolutionarily conserved. Fourth, we show that IgM-ALA levels and their repertoire can vary in normal humans and disease states and this variation may partly explain the observed differences in the inflammatory response after infection, ischemic injury, or after a transplant. We also show how protective IgM-NAA can be rendered pathogenic under non-physiological conditions. We also review IgG-NAA that are more abundant than IgM-NAA in plasma. However, we need to understand if the $(Fab)^2$ region of IgG-NAA has physiological relevance in non-disease states, as in plasma, their functional activity is blocked by IgM-NAA having anti-idiotypic activity. Some IgG-NAA are produced by B2 cells that have escaped tolerance mechanisms and we show how such pathogenic IgG-NAA are regulated to prevent autoimmune disease. The Fc region of IgG-NAA can influence inflammation and B cell function *in vivo* by binding to activating and inhibitory FcγR. IgM-NAA has therapeutic potential. Polyclonal IgM infusions can be used to abrogate on-going inflammation. Additionally, inflammation arising after ischemic kidney injury, e.g., during high-risk elective cardiac surgery or after allograft transplantation, can be prevented by pre-emptively infusing polyclonal IgM or DC pretreated *ex vivo* with IgM or by increasing *in vivo* IgM with a vaccine approach. Cell therapy is appealing as less IgM will be required.

Keywords: natural IgG autoantibodies, natural IgM autoantibodies, natural autoantibodies, natural IgM anti-leukocyte antibodies, allograft rejection, TH-17, renal IRI, autoimmune insulitis, regulation of co-stimulatory receptors, regulatory dendritic cells

OPEN ACCESS

Edited by:
Nichol E. Holodick, The Feinstein Institute for Medical Research, USA

Reviewed by:
Ziaur S. M. Rahman, Pennsylvania State University, USA
Christoph J. Binder, Medical University of Vienna, Austria

***Correspondence:**
Peter Isaac Lobo
pil@virginia.edu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *16 December 2015*
Accepted: *06 May 2016*
Published: *06 June 2016*

Citation:
Lobo PI (2016) Role of Natural Autoantibodies and Natural IgM Anti-Leucocyte Autoantibodies in Health and Disease. Front. Immunol. 7:198. doi: 10.3389/fimmu.2016.00198

INTRODUCTION

The study of natural IgM autoantibodies (IgM-NAA) has given us a good insight on how nature, by creating polyreactive pentavalent antibodies, has accomplished a difficult task of trying to protect the host from both diverse foreign pathogens and from diverse self neo-antigens that are constantly produced within the host. Poly-reactivity with low binding affinity, but with high avidity, has enabled these IgM-NAA-producing B cell clones to be rapidly activated by a foreign or an auto neo-antigen for deploying protective mechanisms to the host. Such a response provides time for the adaptive immune system to mount a highly specific immune response to foreign antigens and, in addition, lessens the burden on the host to maintain diverse B cell clones producing highly specific IgG auto-antibodies, which has the potential of causing autoimmune disease owing to their high-affinity binding. Second, since both foreign and self neo-antigens can induce an inflammatory response, these IgM-NAA have taken over another task of subduing an excessive inflammatory response that can injure the host. This regulation of inflammatory cells without damage is made possible by the low binding affinity of IgM-ALA to live leukocytes, combined with its inability to effectively activate the lytic component of complement at body temperature (37°C). Hence, it is not surprising why these IgM-NAA antibodies, which first arose in cartilaginous fish, have been conserved during evolution [reviewed in Ref. (1)] and why IgM-NAA makes up about 70–80% of circulating IgM (2, 3). Additionally, some studies have shown that the binding of natural IgM to autologous receptors is also evolutionarily conserved among mammalian species as human IgM has the same functional effect as murine IgM on murine cells *in vitro* or when used *in vivo* in mice (4–6).

Natural autoantibodies of different isotypes have been intensively studied during the last 40 years (7–17). These autoantibodies have been termed "natural antibodies" as they are produced at birth in the absence of exposure to foreign antigens. The full repertoire of NAA develops by early childhood. In mice, NAA are predominantly produced by the CD5+ B1 cells, while marginal-zone splenic B (MZB) cells contribute the remainder. These B1 cells produce predominantly IgM, IgA, and IgG3 autoantibodies (18, 19), independently of T cell help, and exhibit an enhanced response to innate immune signals, such as TLR agonist. Hence, B1 and MZB cells differ from B2 cells in that the response of these cells *in vivo* is rapid and can be driven by TLR agonists independently of antigen binding to their BCR (20–23). Additionally, there are data to indicate that autoantibody-producing B1 cells, unlike self-reactive T cells, are positively selected for their self-reactivity, thus implying that NAA are conserved by design (24–27). Further support for their importance comes from studies in chimeric mice demonstrating that IgM-NAA comprise the majority of circulating IgM (2, 3). Most cross-sectional studies in humans and rodents would indicate that IgM-NAA decrease with age (28–31) or lose their effectiveness with age (32) except for one report where follow-up of five healthy individuals for 25 years revealed no change in IgM-NAA levels (33). However, IgG-NAA can increase (34) but do not decrease with age (35, 36).

Innately produced natural IgM-NAA should not be confused with immune IgM and IgG that are produced several days later after exposure to foreign antigens or pathogens. Such immune IgM and IgG are not natural autoantibodies and in general are antigen specific and are produced by B2 cells that require antigen binding to BcR and additional T cell help to generate anti-protein antibodies. However, production of immune IgM is limited as these IgM secreting B2 cells migrate to B cell follicles, where with the help of T cells, these B2 cells undergo isotype switching and somatic hyper-mutation, thus generating long-lived memory B cells and differentiating into plasma cells that produce IgG antibodies with high-affinity binding.

The human equivalent of the murine CD5+ B1 subset has been recently identified and characterized. This CD20+ CD43+ CD27+ human B1 subset that can spontaneously secrete antibody represents about 50% of umbilical cord B cells and 15–20% of circulating adult B cells, and is the predominant source of human IgM-NAA (29). In humans, CD5 is not a specific marker of B1 as this marker is expressed by both B1 and B2 cells. Similarly, CD43 and CD27 are not specific markers for human B1 as about 20% of CD43+ CD 27+ B cells have characteristics of pre-plasmablasts that are derived from T-dependent B cells present in germinal centers (37, 38). Human IgM-NAA are also polyreactive and bind similar autoantigens as in mice, including oxidized neo-determinants and leukocyte receptors (4, 39–41).

IgM-NAA, IgG-NAA, and Pathogenic IgG Autoantibodies

One physiological role of NAA is to protect the host from pathogenic IgG autoantibodies. We will, therefore, briefly describe the biology of natural IgM and IgG-NAA and pathogenic IgG autoantibodies in health and disease and then discuss the different mechanisms used by NAA to counter pathogenic IgG. B1 cells have been shown to secrete IgM, IgA, and IgG3-NAA that are encoded by minimally or non-mutated germ line genes that are enriched for heavy chain variable region rearrangements and with H-L pairings that allow for poly-reactivity (18, 19). NAA generated by B1 cells rarely undergo isotype switching or somatic hyper-mutation to acquire antigen specificity. IgA in the gut is predominantly produced by B1 cells present in the gut lymphoid tissue but there is increasing evidence to show that B2 cells can also participate in gut mucosal immunity and this could account for the observed somatic hyper-mutation and antigen specificity of gut mucosal IgA (42–46).

Natural IgM Autoantibodies

In mice, the majority of IgM-NAA are produced by splenic B1 and MZB cells (47–49). B1 and MZB cells, such as memory B cells, express CD27 and spontaneously produce IgM, but IgM production can also be increased by TLR activation [e.g., lipopolysaccharide (LPS)] or via the BCR in response to pathogens. Normal levels of IgM-NAA are present at birth even under germ-free conditions and in nude mice, indicating that IgM-NAA production is not dependent on exposure to foreign antigens, TLR or T cell activation (50). IgM-NAA repertoire is shaped by T-independent antigen activation, especially of MZB cells (51, 52). An important characteristic of these antibodies is their low binding affinity (53). It is possible that the membrane expressed IgM or BCR of B1 and

MZB cells producing NAA also exhibit low binding affinity and perhaps this latter characteristic may be involved in preventing autoreactive B1 cells from being deleted or undergoing negative selection. There are data to show that autoreactive B1 cells are positively selected and this process requires both the autoantigen and the relevant BCR (24–27). The need to positively select B1 cells secreting IgM-NAA would indicate that these antibodies have an important physiological role that will be reviewed later.

Natural IgM autoantibodies have been shown to be polyclonal with clones having specificity for some, but not all self-antigens, i.e., certain common epitopes present on phylogenetically conserved self-antigens. Some of these IgM clones with reactivity to self-antigens have been identified, e.g., IgM clones with specificity for leukocyte receptors [IgM anti-leukocyte antibodies (IgM-ALA)] (4, 39), Fc domain of IgG (rheumatoid factor) (14, 15), complement components (17), collagen, thyroglobulin, intracellular constituents, such as cytoskeletal proteins, cytosolic enzymes, dsDNA, or nucleosomes, neutrophil cytoplasmic enzymes (ANCA) (50, 54) and oxidized neo-determinants [e.g., phosphorylcholine (PC)] that are exposed when lipids are oxidized or cells undergo apoptosis (55, 56). While some IgM-ALA have mono-reactivity, e.g., to some cytokines, most are polyreactive with each polyreactive IgM-NAA clone having a selective binding profile (50). For example, IgM anti-PC NAA will bind to ABO blood type antigens, endotoxins, and oxidized neo-determinants on apoptotic cells but this autoantibody has no binding reactivity to nuclear antigens or to IgG (57). Additionally, these IgM-NAA, by virtue of being polyreactive, also cross-react with pathogen-expressed molecules, for example, PC on *Streptococcus pneumoniae* and other antigens expressed by various viruses and parasites (55–57) Hence, it has been suggested that these natural IgM antibodies are protective, serving as a first line of defense against infections and protecting the host from pathogen-mediated apoptotic cells and oxidized neo-determinants that can induce pathogenic IgG autoantibodies (55, 56). Additionally, polyreactive IgM-NAA have been shown to bind to idiotypic determinants on self-reactive IgG, thus providing another mechanism to protect the host from high-affinity binding IgG autoantibodies that are potentially pathogenic (31, 50).

In mice, B1 cells are rare in the bone-marrow, lymph nodes, and splenic B cell follicles (white pulp) while significant numbers of B1 cells are located in the splenic marginal zone as well as in the peritoneal and pleural cavities. However, under normal conditions, these peritoneal B1 cells do not contribute significantly to circulating IgM-NAA, but during sepsis, peritoneal B1 cells rapidly migrate to the splenic marginal zone (23, 58) where most of the circulating IgM-NAA are produced (59). B1 cells are distinct from B2 cells in many respects and they are derived from different progenitors (18, 19, 60). Importantly B1cells express CD27, a memory B cell marker, and such as memory B cells, on encountering antigen, spontaneously secrete IgM without requiring signaling via co-stimulatory molecules (60). B1 cells do not require to traffic or reside in the splenic B2/T cell follicles as they are T independent and secrete IgM without isotype switching or somatic mutation (25, 59, 61). In fact, isotype switching and somatic hyper-mutation of immunoglobulins in B1 cells is kept in check during an immune response to prevent the development of high affinity, anti-self IgG antibodies (61, 62). In this regard, SPA-1 in B-1 cells inhibits Rap-1 GTP, which enhances somatic hyper-mutation and hence SPA-1 deficient mice generate high-affinity IgG anti-dsDNA and anti-red blood cell autoantibodies and develop lupus nephritis as they age (62). Recent data would indicate that levels of peritoneal B1 and splenic B1 and MZB cells is regulated by serum levels of polyclonal (but not monoclonal) IgM (63–65) that interacts with FcμR expressed by these cells (66) as well as by IgG binding to FcγRIIB (67).

IgG-NAA

One can demonstrate the existence of IgG-NAA in normal adult murine or human serum under conditions where serum IgM is either removed or diluted out (31, 68–70), indicating therefore that in serum the functional activity of IgG-NAA is blocked by polyreactive IgM with anti-idiotypic activity (31, 50, 54). There is, however, a large amount of IgG-NAA as significant levels of IgG-NAA can be detected even after diluting serum at 1:500 (34). Both IgG-NAA and IgM-NAA bind to the same phylogenetically conserved self-antigens and 15–20% of normal mouse IgG has been found to be polyreactive (50, 68). IgG produced by B1 cells are characteristically of the IgG3 isotype that have been shown to be polyreactive (18, 19). Clones of B cells secreting IgG4κ with high-affinity binding to dsDNA has been shown to exist in human umbilical cord blood and in the same umbilical cord there were other B cell clones producing IgM antibodies with binding reactivity to idiotypic determinants on IgG4 anti-dsDNA but not to idiotypic determinants on other IgG4 antibodies (34). The above observations would suggest that B cells generating IgG-NAA of different isotypes and affinities are present at birth and the IgG-NAA are rendered functionally inactive by polyreactive IgM with anti-idiotypic activity.

Importantly, IgG-NAA-producing B cells, unlike IgM-NAA-producing B cells, are in an inactive state at birth and in mice these B cells start producing IgG-NAA after exposure to bowel bacteria or foreign antigens (71–73). In humans, it may take more than 2 years before significant levels of IgG-NAA can be detected in the serum (74). It is also possible that autoreactive T cells, which have been shown to exist in normal individuals (75) or infectious agents could activate B cells to produce IgG-NAA later on in life. For example, levels of polyreactive IgG anti-dsDNA have been shown to increase after various infections and these polyreactive IgG anti-dsDNA have been found to cross-react with antigens on micro-organisms, including bacteria (76). It is, therefore, not surprising that low levels of IgG-NAA at birth increase after exposure to infections (71–73).

Studies on IgG-NAA isotypes in normal sera or on the B cell subsets that produce these antibodies are lacking. There are, however, extensive data on an IgG-NAA, i.e., IgG anti-DNA, that is both mono-reactive and polyreactive and present in both normal sera and SLE sera and some of the findings with IgG anti-DNA may apply to other IgG-NAA. Several lines of evidence would favor that B2 cells, with the help of T cells, can generate IgG-NAA. Wardemann et al. clearly demonstrated that 55–75% of early B cell precursors, present in human adult bone-marrow, display auto-reactivity to conserved intracellular cytoplasmic and nuclear constituents. About 80% of these autoreactive B cells are removed at two check points, i.e., centrally in the bone-marrow and in the periphery (77). Second, in human umbilical cord, B cells secreting high-affinity

binding IgG4 anti-dsDNA have been isolated and IgG1 anti-myeloperoxidase (ANCA) antibodies have been found in normal human sera, indicating that these high-affinity isotype-switched antibodies are produced by the T cell-dependent B2 cells (34, 78). Furthermore, in the MLR/*lpr* murine model of SLE, IgG anti-dsDNA has been shown to be produced by B2 cells under the influence of excess T helper cells as a result of Fas deficiency (79). Hence, removing T cells ameliorates SLE and reduces production of IgG anti-DNA in this model of SLE (80–82).

Under normal conditions, it is unclear whether the $(Fab)^2$ region of IgG-NAA is physiologically active especially since their activity is blocked by poly reactive IgM with anti-idiotypic activity (31, 50). Hence, most of the physiological functions of IgG-NAA have been derived from purified IgG or pooled purified IgG (IVIG). *In vivo* infusion of IVIG has been shown to ameliorate cell-mediated inflammatory processes, e.g., in Kawasaki's disease or autoantibody-mediated disorders, e.g., in idiopathic thrombocytopenic purpura, anti-factor VIII autoimmune disease, or myasthenia gravis [reviewed in Ref. (83)]. One can show, in *in vitro* studies, that the beneficial effects of purified IgG-NAA can be mediated by both the $(Fab)^2$ region and the Fc region of IgG-NAA. In these *in vitro* studies, the $(Fab)^2$ region has been shown to (i) block function of activating FcγR receptors expressed by monocytes/macrophages. Such activating FcγR are involved in phagocytosis of IgG autoantibody complexed to platelets, (ii) block idiotypic determinants on IgG autoantibodies, thus neutralizing these antibodies, e.g., their binding to Factor VIII or platelets, (iii) inhibit function of certain pro-inflammatory cytokines by binding to these cytokines or their receptors, and (iv) provide protection against bacterial infection by binding of IgG-NAA to lectins on bacterial surfaces thus enhancing opsonization (84). Similarly, in *in vitro* studies, the Fc region of IgG-NAA has been shown to inhibit the function of B cells, plasma cells, dendritic cells (DC), macrophages, and neutrophils by binding to the inhibitory FcγRIIB receptor expressed by these cells. Activation of FcγRIIB by Fc region of IgG-NAA inhibits production of autoantibodies by B cells and plasma cells. Additionally IVIG ameliorates cell-mediated inflammation, e.g., in Kawasaki's disease, by activating FcγRIIB and inhibiting the function of dendritic cells, macrophages, and neutrophils.

IgG with anti-idiotypic activity have been found in pathological conditions especially when there is minimal or no IgM with anti-idiotypic activity (85) and increased levels of *in vivo* anti-idiotypic IgG has been associated with amelioration of disease activity mediated by pathogenic IgG autoantibodies, thus indicating that under pathological conditions, *in vivo* IgG with anti-idiotypic activity is functionally active (85, 86). There are data to show that normal levels of IgG autoantibodies, e.g., to myeloperoxidase (MPO) increase >10-fold during disease states (87) and it is possible that under pathological conditions when IgM-NAA with anti-idiotypic activity is low, there is initially a compensatory increase in IgG antibodies with anti-idiotypic activity and the disease does not manifest clinically (85). However, with time, the data would also indicate that there is a decrease in the protective IgM/IgG anti-idiotypic antibodies and the disease manifests clinically (85). Hence, administering IVIG, which is known to have anti-idiotypic activity, could be beneficial in this situation especially if levels of both IgG and IgM-NAA are relatively low.

Pathogenic IgG Autoantibodies

Pathogenic autoantibodies that cause disease differ from IgM and IgG3-NAA in that they are predominantly of the IgG isotype, undergo somatic mutation with addition of N regions and these IgG autoantibodies can be either polyreactive or exhibit mono-reactivity. IgG autoantibodies, unlike IgM-NAA, have high-binding affinity (53). However, not all IgG autoantibodies with high-binding affinity are pathogenic *in vivo* and this is best exemplified by IgG anti-dsDNA autoantibodies (88–91). There are conflicting data on the B cell precursors that generate these pathogenic IgG autoantibodies. Data would indicate that both B1 and B2 cells can generate pathogenic IgG autoantibodies depending on the animal model used. For example, studies in the Fas-deficient MRL-*lpr* murine SLE model would indicate that pathogenic self-reactive IgG antibodies are generated by B2 cells (79). However, in the NZB/W disease model of SLE and in a murine transgenic model of hemolytic anemia, the pathogenic murine IgG anti-dsDNA and anti-erythrocyte transgenic autoantibodies were shown to be produced by B1 lymphocytes (21, 92–94). Hence, removing T cells did not affect production of IgG anti-dsDNA or disease activity in the NZB/W model of SLE (80).

There are several mechanisms that increase pathogenic IgG antibody production and IgG anti-dsDNA has been most studied in this regard. In adolescent females with SLE and rheumatoid arthritis (RA) patients, there are data to show that production of IgG polyreactive autoantibodies results from breakdown of B cell tolerance mechanisms where during B cell development, B cells producing high-affinity IgG autoantibodies have not been removed or silenced either centrally in the bone-marrow or in the periphery before they mature into naïve immuno-competent lymphocytes (95, 96). Studies in healthy individuals indicate that 55–70% of newly generated bone-marrow B cells express self-reactive antibodies and most of these B cells are removed or silenced such that in healthy humans there remains 5–20% of circulating naïve B cells that continue to generate self-reactive IgG autoantibodies (77). However, in SLE and RA patients, owing to breakdown of B cell tolerance mechanisms, 25–50% of mature naïve B cells were found to generate self-reactive IgG autoantibodies with diverse specificities (95, 96). Second, excess BAFF could also contribute to the breakdown of tolerance in the periphery as BAFF has been shown to inhibit deletion or apoptosis of self-reactive B cells (92, 97). Elevated BAFF levels have been shown to be present in serum of RA and Sjogren's syndrome (98). Third, there are murine studies to show that IgM-NAA-producing B1 cells can be induced, especially with repeated immunization, to generate polyreactive IgG anti-self that can also react to the immunizing antigen. For example, B1 cells can be induced to generate pathogenic polyreactive IgG anti-DNA and anti-myosin antibodies by immunization with a hapten or an antigen derived from a micro-organism (99–101). Other mechanisms may, however, operate in certain other autoimmune disorders where highly specific IgG autoantibodies are produced, e.g., in autoimmune hemolytic anemia and in Factor VIII deficiency. It is possible that such autoimmune disorders could arise from a breakdown

in peripheral mechanisms such as specific deficiency of anti-idiotypic IgM-NAA that blocks pathogenic autoantibody or specific deficiency of IgM-NAA that binds and masks autologous neo-determinants such as dsDNA.

Based on observations in the preceding paragraph, it appears that different mechanisms could operate in generating pathogenic IgG autoantibodies depending on the murine disease model. Additionally, the mechanisms may differ in humans even though the disease has similar disease manifestations. A summary of the existing evidence would indicate that IgG autoantibodies can be induced by the following mechanisms:

(i) An excessive increase in production of pathogenic IgG autoantibodies relative to IgM-NAA-producing B1 cells. Potential mechanisms that increase self-reactive IgG antibodies include lack of helper T cell apoptosis [e.g., in Fas-deficient MRL-*lpr* mice (79)], excess neoantigen-driven B cells [e.g., in human C1q deficiency with decreased removal of apoptotic cells (102)], excess BAFF production [e.g., in patients with Sjogren's syndrome (98)], a breakdown in B cell tolerance mechanisms resulting in excess naïve self-reactive B cells [e.g., in SLE affecting young female patients (95)] and a deficiency in the inhibitory FcγRIIB receptor that normally inhibits B cells and plasma cells from producing IgG antibodies (103, 104).

(ii) Genetic predisposition that permits some antigen-driven B1 cell clones to acquire the ability for isotype switching and somatic mutation as, for example, in the NZB/W murine lupus model and in SPA-1 deficient mice (105, 106, 62). Hence, diseases induced by such defective B1 cells are ameliorated with administration of either polyclonal IgM or the specific IgM-NAA (107) or depleting B1 cells (94).

(iii) Repeated immunization that induces isotype switching in B1 cells that normally generate IgM autoantibodies with germline genes. For example, repeated immunization with hapten or a streptococcal antigen can induce B1 cells to generate IgG antibodies of different isotypes that are polyreactive and bind to the immunizing antigen as well as to self-antigens, such as DNA or myosin (99–101).

(iv) Excess production of a highly specific pathogenic IgG autoantibody by autoreactive B2 cell clones as, for example, in myasthenia gravis (108). Expansion of such B2 cell clones could have been driven by antigen, e.g., with cross-reactive micro-organisms in patients without thymoma (109) or be driven by acetylcholine receptor specific autoreactive helper $CD4^+$ T cells in thymoma-associated myasthenia gravis where such autoreactive T cells have escaped tolerance mechanisms (110, 111).

(v) Deficiency of IgM-NAA, especially IgM-NAA with anti-idiotypic activity to a specific IgG autoantibody, thus incompletely neutralizing the increase in pathogenic IgG autoantibodies, such as antibodies to neutrophil cytoplasmic enzymes (myeloperoxidase, proteinase 3), which are commonly referred to as ANCA, dsDNA, and glomerular basement membrane (GBM) that are present in normal individuals (31, 50, 87, 112). Additionally, certain autoimmune disorders, such as ANCA vasculitis and anti-GBM nephritis, frequently occur in elderly patients who, with aging, can develop lower levels of IgM-NAA (28–32) but not IgG-NAA (33, 35, 36), which can increase with aging (34).

Figure 1 summarizes the role of natural and immune antibodies in normal and pathological states.

Role of IgM-NAA in Protecting Against Pathogenic IgG Autoantibodies

SLE is the most studied autoimmune disorder where high levels of IgM and IgG autoantibodies, directed against nuclear antigens, especially dsDNA, are produced in the majority of patients. Before we discuss the role of IgM-NAA in protecting against pathogenic IgG anti-dsDNA, it would be important to indicate that a subset of lupus nephritis (approximately 25%) can occur in the absence of detectable circulating IgG anti-DNA and this is commonly referred to as C1q nephropathy (113, 114). The latter observations have led some investigators to question whether IgG anti-dsDNA is indeed pathogenic (115). In some patients with C1q nephropathy, circulating IgG anti-dsDNA can be detected several years later but this subset of patients with C1q nephropathy have in general a better prognosis even though histologically there is similar glomerular immunoglobulin staining and immune deposits as in classical lupus nephritis (114). It is tempting to speculate that patients with C1q nephropathy may have high IgM-NAA and IgM anti-dsDNA that may be preventing detection of circulating IgG anti-dsDNA. A murine model with lupus nephritis but with no IgG anti-dsDNA in both the circulation and in kidney eluates has also been described (116). It is possible that in this murine model, the immune deposits detected in the kidney may be caused by

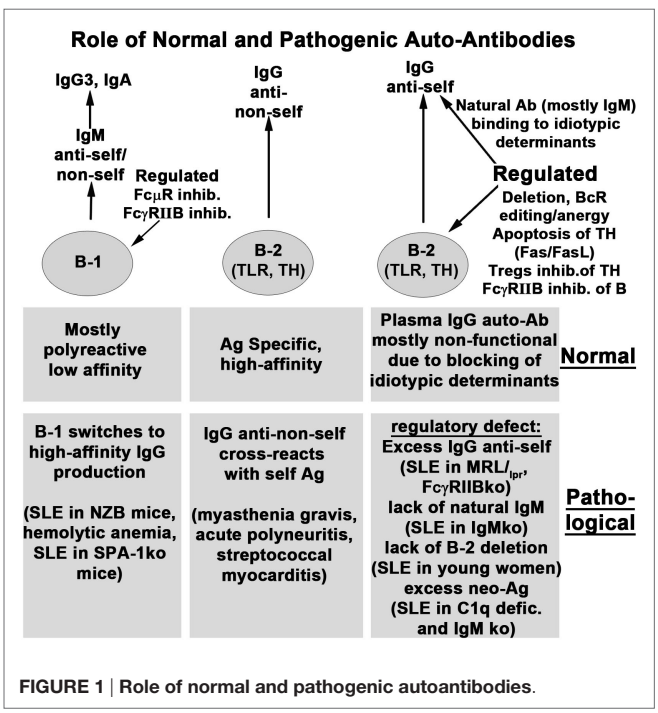


FIGURE 1 | **Role of normal and pathogenic autoantibodies.**

IgG complexes containing another antigen, other than dsDNA, and a similar mechanism could also operate in some patients with C1q nephropathy without circulating IgG anti-dsDNA.

However, there is evidence indicating that IgG anti-dsDNA, together with C1q, is an active participant in inducing or initiating glomerular damage in classical lupus nephritis with circulating IgG anti-dsDNA. First, SLE in the MRL/*lpr* lupus-prone mice is ameliorated when rendered IgG deficient by crossing these mice with either activation-induced deaminase (AID) or CD40L-deficient mice (117, 118). Second, one can induce lupus nephritis in normal mice by either infusing purified IgG anti-dsDNA or by increasing *in vivo* IgG anti-dsDNA with use of transgenic cells or hybridoma cells (88–91). Importantly, not all IgG anti-dsDNA are pathogenic as defined by induction of lupus nephritis *in vivo* and this also may explain why certain patients with high levels of circulating IgG anti-DNA are spared from lupus nephritis (88–91). In SLE, a potential protective mechanism involves binding of IgM anti-dsDNA to DNA with masking of the antigenic determinants that bind to IgG anti-dsDNA. Hence, lupus nephritis can occur if there is excess IgG anti-dsDNA or if there is a relative deficiency of IgM anti-dsDNA. Such a concept will explain the accelerated development of lupus nephritis in MRL/*lpr* mice lacking IgM and will also explain the lack of lupus nephritis in patients having high IgG anti-dsDNA and high IgM anti-dsDNA or IgM-NAA (119, 120). This latter possibility may also explain why FcγRIIB-deficient Balb/c mice are more resistant to developing lupus nephritis when compared to FcγRIIB-deficient C57BL/6 mice (121). In both mice, there is excess IgG anti-dsDNA production due to lack of the inhibitory FcγRIIB receptor but Balb/c mice have significantly higher levels of plasma IgM when compared to C57BL/6 mice (122–124).

Several observations would suggest a role of IgM-NAA in protecting normal individuals and patients with autoimmune disorders from such self-reactive IgG antibodies. The protective role of IgM-NAA is best exemplified in murine models of SLE, where enhancing IgM anti-dsDNA levels or infusing IgM anti-dsDNA, ameliorates disease activity (107, 125, 126). Well studied mechanisms by which IgM-NAA can induce protection and prevent inflammation include (i) removing neo-antigens by binding of IgM-NAA to oxidized neo-determinants and removal of IgM/C1q bound antigens by phagocytosis, e.g., IgM anti-PC (127), (ii) inhibition of the pathogenic IgG autoantibody induced inflammatory response by IgM-NAA, e.g., IgM-ALA (128), (iii) neutralization of pathogenic IgG autoantibodies by IgM-NAA having anti-idiotypic activity (129), (iv) preventing binding of IgG to antigenic determinants by competitive inhibition even though these IgM-NAA have lower binding affinity (129), (v) inhibiting complement activity by binding of IgM to complement components (17), and (vi) inhibiting generation of IgG autoantibodies possibly by binding of IgM-NAA to endogenous autoantigens/neo-antigens or to the B cell FcμR (64, 65, 119, 130–132).

The above mechanisms may explain why mice without secretory IgM have increased IgG autoantibodies, including anti-dsDNA, with aging (119, 120). This would also explain why B1-deficient SJL mice are more susceptible to induction of experimental autoimmune thyroiditis or allergic encephalitis cells (133). Conversely, such a concept would also explain why disease activity in SLE patients is less severe with high levels of IgM anti-dsDNA and other IgM-NAA (125, 134, 135). The above mechanisms may also explain why several autoimmune disorders are helped by either increasing production of *in vivo* IgM-NAA (126) or administering either pooled polyclonal IgM or a specific IgM-NAA (110, 129, 136). The latter is best exemplified by IgM anti-dsDNA where infusion of a specific IgM-NAA ameliorated SLE-induced nephritis in NZB mice (107).

Presence of pathogenic IgG autoantibodies leads to a compensatory increase in IgM–NAA, such as IgM anti-PC, IgM-ALA, and IgM/IgG anti-idiotypic antibodies, to counter these pathogenic IgG antibodies as well as inflammation initiated by the excess IgG self-reactive antibody. It is also possible, that over time, the production of IgM and IgG anti-idiotypic antibodies may not be able to keep up with the relatively high production of pathogenic IgG autoantibody and the disease becomes symptomatic. Hence, the initial increase in IgM-NAA and IgG anti-idiotypic antibody levels could explain the asymptomatic nature of the disease despite increased levels of IgG anti-dsDNA and other anti-nuclear antibodies several years before clinical onset of disease (85, 137). Additionally, there could be a heightened compensatory response of other immuno-regulatory mechanisms, e.g., increase in T-regs or IL-10 in B1 cells, which could inhibit the clinical onset of disease or ameliorate disease activity in these patients and other animal models. The latter is best exemplified in mice with secretory IgM deficiency, where despite total lack of circulating IgM and increased IgG3 anti-dsDNA (120), these cellular compensatory mechanisms inhibit development of autoimmune disease including lupus nephritis (63, 119). It is also possible that IgG3 anti-DNA is not pathogenic and their increased production by B1 cells, in secretory IgM deficiency (120), may be protective. However, in situations where inflammation has been experimentally induced or there is an excessive increase in production of pathogenic IgG autoantibodies or increased apoptosis, then these cellular regulatory mechanisms cannot effectively compensate for lack of IgM and this leads to a more severe and rapid worsening in disease activity. For example, SLE disease is more severe in the MRL-*lpr* mice when these mice are crossed with secretory IgM deficient mice (120). Similarly, secretory IgM-deficient mice are prone to develop a more rapid and severe inflammatory response after renal ischemia or allogeneic cardiac transplantation (138).

Physiological Role of IgM-NAA

IgM–NAA, which are spontaneously secreted by B1 and MZB cells, are polyclonal and exhibit a diverse repertoire of antigen specificities, many of which are polyreactive and, hence, cross-react with foreign antigens or pathogens. Additionally, B1 and MZB cell clones can be activated either by their TLR or BCR by both endogenous and foreign antigens. These special characteristics of IgM-NAA, together with the low-binding affinity (but with high-binding avidity owing to its pentameric structure), have allowed these antibodies to acquire functions that are not characteristic of the highly specific immune IgM and IgG antibodies. Physiological functions that have been attributed to IgM-NAA have included the following:

(i) *Countering the pathogenic effects of the normally present IgG autoantibodies that escape B cell tolerance mechanisms.* Potential strategies employed by IgM-NAA for this purpose include (a) neutralization of these IgG autoantibodies by anti-idiotypic mechanisms (129), (b) masking endogenous antigens (129), and (c) inhibiting the expansion of B2 cell clones that produce these IgG autoantibodies (79, 126). We have discussed these mechanisms in an earlier section of this review. However, the most compelling data demonstrating that IgM-NAA can counter pathogenic IgG autoantibodies are studies in murine models of SLE where administration of IgM anti-DNA or increasing expression of IgM anti-DNA in MRL-*lpr* mice (by crossing MRL-*lpr* mice with transgenic mice expressing IgM anti-DNA) ameliorated disease activity (107, 126).

(ii) *Providing the first line of defense against pathogens while the adaptive immune system*, i.e., *B2 and T cells, are being deployed to mediate a more specific and effective immune response that is long lasting and has memory*. The role of NAA in mediating protection against pathogens was first brought to light by the observation that NAA recognize bacterial toxins and several viral antigens (139, 140). Second, the creation of mice deficient in secretory IgM clearly aided in analyzing the protective role of both natural and immune IgM (63, 141). This topic will be briefly discussed as there are several excellent and detailed reviews on this subject (55, 141). Briefly, IgM-NAA mediates this protection through several mechanisms. First, each natural IgM clone, by having a pentavalent structure and by being polyreactive, can simultaneously bind to different conserved structures, such as nucleic acids, phospholipids, and carbohydrates, on the same pathogen and inhibit the pathogen from invading cells and disseminating into different organs. Second, the early components of complement, such as C1q, bind to the IgM complexed to the invading organism. Complement binding to IgM not only enhances neutralization of pathogens, e.g., certain viruses and bacteria, but also enhances phagocytosis of pathogens by macrophages and dendritic cells. The importance of either natural or immune IgM in limiting infection varies with different pathogens and this subject has been well reviewed (55, 141). Third, normally present IgM-NAA has significant levels of IgM anti–PC, which can readily bind to PC that is attached to sugar residues on the cell membranes and cell walls of many invading organisms (142). PC was first detected in 1967 on *S. pneumoniae* (143) and subsequently found to be present on many bacteria, protozoa, fungi, and nematodes (142). Infection with many of these organisms increases levels of IgM anti-PC, which in the presence of C1q enhances phagocytosis of these organisms and inhibits an excess inflammatory response by mechanisms discussed below. Finally, we have shown that IgM-NAA can limit HIV infection, both *in vitro* and in humanized SCID mice through another mechanism, that is by inhibiting T cell activation (39) as well as by binding of natural IgM to CD4 and chemokine receptors and inhibiting HIV entry into cells (**Figure 2**) (39, 144).

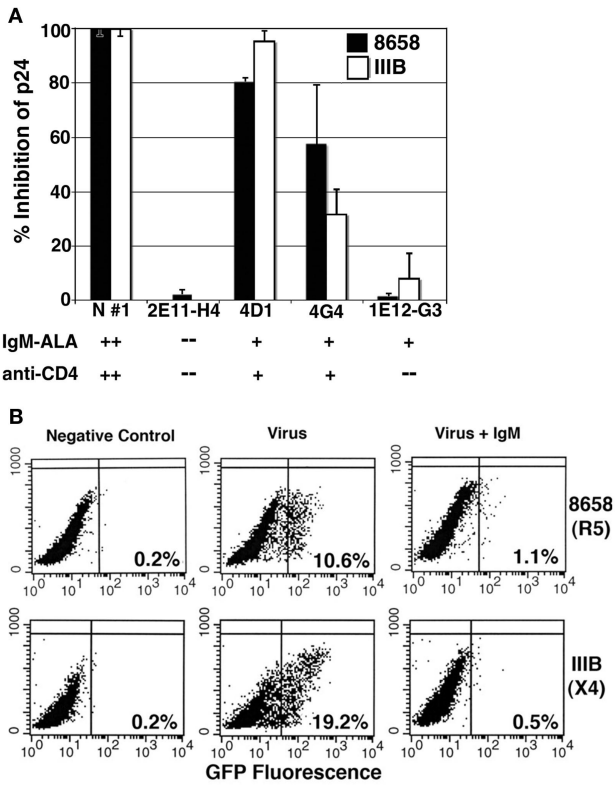


FIGURE 2 | Evaluating the inhibitory effect of human monoclonal IgM and polyclonal IgM on R5-8658 and X4-IIIB HIV viruses. **(A)** Specificity of umbilical cord B cell clone IgM was characterized by binding of IgM, to recombinant CD4 and to leukocytes, which is depicted below each clone. IgM from B cell clones was used at concentration of 3 μg/ml, while purified IgM from normal serum (positive control) was used at 10 μg/ml. B cell clone 2E11-H4 with no IgM-ALA and no anti-CD4 activity was used as a negative control. Without IgM, p-24 levels for R5-8658 and X4-IIIB were 87,500 and 126,000 pg/ml, respectively. These data are representative of three different experiments. **(B)** The inhibitory effect of normal polyclonal IgM (5 μg/ml) on HIV-1 infection (single cycle replication) of GHOST cells that express GFP upon integration on viral HIV-1 DNA. IgM was present throughout the culture period and added to cells 15 min before adding virus. These are representative examples of five separate experiments. Figure and legend reproduced with permission from Ref. (144). Copyright 2008. The American Association of Immunologists, Inc.

(iii) *Inhibiting IgG autoantibody production and inflammatory responses by clearing apoptotic cells and binding to oxidized neo-determinants.* Apoptotic cells that are normally produced in large numbers, express oxidized neo-determinants. Several homeostatic mechanisms, including binding of C1q and mannose binding lectin (MBL) to apoptotic cells, are involved in inducing macrophages and DC to phagocytose apoptotic cells and nuclear antigens [reviewed in Ref. (145)]. IgM-NAA are also involved in enhancing this phagocytic process, especially after tissue injury of nerves (69), and in inducing an anti-inflammatory effect (56). Several IgM-NAA antibodies have been identified, each with binding specificity to certain oxidized neo-determinants, including PC,

malondialdehyde (MDA), cardiolipin, phosphatidylserine, and Annexin IV present on apoptotic cells and not live cells (146). The best-studied IgM-NAA reactive to apoptotic cells is the IgM anti-PC antibody (also referred to as T-15), which binds to a dominant oxidized neo-determinant on apoptotic cells. Both *in vitro* and *in vivo* studies, using monoclonal IgM anti-PC, have clearly shown that phagocytosis by DC and macrophage is enhanced when C1q or MBL binds to the IgM/apoptotic cell complex. In these *in vivo* studies, either endogenous production of IgM anti-PC was increased by administering large numbers of apoptotic thymocytes (2.5×10^7) or mice were given 1.5–2 mg of a monoclonal IgM anti-PC preparation that bound to endogenous apoptotic cells. Hence, natural IgM anti-PC inhibits production of pathogenic IgG autoantibodies by masking oxidized neo-determinants and enhancing removal of apoptotic cells. However, enhancing apoptotic cell phagocytosis by DC also inhibits DC maturation/activation and induces an anti-inflammatory effect with decreased production of pro-inflammatory cytokines and an increase in levels of splenic regulatory B cells (127, 147). Additionally, repeated weekly infusions of monoclonal IgM anti-PC or apoptotic thymocytes inhibited development of arthritis and induced regulatory B cells in mice (127, 147).

Natural IgM autoantibodies has also been shown to bind to various oxidation-specific neo-determinants, specifically PC and MDA present on oxidized LDL lipids, but not native LDL (146, 148, 149). High levels of IgM anti-PC and IgM anti-MDA were also found in ApoE-deficient mice that have high circulating cholesterol and severe atherosclerosis. It has been suggested that oxidized lipids, in this murine model, induces these high IgM anti-PC/MDA antibodies as a protective mechanism. Supporting such a concept are other observations showing an association of high *in vivo* levels of IgM anti-PC and reduced atherosclerotic plaques in both patients and different animal models [reviewed in Ref. (56)]. Similarly, there are studies showing that immunization of LDL receptor knockout mice with the *S. pneumoniae* vaccine induces IgM anti-PC and this is associated with less atherosclerotic lesions (150). Several mechanisms have been postulated to explain the amelioration of atherosclerotic lesions by IgM anti-PC in these murine models of atherosclerosis. First, IgM anti-PC masks the PC containing neo-determinants on oxidized lipids, thus inhibiting uptake of these lipids by macrophages and this leads to a decrease in foamy macrophages that are part of the atherosclerotic plaque (151). Second, IgM anti-PC, together with C1q, enhances phagocytosis of apoptotic foamy macrophages containing high levels of lipids. Enhanced phagocytosis of apoptotic macrophages, especially by DC, inhibits DC maturation/activation and induces an anti-inflammatory milieu (127, 147). Data demonstrating increased atherosclerosis in secretory IgM-deficient mice or in C1q ko mice would lend support to the role of IgM anti-PC and C1q in inhibiting atherosclerosis (102, 141, 152–154).

The finding that both IgM-deficient mice and C1q knockout mice have increased apoptotic cells as well as develop SLE would indicate that both IgM anti-PC and C1q are required to effectively remove or clear apoptotic cells (155, 156). The latter studies would also indicate that inefficient clearance of apoptotic cells, including nuclear antigens, induces development of pathogenic IgG autoantibodies and SLE. However, ineffectual clearance of apoptotic cells cannot entirely explain the increased propensity of IgM-deficient mice to develop autoimmunity and more severe atherosclerosis when compared to C1q ko mice as both mice have similar number of apoptotic cells in the atherosclerotic plaques and the C1q ko mice has in addition, circulating apoptotic cells that should have worsened atherosclerosis (102, 141, 155). Such observations would indicate that IgM-NAA regulates inflammation by other additional mechanisms (to be presented below) besides enhancing phagocytosis of apoptotic cells.

(iv) *Inhibiting inflammation by binding of IgM-NAA to receptors on live leukocytes, i.e., via IgM-ALA*. The existence of this IgM-NAA subset was known since 1970 (157). Their role in inflammation was recognized when several investigators showed that the level of these antibodies, like IgM anti-PC, increased with diverse infections and inflammatory states [reviewed in Ref. (39)]. However, it was unclear whether IgM-ALA were pathogenic or not. The idea that IgM-ALA may have anti-inflammatory function and may be protective came from observations in allograft recipients where patients with high levels of IgM-ALA were found to have significantly less rejections and developed less alloantibodies after alloimmunization (**Figure 3**) (39, 131). We hypothesized that these natural IgM-ALA increase during inflammatory states to regulate leukocyte function and prevent excess inflammation that may be detrimental to the host. Two characteristics inherent in IgM-ALA allowed us to make this hypothesis. First, we showed that these antibodies bind with low affinity to different leukocyte receptors, including co-stimulatory molecules and chemokine receptors (4, 39). Second, we and other investigators observed that these complement fixing antibodies did not lyse leukocytes at body temperature but readily lysed cells at room temperature or colder temperatures in the presence of complement (131, 157, 159). This non-lytic nature of these antibodies at body temperature led us to hypothesize that IgM-ALA, by binding to receptors on live leukocytes at body temperature, could alter or regulate their function. Initially, with *in vitro* studies using human IgM, we showed that the repertoire of IgM-ALA varies among individuals during health and in disease and, second, that IgM-ALA regulates the function of both human and murine T cells and dendritic cells (4, 39, 138). Additionally, in murine models, we showed that increasing *in vivo* levels of IgM-ALA with physiological doses of purified polyclonal IgM, i.e., 150 μg/mouse, every 3 days, protected mice from (a) renal ischemia reperfusion injury (IRI), (b) ameliorated rejection in a fully mis-matched cardiac allograft model, and (c) protected NOD mice from developing autoimmune insulitis and diabetes. In further experiments, we show that the protective effect in the model of innate inflammation (i.e., renal IRI) is mediated in part by IgM-ALA binding

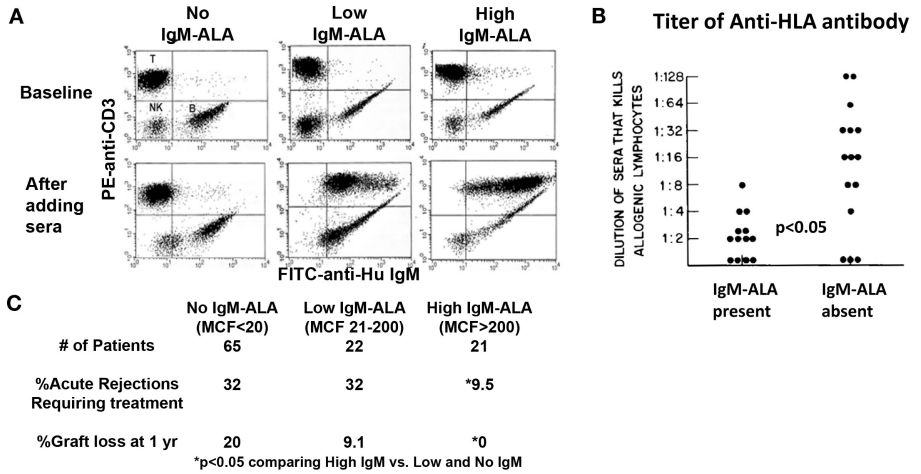


FIGURE 3 | High levels of serum IgM-ALA in transplant recipients are associated with better kidney allograft survival and decreased alloantibody levels. **(A)** Dot plots labeled "baseline" depict IgM staining on B lymphocytes, but not on T cells, in the absence of sera. The *lower panels* (after adding sera) depict differences in the level of IgM bound to donor T lymphocytes (IgM-ALA) after addition of pre-transplant serum from different ESRD patients. **(B)** Alloantibody titer in sera measured by cytotoxicity is correlated to presence of IgM autoantibody to leukocytes. **(C)** The difference in percentage of acute rejections and graft loss comparing high IgM-ALA vs. the no and low IgM-ALA groups. *MCF* indicates increase in mean channel fluorescence of anti-IgM staining after addition of serum. Figure and legend reproduced with permission from Ref. (131) copyright 1981 Wolters Kluwer Health Inc. **(B)** Figure and legend reproduced with permission from Ref. (158). Copyright 2010 Springer Science + Business Media LLC **(A,C)**.

to activated/mature DC and regulating their function (4). In a later section, we will review in more detail the above data on IgM-ALA. We plan to show that the observed anti-inflammatory effects of polyclonal IgM was predominantly from IgM-ALA and not from IgM anti-PC that bound to apoptotic cells.

(v) *Inhibiting expansion of B1 cells and enhancing antigen presentation to B2 and helper T cells in splenic lymphoid follicles.* This physiological function of IgM became obvious in mice with secretory IgM deficiency as well as in mice with FcμR deficiency. B1 cells in the splenic marginal zone increased several fold in mice deficient in IgM while levels of IgM increased in FcμR-deficient mice indicating that IgM maintains B1 cell homeostasis and levels of natural IgM by binding to FcμR (63, 64, 66, 160). Second, both these mice have impaired immune IgG response to protein antigens, especially with low dose antigen, indicating that IgM facilitates antigen trafficking from the splenic marginal zone to the B2 cell rich lymphoid follicles via FcμR bound IgM/antigen complexes (63, 160).

The above observations indicate that IgM-NAA protect the host from invading organisms and more importantly maintain several homeostatic mechanisms primarily aimed at preventing autoimmunity and over exuberant inflammation, which can have detrimental effects on the host. **Table 1** summarizes some of the physiological and pathological concepts outlined above. Several observations indicate that infective and other inflammatory states increase all IgM-NAA subsets, especially IgM anti-PC to clear the increased production of apoptotic cells that could trigger autoimmunity and, second, increase IgM-ALA to subdue excess inflammation that can be detrimental to the host [reviewed in Ref. (56) and next section]. Based on the preceding observations, one could predict that a decrease in IgM-NAA, as can occur in aging (28–30), could predispose to increased autoimmunity and increased morbidity and mortality from an excess inflammatory response, for example with infections.

TABLE 1 | Physiologic function of non-pathogenic autoantibodies.

	IgM natural antibodies	IgG natural antibodies
Protection from micro-organisms	Binds to bacteria and enhances phagocytosis (req. C1q, Fcα/μR) Inhibits HIV by blocking entry and inactivating cells	Binds to bacteria and enhances phagocytosis (req. C1q, FcγRI)
Prevent autoimmunity	Blocks anti-self IgG Ab (anti-idiotypic) IgM masks neo-antigen Binds to apoptotic cells (PC), nuclear and cytoplasmic debris and enhances phagocytosis (req. C1q, MBP)	Blocks anti-self IgG Ab (anti-idiotypic) Binds to nuclear and cytoplasmic debris and enhances phagocytosis (req. C1q, MBP)
Abrogate inflammation	Anti-complement IgM-ALA ↓ iTNF-α, IL-17, IFN-γ IgM-ALA regulates DC and T cells, and enhances Tregs by binding to CD40, CD86, CD4, CD3, and TcR IgM-ALA blocks chemokine receptors	Regulates B cells, plasma cells, DC, macrophages, and neutrophils via FcγRIIB
B cell homeostasis	IgM regulates B1 cell expansions via FcμR	IgG regulates B1 and B2 cell expansion via FcγRIIB

Pathogenic Effects of IgM-NAA Under Non-Physiological Conditions

Certain IgM-NAA which under physiological conditions are protective and anti-inflammatory can under non-physiological

conditions become pathogenic and induce inflammation. In this section, we will present some of the conditions that can induce certain protective IgM-NAA to become pathogenic.

(i) *Induction of pathogenesis by binding of IgM-NAA at cold temperatures*: This is best exemplified in human renal allograft transplant recipients having high IgM-ALA and IgM-anti endothelial cell antibody (IgM-AEA) levels at the time of the kidney transplant. These recipients have a high incidence of delayed kidney graft function (DGF) (161, 162) resulting from high levels of IgM-AEA which cause glomerular endothelial cell injury when, after successful vascular anastomosis, warm blood is allowed to flow into a cold kidney. Fortunately, this self-limiting injury is prevented by warming the kidney prior to re-instituting blood flow. Such observations highlight the nature of IgM-NAA, i.e., their potential for complement mediated cytotoxicity under non-physiological cold conditions (157, 159).

(ii) *Induction of pathogenesis by binding of natural IgM to unmasked neo-antigens.* This is best exemplified by the ubiquitous neo-antigen "non-muscle myosin heavy chain type IIA and C (NMM)" which is unmasked in murine models of acute ischemia to the small bowel, skeletal muscle (hind limb) and heart. About 1–2% of IgM-NAA B1 cell clones in mice secrete IgM anti-NMM probably to protect against NMM derived from infectious organisms (163, 164). In murine models of acute ischemia to the bowel or hind limb, injury is predominantly mediated during reperfusion by innate inflammation triggered by IgM binding to the unmasked NMM and activation of complement (6, 163, 165, 166). RAG-1 ko mice are normally protected from this ischemic injury to the small bowel or hind limb (163, 166). However, RAG-1 ko mice succumb to this ischemic injury after infusion of polyclonal IgM or monoclonal IgM anti-NMM. Additionally, one can clearly demonstrate binding of IgM and complement to NMM in bowel epithelial cells or to hind limb striated muscle cells (163, 165).

Interestingly, one does not observe innate inflammation mediated by IgM anti-NMM after murine renal ischemia even though endothelial cells in glomeruli and peri-tubular capillaries express NMM (167, 168). One possibility is that NMM in the peri-tubular capillaries or in the tubules is not unmasked after ischemia. After renal IRI, most of the ischemia induced kidney injury occurs in the outer medullary renal tubules. However, after renal IRI, one can detect increased IgM binding to glomeruli but not to the extensive network of NMM containing capillaries that surround the tubules or the renal tubules (168). Additionally, depleting B1 cells, decreased binding of IgM to glomeruli, and decreased glomerular injury, but did not protect tubules from inflammation-mediated renal injury, thus indicating that the innate inflammation seen after renal IRI is not mediated by natural IgM and complement (168). Other studies would indicate that the inflammatory response after renal IRI is mediated by ischemia-induced renal tubular injury that activates innate immune cellular mechanisms involving NK and NKT cells (169). This latter study would also explain why Rag-1 ko mice and IgM ko mice without secretory IgM are not protected from renal IRI (4, 138, 170–172).

(iii) *Induction of pathogenesis by non-physiologic expansion of specific IgM-NAA clones*. This is best exemplified by expansion of certain B1 cell clones that specifically secrete rheumatoid factor (RhF), an IgM-NAA that binds to self IgG. Excess production of RhF predisposes to generation of large circulating IgM/IgG complexes (referred to as cryoglobulins as these complexes precipitate *ex vivo* in the cold) that cause thrombosis of small blood vessels especially in the kidney glomeruli and skin (173, 174). This serious clinical problem is treated by plasmapheresis (to remove cryoglobulins) and agents to deplete B cells. The most common etiology for monoclonal or polyclonal expansion of RhF secreting B1 cell clones is chronic hepatitis C infection. Currently, it is unclear why this viral infection is associated with clonal expansion of RhF secreting B1 clones. More importantly, even though RhF was the first IgM-NAA to be discovered, we do not understand their normal physiological role.

Nature of IgM-ALA and Understanding Their Anti-Inflammatory Function

In this section, we review some of our pertinent observations on IgM-ALA that initially were discovered because of their lymphocytotoxic activity at room temperature in the presence of complement [reviewed in Ref. (39)]. Here, we show (a) that a substantial subset (8–10%) of IgM secreting human B cell clones, obtained from the umbilical cord, has IgM-ALA activity, (b) that polyclonal IgM-ALA clones have binding specificity to certain leukocyte subsets and that polyclonal IgM-ALA will bind to only some leukocyte receptors and not others. More importantly, we show that the repertoires of IgM-ALA vary in disease states among different individuals, and (c) that IgM-ALA exhibits anti-inflammatory effects, both *in vitro* and *in vivo.* We show that the anti-inflammatory effects are in part explained by IgM-ALA binding to co-stimulatory receptors and regulating the function of T effector cells and DC without decreasing Tregs. These *in vitro* studies involve human and murine cells, while *in vivo* studies were performed in mice.

Human Umbilical Cord B Cell Clones Produce IgM-ALA that Exhibit Leukocyte Receptor Specificity – Binding of IgM to Leukocytes was Not Mediated by FcμR

We initially wanted to determine if IgM-ALA exhibited leukocyte receptor specificity as natural antibodies are polyreactive and, hence, each monoclonal IgM could non-specifically bind to carbohydrate or other moieties on several leukocyte receptors. We isolated B cell clones from human umbilical cord blood and observed that >90% of B cells were IgM secreting with about 10% of the IgM clones having IgM-ALA binding activity when examined by flowcytometry on a cell mixture comprising of B (Daudi), T (Jurkat, Sup T1), and macrophage (U937) human cell lines. The lack of demonstrable leukocyte reactivity by the majority of IgM clones would indicate that IgM did not bind to FcμR on human B cells and macrophages (175). Second, we

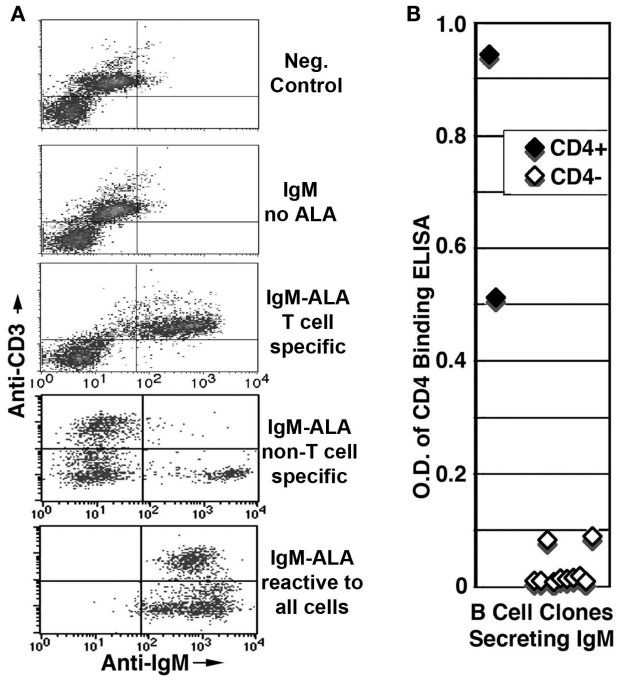


FIGURE 4 | Presence of IgM-ALA in supernatants from umbilical cord B cells. (A,B) *IgM*-ALA reactivity in IgM containing supernatants from B cell clones activated with the EBV virus. IgM-ALA reactivity was detectable in 8 of 79 supernatants. **(A)** Supernatants were interacted with cells containing a mixture of cell lines, i.e., Jurkat, SupT-1, Daudi, and U937. Note that the supernatants have IgM-ALA that is specific for either T or non-T cells or all four cell lines. Subsequent studies revealed that IgM anti-non-T cell had binding reactivity to only U937 cells and not to Daudi cells. **(B)** Data on IgM anti-CD4 reactivity using an ELISA technique. Note that only 2 of 79 IgM-containing supernatants had IgM anti-CD4 reactivity and both these supernatants also had IgM with binding to leukocytes. Figure and legend reproduced with permission from Ref. (39). Copyright 2008. The American Association of Immunologists, Inc.

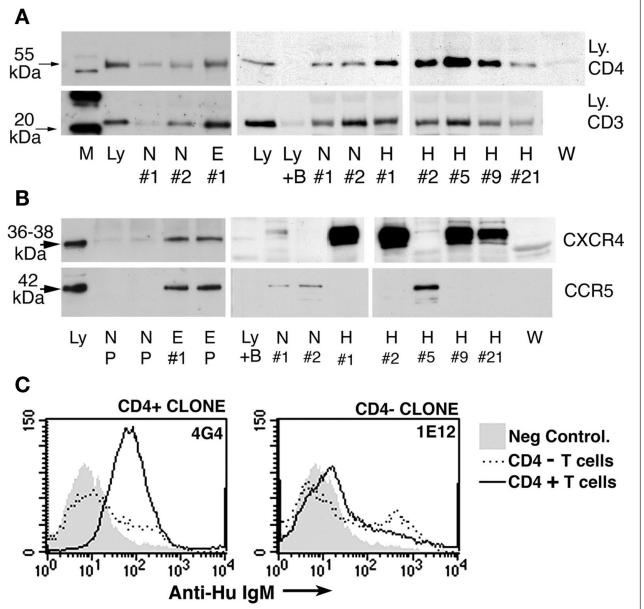


FIGURE 5 | Immunoprecipitation experiments to show binding of human polyclonal IgM to CD3, CD4, CCR5, and CXCR4. (A,B) Identical quantities of individual (labeled no. 1, 2, etc.) or pooled (labeled P) IgM from normal (labeled N), HIV (labeled H), ESRD (labeled E), or Waldenstrom (labeled W) were used to immunoprecipitate leukocyte receptors from equal amounts of whole cell lysates or recombinant soluble CD4. As controls, Western blots were performed with cell lysates in the absence of agarose beads [to control for binding of primary Ab to leukocyte receptor and to determine receptor size (labeled Ly)]. In another control, agarose beads without IgM were added to lysate to determine whether the leukocyte receptor non-specifically bound to the bead (B plus Ly). Note that several fold more receptors were immunoprecipitated by ESRD and HIV IgM when compared with normal IgM. **(C)** Significantly increased binding of IgM from B cell clone 4G4 to $CD4^+$ T cells when compared with $CD4^-$ T cells (MCF 71.6 vs. 16.6). Clone 4G4 secreted IgM with anti-CD4 reactivity. Note that no increased binding was observed on $CD4^+$ T cells using a B cell clone (IE12) secreting IgM without anti-CD4 reactivity (MCF 22.5 vs. 22.3). Figure and legend reproduced with permission from Ref. (39). Copyright 2008. The American Association of Immunologists, Inc.

observed that these IgM-ALA clones exhibited leukocyte subset specificity in that some of the IgM-ALA monoclonal antibodies only bound to receptors expressed by all leukocytes or either T cells (SupT-1, Jurkat) or macrophages (U937) or B cells (Daudi) (**Figure 4**) (39). Further studies revealed that only human monoclonal IgM, having T cell reactivity, immunoprecipitated CD4 from cell lysates and bound to recombinant soluble CD4, thus indicating that IgM-ALA can exhibit both receptor and cell specificity.

Polyclonal IgM from Different Human Sera Differ in Their Repertoire for Receptor Binding. IgM, in Addition, Regulates Human T Effector Cells and DC Without Affecting Tregs or Chemokine Production

We used polyclonal IgM, purified by size exclusion chromatography, from sera of normals, HIV patients and renal dialysis (ESRD) patients (39). Ammonium chloride precipitation was not used as this method affected IgM-ALA binding [see method details in Ref. (39)]. We showed that IgM could immunoprecipitate CD3, CD4, CCR5, and CXCR4 from lysates of cell lines (**Figure 5**). However, the repertoire of IgM-ALA was found to be different among individuals, especially patients as exemplified with HIV patients (see **Figure 5**). Such differences in the repertoires of IgM-ALA may explain why clinical manifestations of inflammation are different among different individuals. It is also possible that prior exposure to different infective agents or foreign antigens may explain the observed differences in the repertoire of IgM-ALA among different individuals (31).

In *in vitro* studies with human peripheral blood mononuclear cells (PBMC), addition of polyclonal human IgM, but not human IgG or Waldenstrom's IgM lacking IgM-ALA, differentially inhibited co-stimulatory receptor upregulation, cytokine production, and proliferation of T cells (39). Both normal and patient IgM downregulated expression of CD4, CD2, and CD86 but not CD8 and CD28 on blood PBMC activated with

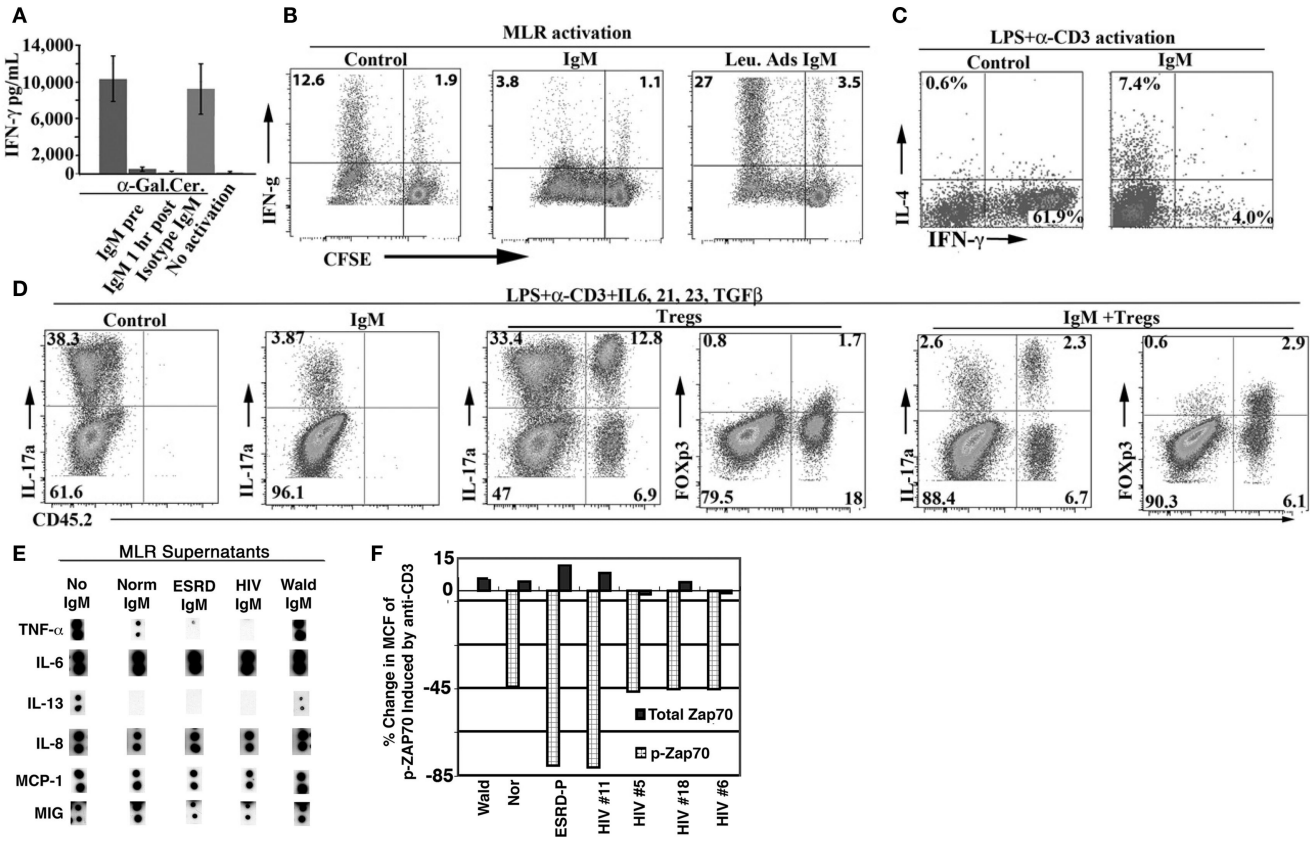


FIGURE 6 | Polyclonal IgM inhibits IFN-γ production and T cell proliferation and differentiation into TH-1 and TH-17 cells of murine splenic cells and specific pro-inflammatory cytokines from human leukocytes activated with alloantigens. **(A)** Supernatant IFN-γ in 48 h culture media of splenic cells activated with a-gal-ceramide that specifically activates NKT-1 cells. IgM was added either 0.5 h before activation (IgM pre) or 1 h post activation. **(B–D)** CFSE labeled WT-B6 splenocytes (2.5×10^5 in 0.5 ml media) were activated either in a one-way MLR (using 7.5×0^5 BALB/c irradiated splenocytes) or LPS (350 ng) and soluble anti-CD3. Cells were cultured for 4 to 5 days. IgM (10–15 μg) was added at the initiation of culture unless otherwise indicated. In **(D)**, the effect of Tregs was evaluated by co-culturing 2.5×10^5 CD45.1 WT-B6 splenic leukocytes, containing 1.8% $CD4^+$ $Foxp3^+$ cells, with 0.5×10^5 CD45.2 WT-B6 Tregs (76% $Foxp3^+$) under cytokine conditions favoring TH-17 differentiation. **(E)** Pooled human normal, ESRD, and HIV IgM but not Waldenstrom IgM significantly inhibit the increase in TNF-α and IL-13 but not that of IL-6, IL-8, MIG, and MCP-1 produced in response to alloantigen activation of T cells. Supernatants were obtained from day 5 MLR cultures stimulated in the presence or absence of pooled IgM (15 μg/ml), added on day 0. **(F)** Pooled human IgM inhibits anti-CD3-mediated Zap-70 phosphorylation of human T cells. IgM was added 30 min before anti-CD3/28 and cells were cultured overnight before quantitation with flowcytometry. Figures and legend reproduced with permission from Ref. (138) **(A–D)** and (39) **(E,F)**. Copyright 2008 and 2012. The American Association of Immunologists, Inc.

alloantigens (MLR) (39). Additionally, both normal and patient IgM inhibited production of the same set of cytokines, i.e., TNF-α, IL-13, and IL-2 but not IL-6 and chemokines when human PBMC were activated by alloantigens [**Figure 6E**; Ref. (39)]. Other investigators working with a monoclonal IgM-ALA with reactivity to TcR have shown that IgM-ALA can inhibit IL-2 production and T cell proliferation by binding to the TcR (5, 176). Similarly, we showed that IgM inhibited T cell proliferation induced by alloantigens or CD3 ligation and also inhibited Zap-70 phosphorylation induced by anti-CD3 [**Figure 6F**; Ref. (39)]. However, IgM did not alter chemokine production by activated PBMC but inhibited chemokine-induced chemotaxis by binding to the receptor and blocking chemokine binding (39). Importantly, IgM did not alter Treg levels in an MLR, despite inhibiting T cell proliferation. In general, identical quantities of patient IgM had a more inhibitory effect in the above studies when compared to normal IgM (39). These functional differences between normal and patient IgM may be explained by differences in their quantity and IgM-NAA repertoire (**Figure 5**). Other investigators have also shown that polyclonal human IgM can inhibit proliferation of human T cells (5, 177).

In summary, the data indicate that polyclonal IgM, in physiological doses, inhibit human T effector cell activation and proliferation as well as regulates production of certain cytokines by binding to certain co-stimulatory molecules (CD4, CD3, TcR). IgM does not inhibit T regs. We also show that the quantity and repertoire of IgM-ALA varies in different individuals especially in disease. Interestingly, IgM-ALA does not appear to affect the production of chemokines by leukocytes, but interferes with their action by binding to chemokine receptors.

Polyclonal Murine IgM Binds to Specific Co-Stimulatory Receptors and Regulates the Function of Murine T Effector Cells, DC, and NKT Cells but Not Tregs

In murine studies, we initially observed that IgM-ALA bound to pronased splenic leukocytes (**Figures 7B,C**) and that IgM-ALA had several fold increased binding to live splenic granulocytes, DC, and B cells when compared to T cells and IgM binding to all leukocytes was enhanced when cells were activated (see **Figure 7A**). Furthermore, we showed that IgM-ALA bound to splenic leukocytes independently of FcμR and, second, showed that IgM, after binding to leukocytes, could immunoprecipitate several different leukocyte receptors (**Figure 7D**) (138). These observations, together with the above findings with human leukocytes, led us to investigate if IgM had an inhibitory effect on the function of T cells, DC, and NKT cells by binding to receptors, e.g., antigen-presenting receptors and co-stimulatory receptors, that get upregulated during activation. Both T cells and DC have an important role in adaptive immunity, e.g., in transplantation and NKT cells, together with DC, have an important role in innate immunity.

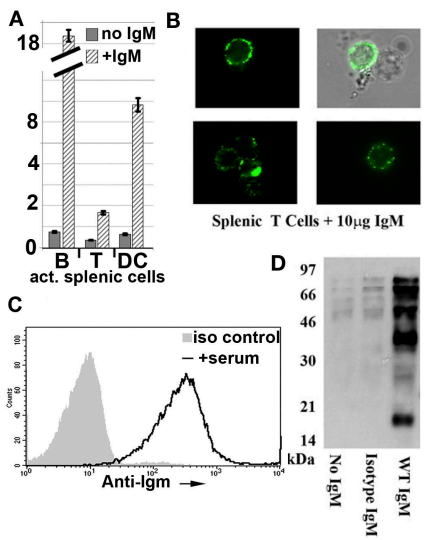


FIGURE 7 | Polyclonal murine WT IgM bind to membrane receptors on leukocytes. **(A)** Polyclonal IgM has several fold increased binding to activated murine splenic B cells and dendritic cells (DC). Splenic leukocytes, activated for 48 h were interacted with purified mouse IgM at 4°C and evaluated for IgM binding using IgG anti-IgM (clone 11/41). Isotype monoclonal IgM with reactivity to KLH did not bind to activated leukocytes (data not shown). IgM binding to B cells was evaluated by blocking intrinsically expressed IgM with unlabeled IgG anti-IgM (clone 11/41). **(B)** It depicts immunofluorescence microscopy images of IgM binding to cell membranes of splenic T lymphocytes. **(C)** It compares binding of IgM and isotype IgM on CD3+WT-B6 pronase pretreated splenic leukocytes. Spleen cells were pronase digested to remove FcμR and to show that IgM-ALA can bind to other receptors on cell membranes. **(D)** It depicts a representative example of a Western blot from two separate experiments demonstrating immunoprecipitation by WT-polyclonal IgM of biotinylated membrane proteins from the murine macrophage cell line J77. In this experiment, WT-polyclonal IgM is compared with an equal amount of isotype IgM that has no binding activity to leukocytes using flow cytometry. Figure and legend reproduced with permission from Ref. (4) **(A)** and Ref. (138) **(B–D)**. Copyright 2012 and 2015. The American Association of Immunologists, Inc.

With murine leukocytes, we showed in *in vitro* studies, that physiological doses of murine polyclonal IgM inhibited naïve T cells from differentiating into TH-1 and Th-17 cells even when IgM was added 48 h after activation (**Figures 6B–D**) (138). This inhibitory effect of IgM-ALA on T cells did not depend on presence of DC as the same inhibitory effect was noted when T cells were activated with insoluble anti-CD3/28. IgM inhibition of anti-CD3-mediated Zap-70 phosphorylation would indicate that the inhibitory effect of IgM on T cell function is mediated by IgM binding to CD3 [**Figure 6F**; Ref. (39)]. IgM did not, however, inhibit differentiation of murine T cells into Foxp3+ cells and, furthermore, under Th-17 differentiating cytokine conditions, IgM inhibited sorted Foxp3+ cells from differentiating into TH-17 cells (**Figure 6D**) (138).

Since there are <1.5% of DC in murine splenic leukocytes, we used 7–8 day cultured murine bone-marrow DC (BMDC) to investigate the functional effects of IgM on DC (4). We showed that polyclonal murine IgM, but not IgM pre-adsorbed with activated splenic leukocytes, bound to recombinant soluble CD40 and PD1 but not PDL-1, CD40L, and CD80, indicating, therefore, that IgM-ALA has binding specificity to certain DC receptors, just as we observed with human T cell receptors where IgM bound to CD4, CD3, and CD2 but not to CD8 (39). The functional effect of IgM binding to certain specific co-stimulatory receptors was tested on LPS-activated BMDC. We show that IgM, even when added 2 h after LPS, inhibited LPS-induced CD40 upregulation, but not upregulation of CD86, PDL-1, and MHC-II of BMDC and downregulated basal expression of PD1 on BMDC. IgM, in addition, downregulated p65NF-κB activation induced by LPS (**Figure 8D**) but not by LPS + anti-CD40 (agonistic Ab), thus indicating that IgM can inhibit p65NF-κB upregulation mediated by TLR4 activation, but not when both TLR4 and CD40 are activated (4). Interestingly, IgM inhibited TLR4 activation by a mechanism that did not involve inhibition of LPS binding to cell receptors (4). However, despite downregulation by IgM of TLR4-induced p65NF-κB, there was no decrease in IL-12 production or increase in IL-10 production indicating that LPS activates other transcription factors, besides p65NF-κB, to upregulate these cytokines and certain other co-stimulatory receptors that were not downregulated with IgM (4). In *in vivo* studies (to be presented in the next section), we show that IgM pretreatment of LPS-activated BMDC switches these activated BMDC to a regulatory phenotype possibly by a mechanism involving down-regulation of CD40 and NF-κB.

We next did *in vitro* studies to test the effect of polyclonal IgM on Type1 NKT cells, which together with DC, have an important role in inducing innate inflammation, e.g., after renal reperfusion injury (IRI). In these studies, we used α-gal-ceramide, a glycolipid that is taken up by DC and presented via the CD1d receptor to Type 1 NKT cells, which get activated and secrete IFN-γ. This assay is specific for determining Type 1 NKT function as only Type 1 NKT, but not T effector cells, will secrete IFN-γ after exposure to α-gal-ceramide, which is recognized, in context of CD1d presentation, by the invariant TcR on Type 1 NKT cells. We show that physiological doses of IgM inhibits α-gal-ceramide

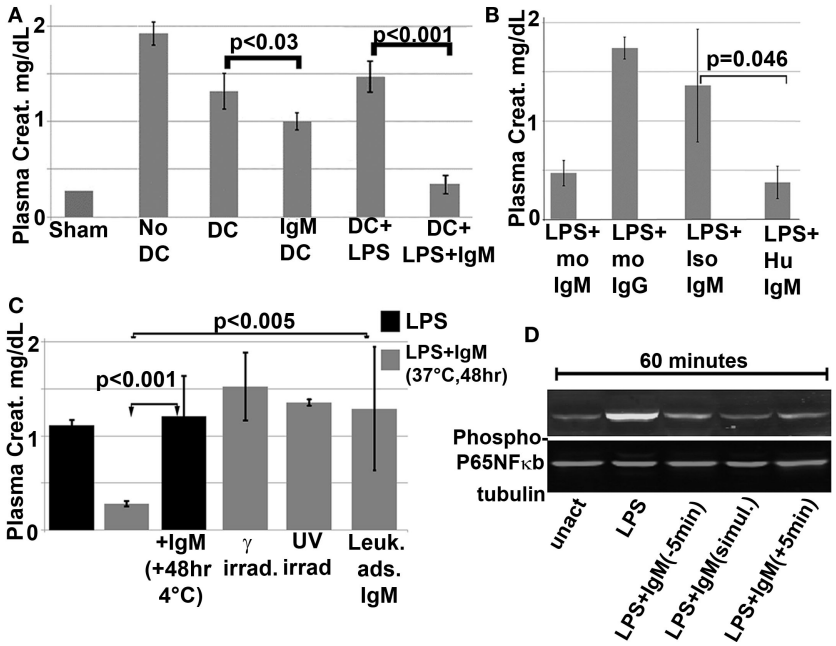


FIGURE 8 | *In vivo* infusion of polyclonal human or murine IgM pretreated WT-BMDC with downregulated NF-κB protects mice from renal IRI. **(A)** There is more protection when using WT-BMDC pretreated with both IgM and LPS as evaluated by SCr. WT-BMDC were pretreated with IgM for 1 h before adding LPS and culturing BMDC at 37°C for 48 h. **(B)** LPS-activated BMDC pretreated with either mouse or human IgM are protective in renal IRI. **(C)** Pretreating 48 h LPS-activated WT-BMDC with IgM for 1 h at 4°C is not protective in IRI. Fourty-eight hour IgM/LPS-pretreated WT-BMDC are non-protective in IRI after being irradiated (3000 Rad) or rendered apoptotic by exposure to UV light. Additionally, polyclonal IgM adsorbed with splenic leukocytes to deplete IgM-ALA clones was not protective in IRI. **(D)** Western blot depicting IgM-mediated downregulation of LPS-induced phosphorylation of p65 NF-κB in BMDC. IgM was added either before or after LPS activation. Figure and legend reproduced with permission from Ref. (4) Copyright 2015. The American Association of Immunologists, Inc.

TABLE 2 | *In vitro* effects of IgM-ALA on human and murine leukocytes.

	T cells	NKT-1 cells	BMDC
Degree of membrane binding	Moderate	Moderate	Very high
Cell receptor binding	CD4, CD3, TcR, downregulation of CD4; inhibits HIV entry	CD4	Binds CD86, CD40
Intracellular signaling	Inhibits ZAP-70 activation		Inhibits LPS-induced NFκB activation
Pro-inflammatory mediators	Inhibits production of IFNγ, IL-17, TNFα, IL-2	Inhibits production of IFNγ	No effect on IL-12 or IL-10
Anti-inflammatory mediators	Enhances production of IL-4, enhances T regs		Switches BMDC to regulatory phenotype (PD-1, IL-10 dependent)
Proliferation	Inhibits proliferation (alloantigen and anti-CD3/28)		

induced IFN-γ production of splenic leukocytes even when IgM is introduced 1 h after α-gal-ceramide (**Figure 6A**) (138). We have not defined the mechanism for the inhibitory effect of IgM on Type 1 NKT function.

In summary (see **Table 2**), the *in vitro* data with both human and murine cells would indicate that IgM-ALA regulates leukocyte function by binding and downregulating certain leukocyte receptors (e.g., CD4 and CD2 on T cells, CD40, and CD86 on DC) and inducing regulatory function in DC. Importantly, IgM does not decrease Foxp3+ Tregs. IgM regulates leukocyte activation, proliferation, and chemotaxis to attenuate excess inflammation [**Figure 6**; Ref. (39)]. The marked individual variation in the repertoire of IgM-ALA, with specificity to the different leukocyte receptors, observed in both normal and disease states could potentially explain the differences in the vigor and character of inflammatory responses in different individuals exposed to the same inciting agent. Additionally, differences in inflammatory response may also be influenced by total levels of IgM-NAA or IgM-ALA as we observed in transplant recipients (**Figure 3**). Finally, IgM-ALA, by binding to leukocyte receptors and inhibiting cell activation, can provide another mechanism to limit viral entry into cells and replication as we have shown with the HIV-1 virus (**Figure 2**) (144).

IgM-ALA Inhibits the Innate Immune Inflammatory Response in Renal Ischemia Reperfusion Injury

To test the *in vitro* inhibitory effects of IgM-ALA on DC and NKT cells, we used an *in vivo* murine model of renal IRI (169). In this model, renal vessels to both kidneys are completely occluded with clamps for 26 or 32 min to induce either mild or severe ischemic

renal tubular injury. The kidneys are then allowed to re-perfuse by unclamping the blood vessels and the extent of renal injury or decrease in renal function is quantitated at 24 h of reperfusion by measuring for accumulation in the plasma of waste products (e.g., creatinine) that are normally only removed by the kidneys. In this model, the initial ischemic injury is not sufficient to impair renal function as quantitated by measuring plasma creatinine, but it is the innate inflammatory response to products released by ischemic renal cells (e.g., DAMPS and glycolipids) that significantly worsen kidney injury that leads to loss of function. In this model, DAMPS and glycolipids released by ischemic renal cells are taken up by DC and in the splenic marginal zone, DC present glycolipids in the context of CD1d to activate NKT cells, which rapidly release IFN-γ to activate innate effector cells especially granulocytes, macrophages, and NK cells (169). Activated innate effectors migrate to the kidney, where chemokines, released by ischemic cells, enhance extravasation of inflammatory cells into the kidney interstitium. The inflammatory effector cells in the kidney interstitium cause further renal tubular injury with loss of kidney function that leads to an increase in plasma creatinine. This acute loss in kidney function is referred to as acute kidney injury (AKI).

We used two approaches to test the protective role of IgM in the suppression of this ischemia-induced innate inflammatory response. First, we performed renal IRI in B6/S4-IgMko mice (referred to as IgM ko) that lack circulating IgM but have normal levels of other immunoglobulins. These mice have normal or increased levels of Tregs, B regs, and IL-10 and their normal functioning B cells express membrane IgM or BcR but are unable to secrete IgM. Unlike their WT counterpart, we demonstrated that these mice are very sensitive to renal ischemia, developing AKI with mild ischemia (26 min clamp time) that is insufficient to cause AKI in their WT counterparts (**Figures 9A,B**). Replenishing IgM in the IgM ko mice with a single 240 μg dose of polyclonal IgM, to achieve plasma levels similar to that in their WT counterparts, protected these IgM ko mice from developing AKI with mild ischemia, thus indicating that sensitivity to ischemia in the IgM ko mice resulted from a lack of circulating IgM (**Figures 9A,B**).

In the second approach, a single dose (150 μg) of normal purified polyclonal IgM was administered intravenously to wild-type C57BL6 (WT-B6) mice to increase baseline circulating IgM by about 30–50% and to determine if increasing IgM would protect these WT-B6 from severe renal ischemia (32 min clamp time). These studies clearly indicated that increasing circulating IgM levels protected mice from severe renal IRI (**Figure 9C**). We next determined that this protection was mediated by IgM-ALA as administering similar quantity of polyclonal IgM pre-adsorbed with activated splenic leukocytes to remove IgM-ALA failed to protect these WT-B6 mice from severe renal IRI (**Figure 9C**).

In both approaches, physiological doses of intravenous polyclonal IgM mediated protection by decreasing the innate ischemia-induced inflammatory response. Protected kidneys had a very minimal inflammatory response with no or minimal tubular injury that could be detected on histology. Based on our *in vitro* data, *in vivo* IgM-ALA could mediate protection through several mechanisms, including regulation of NKT and DC and maintaining or enhancing Tregs, which also mediates protection in this model of innate inflammation (178). Our prior observations showing that IgM-ALA had several fold increased binding to splenic DC, when compared to T cells, prompted us to investigate the role of IgM-ALA in regulating DC in this model. These studies are presented in the next section.

Protection from Renal Ischemia is Mediated by IgM Induced Regulatory DC. Regulatory DC Require Tregs, B cells, Circulating IgM, and IL-10 to Mediate *In Vivo* Protection

To examine the *in vivo* role of IgM on DC, we used 7 to 8-day-old cultured BMDC. In these studies, BMDC were activated *ex vivo* for 48 h with LPS with or without polyclonal IgM. Activated BMDC were washed and then 0.5×10^6 BMDC were infused intravenously into mice 24 h before performing renal ischemia. In these studies, IgM + LPS-pretreated BMDC protected mice from ischemia induced AKI (**Figures 8A**) by inhibiting the increased generation of circulating granulocytes and inhibiting innate activated leukocytes from infiltrating the ischemic kidney [**Figure 10**, Ref. (4)]. Importantly, protection with LPS-activated BMDC was only observed when IgM was present during the 48-h culture and not when IgM was added at the end of the 48 h LPS activation (**Figure 8C**), indicating therefore that regulation of BMDC by IgM is an active process requiring both NF-κB and CD40 downregulation induced by IgM (**Figure 8D**). Preventing downregulation of NF-κB and CD40 by adding the agonistic anti-CD40 antibody to LPS + IgM during activation, negated the protective effect, thus supporting that NF-κB and CD40 downregulation are required to switch activated BMDC to a regulatory phenotype (4). It is possible that IgM by binding to CD40 induces this regulatory phenotype. Ex-vivo pretreatment of murine BMDC with human IgM was also protective in renal IRI (**Figure 8B**), indicating that the function of IgM-NAA is evolutionarily conserved among species.

However, since LPS activation of BMDC generates apoptotic cells, it is possible that protection from ischemia could be mediated by complexes of IgM anti-PC and apoptotic cells, which when injected, regulate endogenous DC (127). Such a possibility seemed unlikely, as in the *in vivo* murine studies to demonstrate the anti-inflammatory role of apoptotic cells, large quantities of apoptotic cells (2.5×10^7 thymocytes) were used (127), while in our studies, we only used 0.5×10^6 BMDC (4). However, to exclude the possibility of apoptotic cell-mediated protection, we used apoptotic BMDC by subjecting activated LPS + IgM pretreated BMDC (0.5×10^6 cells) to UV irradiation. Such apoptotic LPS + IgM pretreated BMDC failed to protect mice from ischemia induced AKI, thus excluding the role of apoptotic cell/IgM complexes in inducing protection (**Figure 8C**). The latter experiments indicated that IgM-ALA mediated protection by switching LPS-activated BMDC to a regulatory phenotype. BMDC required IL-10 but not IDO (indoleamine 2, 3-dioxygenase) to switch to a regulatory phenotype as IgM + LPS pretreatment of IL-10 ko BMDC, but not IDO ko BMDC, failed to protect mice from developing AKI after renal ischemia (4).

We hypothesize that intravenously injected regulatory BMDC inhibit the innate inflammatory response by entering the splenic marginal zone where they inhibit NKT function. However, in further studies, we show that injected regulatory

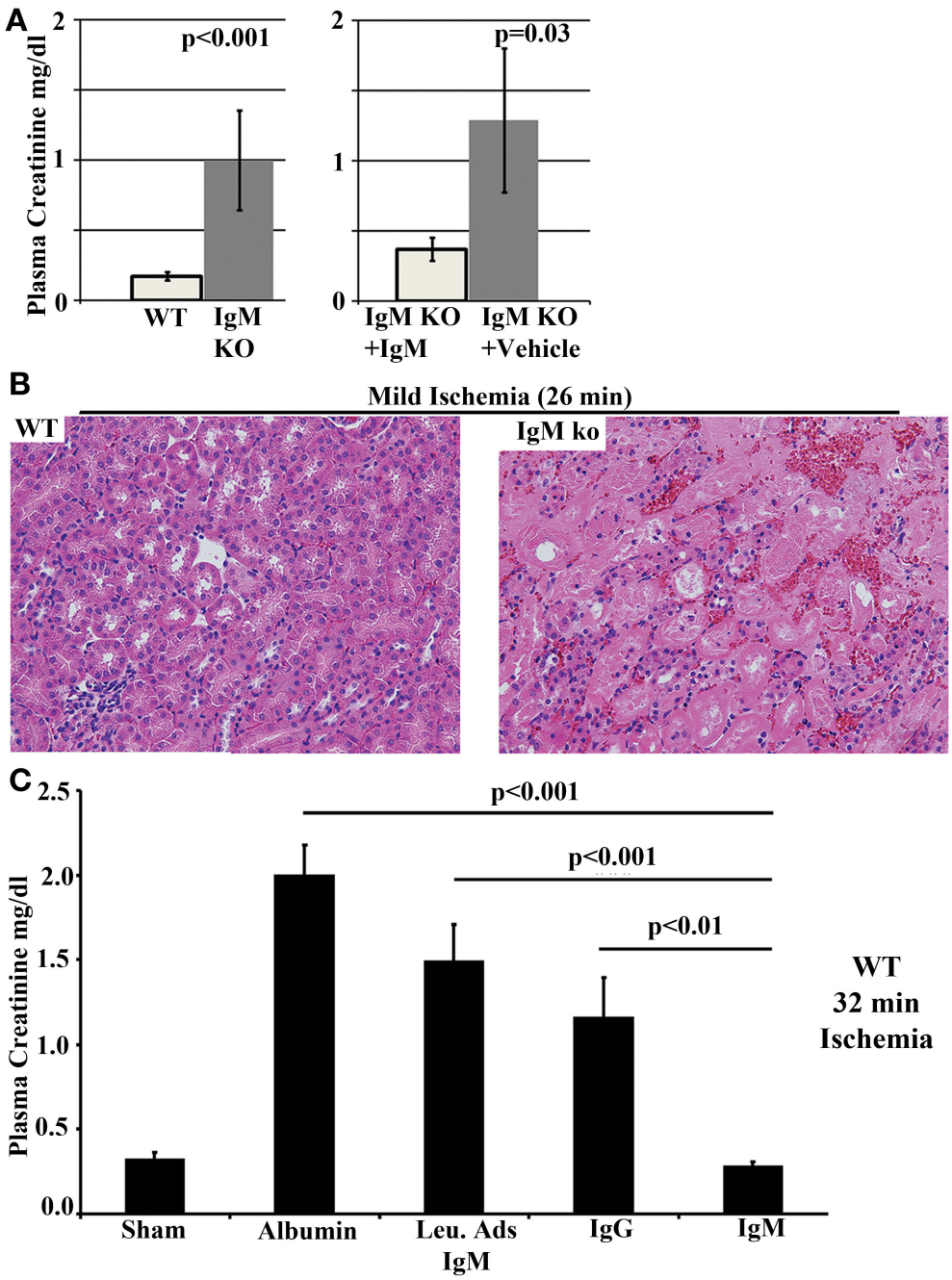


FIGURE 9 | B6/S4-IgMko mice are more sensitive to renal IRI when compared to their WT counterparts (WT-B6/S4). **(A, B)** Kidneys from B6/S4-IgMko mice and their WT counterparts (WT-B6/S4) were subjected to mild ischemia (26 min) and then reperfused. Data depict 24 h plasma creatinine comparing (WT-B6/S4) WT mice with B6/S4-IgMko mice, and B6/S4-IgMko pretreated with 240 μg IgM, 24 h before ischemic injury. Histology depicts H&E staining of renal outer medulla after 24 h of reperfusion. **(C)** Polyclonal IgM, but not leukocyte adsorbed IgM (Leu-Ads IgM), protects against renal IRI in WT-B6 mice. In these studies, WT-B6 mice were pretreated with equal quantities (150 μg in 0.75 ml) of IgM or Leu-Ads IgM or IgG, 24 h before subjecting the kidneys to severe ischemia (32 min). Kidneys were reperfused for 24 h prior to determining plasma creatinine **(C)**. Control mice were pretreated with 0.75 ml RPMI containing 150 μg bovine albumin to exclude variables, such as volume/colloid, that can protect against ischemic injury. Figure and legend reproduced with permission from Ref. (138). Copyright 2012. The American Association of Immunologists, Inc.

BMDC require the presence of other *in vivo* suppressive mechanisms, such as circulating IgM, IL-10, Tregs, and B cells, to mediate protection (4).

In summary, IgM-ALA inhibits the ischemia-induced innate inflammatory response by several mechanisms, including switching activated DC to a regulatory phenotype, inhibiting

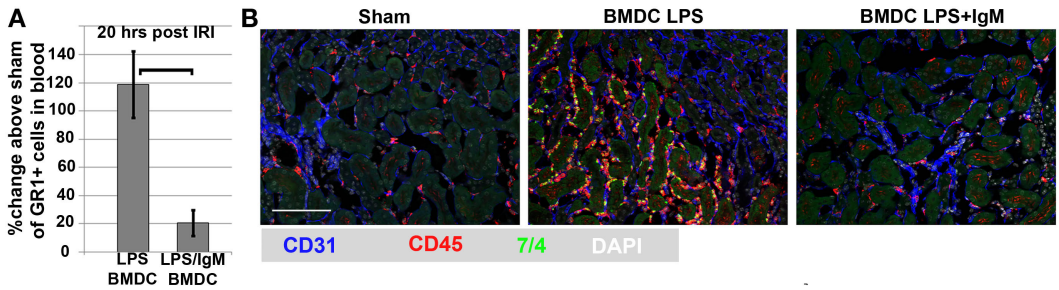


FIGURE 10 | Murine IgM/LPS-pretreated BMDC inhibit the inflammatory response after renal IRI. There is significantly less inflammatory cell response 20 h post ischemia in mice administered IgM/LPS-pretreated BMDC as determined by circulating granulocytes (CD45+, GR1+) in blood **(A)** and infiltrating CD45+ and GR1+ leukocytes in kidneys **(B)**. CD31 is a marker for endothelial cells. 7/4 is a marker for GR1+ granulocytes. Figure and legend reproduced with permission from Ref. (4). Copyright 2015. The American Association of Immunologists, Inc.

NKT cell IFN-γ production, and inhibiting chemotaxis of leukocytes by binding to chemokine receptors. However, IgM-ALA is in-effective on its own in inhibiting the ischemia-induced innate inflammatory response and requires the presence of other *in vivo* suppressive mechanisms, such as IL-10, Tregs and B cells. Conversely, these other *in vivo* suppressive mechanisms, such as Tregs and Bregs, cannot effectively protect against ischemia induced innate inflammation without IgM-NAA as evidenced in our renal ischemia experiments using IgM ko mice that lack secretory IgM (**Figures 9A,B**).

Polyclonal IgM Inhibits Inflammation Mediated by Adaptive Immune Mechanisms in Allograft Transplantation

Because of our clinical observations (**Figure 3**) and the *in vitro* studies demonstrating that IgM (a) inhibited alloantigen-activated T cell proliferation and differentiation into Th-1 and Th-17 independently of DC (**Figure 6**) and (b) could induce regulatory function in DC, we performed experiments aimed at determining whether IgM could also inhibit allograft rejection, which is an *in vivo* model of inflammation mediated by alloantigen-activated DC and T cells (138). Two approaches were used to test the role of polyclonal IgM. First, cardiac transplants were performed intra-abdominally in B6/S4-IgM ko mice (referred to as IgM ko) or their WT littermates using B6-bm12 donor hearts, which are minimally incompatible at the MHC class II locus (Ia) with the recipient. In this transplant model, there is a mild chronic form of cellular rejection and a vasculopathy that is initiated by a T cell-mediated inflammatory process and cardiac graft loss (defined as loss of intra-abdominal cardiac pulsation) occurs at >2 months in WT recipients. However, in IgM ko recipients, there is a more severe acute cellular rejection and graft loss occurs in 2–3 weeks, which is significantly earlier compared with their WT-B6/S4 counterparts, where cardiac graft loss occurs after >2 months (**Figure 11**). Histologically, there are considerably more TH-17 cells infiltrating the cardiac allograft in the IgM ko recipient despite no significant difference in infiltrating Tregs between the groups (138). The T cell findings on histology mirror the *in vitro* studies where IgM inhibited naïve T cells and Foxp3+ T cells from differentiating into TH-17 cells without affecting levels of Tregs (**Figure 6D**).

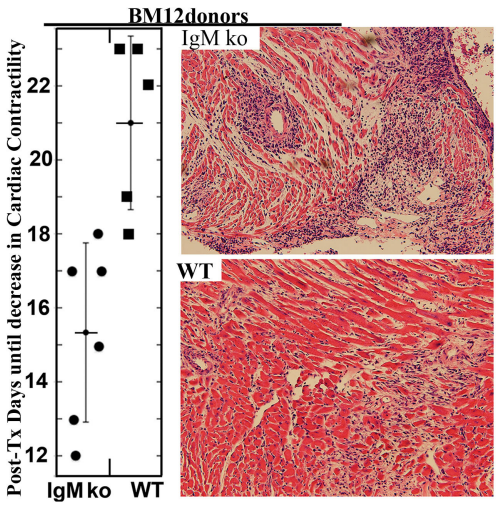


FIGURE 11 | Allograft rejection is more rapid and severe in B6/S4-IgMko mice. B6/S4-IgMko mice and their WT counterparts (WT-B6/S4) received cardiac allografts from B6-bm-12 donors that are only incompatible at the MHC-Ia locus. Graph depicts the post-transplant day when cardiac contractility was found to be decreased by finger palpation. Histology depicts Day 10 post-transplant B6-bm12 cardiac allograft histology. Figure and legend reproduced with permission from Ref. (138). Copyright 2012. The American Association of Immunologists, Inc.

In the second approach, we wanted to determine whether increasing circulating levels of IgM in WT-B6 mice inhibited the severe and rapid rejection that occurs in the setting of fully MHC-incompatible donor hearts (i.e., from BALB/c donors). In this model, rejection in WT-B6 recipients is detectable by day 5 and graft loss occurs by 7–9 days (138). In these studies, 175 μg IgM was intravenously administered 24 h after ascertaining that cardiac surgery was successful, and the dose of IgM was repeated on days 3 and 5. Mice were euthanized on day 6. The data clearly show that IgM inhibited the severe inflammation in the cardiac allograft induced by rejection on day 6, as detected by H&E staining and immunofluorescence staining for neutrophils (7/4) and T cells (CD3). Importantly, with immunohistochemistry, this lack

of leukocyte infiltration in the cardiac parenchyma of IgM-treated recipients was also associated with no or minimal CXCL1+ leukocytes and with no or minimal fragmentation of capillaries, as identified by the endothelial cell marker CD31 (138).

In summary, we show that physiological doses (175 μg) of polyclonal IgM can subdue inflammatory responses mediated by an adaptive immune mechanism. Potential mechanisms include the following (a) a direct inhibitory effect of IgM-ALA on T effector cells, but not Tregs, possibly by binding and down-modulating CD3/TcR and certain specific co-stimulatory receptors, such as CD4 and CD2 but not CD8. As a result, T effector cells are inhibited from proliferating or producing certain specific cytokines (e.g., TNF, IFN-γ, IL-17 but not IL-6, and chemokines) or from differentiating into TH-1 and TH-17 pro-inflammatory cells. Importantly IgM-ALA does not affect levels of Foxp3+ Tregs, but prevents Foxp3 + cells from differentiating into TH-17 cells under pro-inflammatory conditions, (b) by binding to CD40 and switching activated DC to a regulatory phenotype with downregulation of CD40 and p65NF-κB, and (c) by inhibiting chemotaxis. It is highly unlikely that IgM anti-PC could have a significant role in inhibiting allograft rejection in our studies as we used small doses of polyclonal IgM (175 μg) while 1.5–2.0 mg of a monoclonal IgM anti-PC (T15 idiotype) was used to inhibit an arthritis model of inflammation mediated by adaptive immune mechanisms (127).

Polyclonal IgM Antibodies Inhibit Autoimmune-Mediated Insulitis in NOD Mice

Insulitis in the NOD mouse is primarily mediated by autoimmune T cells but there are data to indicate that B cells are also involved. Depleting B cells or only B1 cells ameliorates insulitis indicating that some of the IgG autoantibodies detected in NOD mice may also be pathogenic (179–181). The role of B1 cells in generating pathogenic IgG autoantibodies has also been described in other autoimmune murine models (20, 94). Because our *in vitro* studies demonstrated that polyclonal IgM inhibited T cell proliferation and differentiation into Th-1 and Th-17 cells (**Figures 6B–D**) and can also counter pathogenic IgG autoantibodies (as previously discussed), we performed studies to determine whether IgM could inhibit autoimmune insulitis that results in islet cell destruction and diabetes mellitus (DM) in NOD mice (182). In these mice, the autoimmune inflammatory process begins spontaneously around 4–5 weeks after birth and the initial phase is characterized by a silent and non-destructive leukocyte infiltration of the perivascular and periductal regions in the pancreas as well as the peripheral islet regions by a heterogeneous mixture of CD4 and CD8 T cells, B cells, macrophages, and DC (peri-insulitis). In the invasive phase that begins at 8–12 weeks of age, the immune infiltrate enters the islet inducing beta cell destruction (insulitis). Significant destruction first becomes evident around 12–13 weeks of age with mice exhibiting overt diabetes (DM).

We wanted to determine the effect of increasing IgM levels on development of DM (182). NOD mice were administered bi-weekly intra-peritoneal polyclonal IgM (50 μg/dose) beginning either at 5 or 11 weeks of age and ending when mice were 18 weeks old. At 25 weeks of age, 80% of control mice ($n = 30$) became diabetic, while 0% of mice ($n = 30$) treated with IgM beginning at 5 weeks developed DM. Importantly, only 20% of pre-diabetic mice ($n = 20$) treated with IgM beginning at 11 weeks of age developed DM at 25 weeks of age. This latter observation is particularly notable as prior studies using co-stimulatory blockade failed to prevent DM in this murine model. At 18–25 weeks of age the pancreas revealed no or minimal insulitis in NOD mice treated with IgM beginning at 5 weeks of age. Other investigators using monoclonal polyreactive natural IgM in the neonatal period have also obtained similar results (183, 184).

In summary, these studies indicate that polyclonal IgM inhibits insulitis via several potential mechanisms, including inhibition of autoimmune T effectors and possibly countering IgG autoantibodies via anti-idiotypic mechanisms and by inhibiting the B cells that produce them. Additionally, IgM by switching activated DC to a regulatory phenotype and maintaining Tregs could enhance this protective effect.

CONCLUSION

Figures 12 and **13** summarize our concepts of the inter-relationship between pathogens and natural antibodies. In both murine and human models, the evidence shows that these polyreactive and low-affinity binding IgM-NAA function under physiological conditions to (i) provide a first line of defense against invading organisms, (ii) protect the host from autoimmune inflammation mediated by autoimmune B and T cells that have escaped tolerance mechanisms, (iii) protect the host from endogenous oxidized neo-determinants and other neo-antigens that are unmasked during tissue damage, and (iv) regulate excess inflammation, mediated by both innate and adaptive immune mechanisms. Even though the full repertoire of IgM-NAA develop during the first few years of life, both their levels and repertoire differ in healthy individuals as well as in disease and could contribute to the varying inflammatory response, for example, after an infection or alloantigen exposure (see **Figures 2** and **5**). We hypothesize that high

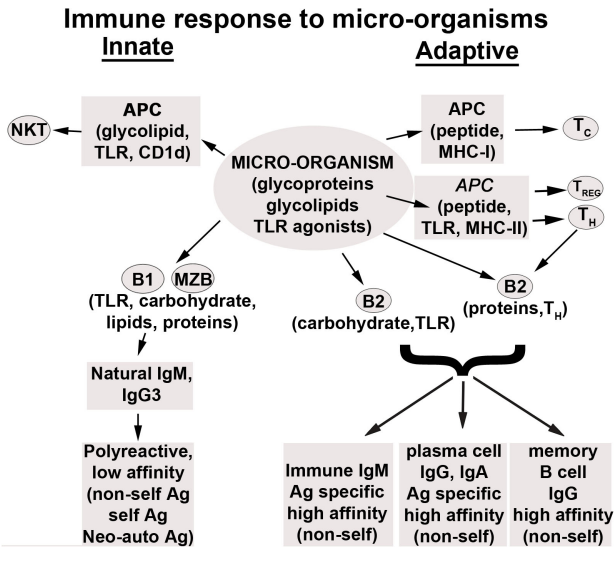


FIGURE 12 | Immune response to micro-organisms.

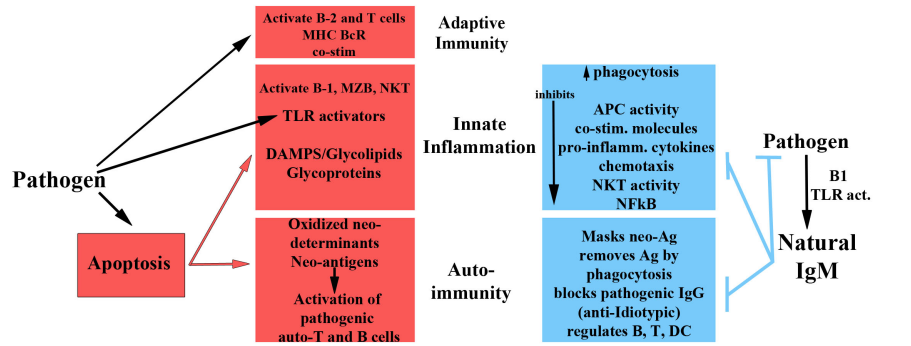


FIGURE 13 | Pathogen-induced natural IgM protects host from pathogen-mediated inflammation and autoimmunity.

protective levels of IgM-NAA are maintained by infections that have been shown to increase IgM-NAA, especially IgM-ALA and anti-PC [reviewed in Ref. (31, 39, 148)]. Such a hypothesis could explain the significantly low incidence of autoimmune disorders, such as SLE or sarcoidosis in rural parts of Africa where malaria and other infections are endemic (185–187). We have shown that IgM-ALA increases in active sarcoidosis (188). There are other suppressive mechanisms (e.g., Tregs, B regs, IL-10, TGF-β) that regulate inflammation and based on the different animal models of inflammation, it would appear that IgM-NAA have a more prominent role in regulating inflammation that involves pathogenic IgG autoantibodies, macrophages, and NKT cells. However, we show that IgM-NAA require Tregs, B cells, and IL-10 to be fully effective in controlling inflammation (4). Conversely, our studies and that of others, using mice deficient in IgM secretion, would also indicate that Tregs and Bregs also require IgM-NAA to effectively control inflammation (39, 119, 120). Finally, it may be easier to develop a vaccine to increase IgM-NAA especially if we understand how diverse infectious agents increase IgM-ALA. Studies are also needed to determine if prolonged high IgM-NAA levels can induce excess immunosuppression. One could also use enriched IgM intravenous preparations to acutely treat patients with uncontrolled inflammation. Cell therapy, especially with IgM pretreated DC, could provide an alternative approach requiring minimal quantities of IgM to prevent ischemic acute renal failure (e.g., in high-risk patients undergoing cardiac surgery) or delayed graft function after renal transplantation (4).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

REFERENCES

1. Dooley H, Flajnik MF. Antibody repertoire development in cartilaginous fish. *Dev Comp Immunol* (2006) **30**(1–2):43–56. doi:10.1016/j.dci.2005.06.022
2. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* (1999) **96**(5):2250–5. doi:10.1073/pnas.96.5.2250
3. Thurnheer MC, Zuercher AW, Cebra JJ, Bos NA. B1 cells contribute to serum IgM, but not to intestinal IgA, production in gnotobiotic Ig allotype chimeric mice. *J Immunol* (2003) **170**(9):4564–71. doi:10.4049/jimmunol.170.9.4564
4. Lobo PI, Schlegel KH, Bajwa A, Huang L, Kurmaeva E, Wang B, et al. Natural IgM switches the function of lipopolysaccharide-activated murine bone marrow-derived dendritic cells to a regulatory dendritic cell that suppresses innate inflammation. *J Immunol* (2015) **195**(11):5215–26. doi:10.4049/jimmunol.1500052
5. Robey IF, Schluter SF, Akporiaye E, Yocum DE, Marchalonis JJ. Human monoclonal natural autoantibodies against the T-cell receptor inhibit interleukin-2 production in murine T cells. *Immunology* (2002) **105**(4):419–29. doi:10.1046/j.1365-2567.2002.01389.x
6. Zhang M, Alicot EM, Carroll MC. Human natural IgM can induce ischemia/reperfusion injury in a murine intestinal model. *Mol Immunol* (2008) **45**(15):4036–9. doi:10.1016/j.molimm.2008.06.013
7. Steele E, Cunningham A. High proportion of Ig-producing cells making autoantibody in normal mice. *Nature* (1978) **274**:483–4. doi:10.1038/274483a0
8. Dighiero G, Guilbert B, Fermand J, Lymberi P, Danon F, Avrameas S. Thirty-six human monoclonal immunoglobulins with antibody activity. *Blood* (1983) **62**(2):264–70.
9. Hardy RR, Hayakawa K. Development and physiology of Ly-1 B and its human homolog, Leu-1 B. *Immunol Rev* (1986) **93**(1):53–80. doi:10.1111/j.1600-065X.1986.tb01502.x
10. Kantor AB, Herzenberg LA. Origin of murine B cell lineages. *Annu Rev Immunol* (1993) **11**(1):501–38. doi:10.1146/annurev.iy.11.040193.002441
11. Nakamura M, Burastero SE, Ueki Y, Larrick JW, Notkins AL, Casali P. Probing the normal and autoimmune B cell repertoire with Epstein-Barr virus. Frequency of B cells producing monoreactive high affinity autoantibodies in patients with Hashimoto's disease and systemic lupus erythematosus. *J Immunol* (1988) **141**(12):4165–72.
12. Mouthon L, Lacroix-Desmazes S, Nobrega A, Barreau C, Coutinho A, Kazatchkine M. The self-reactive antibody repertoire of normal human serum IgM is acquired in early childhood and remains conserved throughout life. *Scand J Immunol* (1996) **44**(3):243–51. doi:10.1046/j.1365-3083.1996.d01-306.x
13. Clarke SH, Arnold LW. B-1 cell development: evidence for an uncommitted immunoglobulin (Ig)M+ B cell precursor in B-1 cell differentiation. *J Exp Med* (1998) **187**(8):1325–34. doi:10.1084/jem.187.8.1325
14. Casali P, Burastero SE, Nakamura M, Inghirami G, Notkins AL. Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. *Science* (1987) **236**(4797):77–81. doi:10.1126/science.3105056

15. Hardy RR, Hayakawa K, Shimizu M, Yamasaki K, Kishimoto T. Rheumatoid factor secretion from human Leu-1+ B cells. *Science* (1987) **236**(4797):81–3. doi:10.1126/science.3105057
16. Kasaian MT, Casali P. Autoimmunity-prone Bl (CD5 B) cells, natural antibodies and self recognition. *Autoimmunity* (1993) **15**(4):315–29. doi:10.3109/08916939309115755
17. Rieben R, Roos A, Muizert Y, Tinguely C, Gerritsen AF, Daha MR. Immunoglobulin M-enriched human intravenous immunoglobulin prevents complement activation in vitro and in vivo in a rat model of acute inflammation. *Blood* (1999) **93**(3):942–51.
18. Sidman CL, Shultz LD, Hardy RR, Hayakawa K, Herzenberg LA. Production of immunoglobulin isotypes by ly-1+ B cells in viable motheaten and normal mice. *Science* (1986) **232**(4756):1423–5. doi:10.1126/science.3487115
19. Solvason N, Lehuen A, Kearney JF. An embryonic source of Ly1 but not conventional B cells. *Int Immunol* (1991) **3**(6):543–50. doi:10.1093/intimm/3.6.543
20. Murakami M, Tsubata T, Shinkura R, Nisitani S, Okamoto M, Yoshioka H, et al. Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. *J Exp Med* (1994) **180**(1):111–21. doi:10.1084/jem.180.1.111
21. Nisitani S, Tsubata T, Murakami M, Honjo T. Administration of interleukin -5 or -10 activates peritoneal B-1 cells and induces autoimmune hemolytic anemia in anti-erythrocyte autoantibody-transgenic mice. *Eur J Immunol* (1995) **25**(11):3047–52. doi:10.1002/eji.1830251110
22. Yang Y, Tung JW, Ghosn EEB, Herzenberg LA, Herzenberg LA. Division and differentiation of natural antibody-producing cells in mouse spleen. *Proc Natl Acad Sci U S A* (2007) **104**(11):4542–6. doi:10.1073/pnas.0700001104
23. Ha S, Tsuji M, Suzuki K, Meek B, Yasuda N, Kaisho T, et al. Regulation of B1 cell migration by signals through Toll-like receptors. *J Exp Med* (2006) **203**(11):2541–50. doi:10.1084/jem.20061041
24. Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, et al. Positive selection of natural autoreactive B cells. *Science* (1999) **285**(5424):113–6. doi:10.1126/science.285.5424.113
25. Martin F, Kearney JF. Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and btk. *Immunity* (2000) **12**(1):39–49. doi:10.1016/S1074-7613(00)80157-0
26. Cancro MP, Kearney JF. B cell positive selection: road map to the primary repertoire? *J Immunol* (2004) **173**(1):15–9. doi:10.4049/jimmunol.173.1.15
27. Tian Q, Beardall M, Xu Y, Li J, Parker DC, Casanova N, et al. B cells expressing a natural polyreactive autoantibody have a distinct phenotype and are overrepresented in immunoglobulin heavy chain transgenic mice. *J Immunol* (2006) **177**(4):2412–22. doi:10.4049/jimmunol.177.4.2412
28. Love SD, Lee W, Nakamura YC, Platt JL, Bollinger RR, Parker W. Natural anti-carbohydrate IgM in mice: dependence on age and strain. *J Immunol Methods* (2000) **246**(1):61–8. doi:10.1016/S0022-1759(00)00296-9
29. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70-. *J Exp Med* (2011) **208**(1):67–80. doi:10.1084/jem.20101499
30. Simell B, Lahdenkari M, Reunanen A, Kayhty H, Vakevainen M. Effects of ageing and gender on naturally acquired antibodies to pneumococcal capsular polysaccharides and virulence-associated proteins. *Clin Vaccine Immunol* (2008) **15**(9):1391–7. doi:10.1128/CVI.00110-08
31. Adib M, Ragimbeau J, Avrameas S, Ternynck T. IgG autoantibody activity in normal mouse serum is controlled by IgM. *J Immunol* (1990) **145**(11):3807–13.
32. Nicoletti C, Yang X, Cerny J. Repertoire diversity of antibody response to bacterial antigens in aged mice. III. Phosphorylcholine antibody from young and aged mice differ in structure and protective activity against infection with *Streptococcus pneumoniae*. *J Immunol* (1993) **150**(2):543–9.
33. Lacroix-Desmazes S, Mouthon L, Kaveri SV, Kazatchkine MD, Weksler ME. Stability of natural self-reactive antibody repertoires during aging. *J Clin Immunol* (1999) **19**(1):26–34. doi:10.1023/A:1020510401233
34. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One* (2013) **8**(4):e60726. doi:10.1371/journal.pone.0060726
35. Lacroix-Desmazes S, Mouthon L, Coutinho A, Kazatchkine MD. Analysis of the natural human IgG antibody repertoire: life-long stability of reactivities towards self antigens contrasts with age-dependent diversification of reactivities against bacterial antigens. *Eur J Immunol* (1995) **25**(9):2598–604. doi:10.1002/eji.1830250929
36. Bachi AL, Suguri VM, Ramos LR, Mariano M, Vaisberg M, Lopes JD. Increased production of autoantibodies and specific antibodies in response to influenza virus vaccination in physically active older individuals. *Results Immunol* (2013) **3**:10–6. doi:10.1016/j.rinim.2013.01.001
37. Covens K, Verbinnen B, Geukens N, Meyts I, Schuit F, Van Lommel L, et al. Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype. *Blood* (2013) **121**(26):5176–83. doi:10.1182/blood-2012-12-471953
38. Tangye SG. To B1 or not to B1: that really is still the question! *Blood* (2013) **121**(26):5109–10. doi:10.1182/blood-2013-05-500074
39. Lobo PI, Schlegel KH, Spencer CE, Okusa MD, Chisholm C, McHedlishvili N, et al. Naturally occurring IgM anti-leukocyte autoantibodies (IgM-ALA) inhibit T cell activation and chemotaxis. *J Immunol* (2008) **180**(3):1780–91. doi:10.4049/jimmunol.180.3.1769
40. Chen ZJ, Wheeler CJ, Shi W, Wu AJ, Yarboro CH, Gallagher M, et al. Polyreactive antigen-binding B cells are the predominant cell type in the newborn B cell repertoire. *Eur J Immunol* (1998) **28**(3):989–94. doi:10.1002/(SICI)1521-4141(199803)28:03<989::AID-IMMU989>3.0.CO;2-1
41. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* (2009) **119**(5):1335–49. doi:10.1172/JCI36800
42. Kroese FG, Butcher EC, Stall AM, Herzenberg LA. A major peritoneal reservoir of precursors for intestinal IgA plasma cells. *Immunol Invest* (1989) **18**(1–4):47–58. doi:10.3109/08820138909112226
43. Murakami M, Honjo T. Involvement of B-1 cells in mucosal immunity and autoimmunity. *Immunol Today* (1995) **16**(11):534–9. doi:10.1016/0167-5699(95)80047-6
44. Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* (2001) **413**(6856):639–43. doi:10.1038/35098100
45. Bowman EP, Kuklin NA, Youngman KR, Lazarus NH, Kunkel EJ, Pan J, et al. The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells. *J Exp Med* (2002) **195**(2):269–75. doi:10.1084/jem.20010670
46. Golby SJ, Spencer J. Where do IgA plasma cells in the gut come from? *Gut* (2002) **51**(2):150–1. doi:10.1136/gut.51.2.150
47. Herzenberg LA. B-1 cells: the lineage question revisited. *Immunol Rev* (2000) **175**(1):9–22. doi:10.1111/j.1600-065X.2000.imr017520.x
48. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* (2002) **20**(1):253–300. doi:10.1146/annurev.immunol.20.100301.064833
49. Rothstein TL. Cutting edge commentary: two B-1 or not to be one. *J Immunol* (2002) **168**(9):4257–61. doi:10.4049/jimmunol.168.9.4257
50. Avrameas S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol Today* (1991) **12**(5):154–9. doi:10.1016/S0167-5699(05)80045-3
51. Kretschmer K, Jungebloud A, Stopkowicz J, Kleinke T, Hoffmann R, Weiss S. The selection of marginal zone B cells differs from that of B-1a cells. *J Immunol* (2003) **171**(12):6495–501. doi:10.4049/jimmunol.171.12.6495
52. Martin F, Kearney JF. Marginal-zone B cells. *Nat Rev Immunol* (2002) **2**(5):323–35. doi:10.1038/nri799
53. Zhou Z, Tzioufas AG, Notkins AL. Properties and function of polyreactive antibodies and polyreactive antigen-binding B cells. *J Autoimmun* (2007) **29**(4):219–28. doi:10.1016/j.jaut.2007.07.015
54. Vittecoq O, Brard F, Jovelin F, Le Loet X, Tron F, Gilbert D. IgM anti-myeloperoxidase antibody-secreting lymphocytes are present in the peripheral repertoire of lupus mice but rarely differentiate into IgG-producing cells. *Clin Exp Immunol* (1999) **118**(1):122–30. doi:10.1046/j.1365-2249.1999.00995.x
55. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) **11**(1):34–46. doi:10.1038/nri2901
56. Gronwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. *Front Immunol* (2012) **3**:66. doi:10.3389/fimmu.2012.00066
57. Baxendale HE, Johnson M, Stephens RC, Yuste J, Klein N, Brown JS, et al. Natural human antibodies to pneumococcus have distinctive molecular characteristics and protect against pneumococcal disease. *Clin Exp Immunol* (2008) **151**(1):51–60. doi:10.1111/j.1365-2249.2007.03535.x

58. Rauch PJ, Chudnovskiy A, Robbins CS, Weber GF, Etzrodt M, Hilgendorf I, et al. Innate response activator B cells protect against microbial sepsis. *Science* (2012) **335**(6068):597–601. doi:10.1126/science.1215173
59. Holodick NE, Tumang JR, Rothstein TL. Immunoglobulin secretion by B1 cells: differential intensity and IRF4-dependence of spontaneous IgM secretion by peritoneal and splenic B1 cells. *Eur J Immunol* (2010) **40**(11):3007–16. doi:10.1002/eji.201040545
60. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. *Nat Immunol* (2006) **7**(3):293–301. doi:10.1038/ni1301
61. Matejuk A, Beardall M, Xu Y, Tian Q, Phillips D, Alabyev B, et al. Exclusion of natural autoantibody-producing B cells from IgG memory B cell compartment during T cell-dependent immune responses. *J Immunol* (2009) **182**(12):7634–43. doi:10.4049/jimmunol.0801562
62. Ishida D, Su L, Tamura A, Katayama Y, Kawai Y, Wang SF, et al. Rap1 signal controls B cell receptor repertoire and generation of self-reactive B1a cells. *Immunity* (2006) **24**(4):417–27. doi:10.1016/j.immuni.2006.02.007
63. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* (1998) **160**(10):4776–87.
64. Lino AC, Mohr E, Demengeot J. Naturally secreted immunoglobulins limit B1 and MZ B-cell numbers through a microbiota-independent mechanism. *Blood* (2013) **122**(2):209–18. doi:10.1182/blood-2012-08-447136
65. Baker N, Ehrenstein MR. Cutting edge: selection of B lymphocyte subsets is regulated by natural IgM. *J Immunol* (2002) **169**(12):6686–90. doi:10.4049/jimmunol.169.12.6686
66. Choi SC, Wang H, Tian L, Murakami Y, Shin DM, Borrego F, et al. Mouse IgM Fc receptor, FCMR, promotes B cell development and modulates antigen-driven immune responses. *J Immunol* (2013) **190**(3):987–96. doi:10.4049/jimmunol.1202227
67. Amezcua Vesely MC, Schwartz M, Bermejo DA, Montes CL, Cautivo KM, Kalergis AM, et al. FcγRIIb and BAFF differentially regulate peritoneal B1 cell survival. *J Immunol* (2012) **188**(10):4792–800. doi:10.4049/jimmunol.1102070
68. Berneman A, Ternynck T, Avrameas S. Natural mouse IgG reacts with self antigens including molecules involved in the immune response. *Eur J Immunol* (1992) **22**(3):625–33. doi:10.1002/eji.1830220303
69. Vargas ME, Watanabe J, Singh SJ, Robinson WH, Barres BA. Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury. *Proc Natl Acad Sci U S A* (2010) **107**(26):11993–8. doi:10.1073/pnas.1001948107
70. Hurez V, Kaveri S, Kazatchkine MD. Expression and control of the natural autoreactive IgG repertoire in normal human serum. *Eur J Immunol* (1993) **23**(4):783–9. doi:10.1002/eji.1830230402
71. Pereira P, Forni L, Larsson E, Cooper M, Heusser C, Coutinho A. Autonomous activation of B and T cells in antigen-free mice. *Eur J Immunol* (1986) **16**(6):685–8. doi:10.1002/eji.1830160616
72. Coutinho A, Kazatchkine MD, Avrameas S. Natural autoantibodies. *Curr Opin Immunol* (1995) **7**(6):812–8. doi:10.1016/0952-7915(95)80053-0
73. Bos NA, Kimura H, Meeuwsen CG, Visser HD, Hazenberg MP, Wostmann BS, et al. Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. *Eur J Immunol* (1989) **19**(12):2335–9. doi:10.1002/eji.1830191223
74. Hamanova M, Chmelikova M, Nentwich I, Thon V, Lokaj J. Anti-gal IgM, IgA and IgG natural antibodies in childhood. *Immunol Lett* (2015) **164**(1):40–3. doi:10.1016/j.imlet.2015.02.001
75. Cohen IR, Young DB. Autoimmunity, microbial immunity and the immunological homunculus. *Immunol Today* (1991) **12**(4):105–10. doi:10.1016/0167-5699(91)90093-9
76. Watts RA, Isenberg DA. Autoantibodies and antibacterial antibodies: from both sides now. *Ann Rheum Dis* (1990) **49**(12):961–5. doi:10.1136/ard.49.12.961
77. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science* (2003) **301**(5638):1374–7. doi:10.1126/science.1086907
78. Xu PC, Cui Z, Chen M, Hellmark T, Zhao MH. Comparison of characteristics of natural autoantibodies against myeloperoxidase and anti-myeloperoxidase autoantibodies from patients with microscopic polyangiitis. *Rheumatology (Oxford)* (2011) **50**(7):1236–43. doi:10.1093/rheumatology/ker085
79. Reap EA, Sobel ES, Jennette JC, Cohen PL, Eisenberg RA. Conventional B cells, not B1 cells, are the source of autoantibodies in chronic graft-versus-host disease. *J Immunol* (1993) **151**(12):7316–23.
80. Wofsy D, Ledbetter JA, Hendler PL, Seaman WE. Treatment of murine lupus with monoclonal anti-T cell antibody. *J Immunol* (1985) **134**(2):852–7.
81. Jevnikar AM, Grusby MJ, Glimcher LH. Prevention of nephritis in major histocompatibility complex class II-deficient MRL-lpr mice. *J Exp Med* (1994) **179**(4):1137–43. doi:10.1084/jem.179.4.1137
82. Steinberg AD, Roths JB, Murphy ED, Steinberg RT, Raveche ES. Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. *J Immunol* (1980) **125**(2):871–3.
83. Kaveri S, Dietrich G, Hurez V, Kazatchkine M. Intravenous immunoglobulins (IVIg) in the treatment of autoimmune diseases. *Clin Exp Immunol* (1991) **86**(2):192–8. doi:10.1111/j.1365-2249.1991.tb05794.x
84. Panda S, Zhang J, Tan NS, Ho B, Ding JL. Natural IgG antibodies provide innate protection against ficolin-opsonized bacteria. *EMBO J* (2013) **32**(22):2905–19. doi:10.1038/emboj.2013.199
85. Abdou NI, Wall H, Lindsley HB, Halsey JF, Suzuki T. Network theory in autoimmunity. in vitro suppression of serum anti-DNA antibody binding to DNA by anti-idiotypic antibody in systemic lupus erythematosus. *J Clin Invest* (1981) **67**(5):1297–304. doi:10.1172/JCI110158
86. Dwyer DS, Bradley RJ, Urquhart CK, Kearney JF. Naturally occurring anti-idiotypic antibodies in myasthenia gravis patients. *Nature* (1983) **301**(5901):611–4.
87. Cui Z, Zhao M, Segelmark M, Hellmark T. Natural autoantibodies to myeloperoxidase, proteinase 3, and the glomerular basement membrane are present in normal individuals. *Kidney Int* (2010) **78**(6):590–7. doi:10.1038/ki.2010.198
88. Ohnishi K, Ebling FM, Mitchell B, Singh RR, Hahn BH, Tsao BP. Comparison of pathogenic and non-pathogenic murine antibodies to DNA: antigen binding and structural characteristics. *Int Immunol* (1994) **6**(6):817–30. doi:10.1093/intimm/6.6.817
89. Ehrenstein MR, Katz DR, Griffiths MH, Papadaki L, Winkler TH, Kalden JR, et al. Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. *Kidney Int* (1995) **48**(3):705–11. doi:10.1038/ki.1995.341
90. Tsao BP, Ohnishi K, Cheroutre H, Mitchell B, Teitell M, Mixter P, et al. Failed self-tolerance and autoimmunity in IgG anti-DNA transgenic mice. *J Immunol* (1992) **149**(1):350–8.
91. Radic MZ, Ibrahim SM, Rauch J, Camper SA, Weigert M. Constitutive secretion of transgene-encoded IgG2b autoantibodies leads to symptoms of autoimmune disease. *J Immunol* (1995) **155**(6):3213–22.
92. Murakami M, Tsubata T, Okamoto M, Shimizu A, Kumagai S, Imura H, et al. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature* (1992) **357**:77–80. doi:10.1038/357077a0
93. Takahashi T, Strober S. Natural killer T cells and innate immune B cells from lupus-prone NZB/W mice interact to generate IgM and IgG autoantibodies. *Eur J Immunol* (2008) **38**(1):156–65. doi:10.1002/eji.200737656
94. Murakami M, Yoshioka H, Shirai T, Tsubata T, Honjo T. Prevention of autoimmune symptoms in autoimmune-prone mice by elimination of B-1 cells. *Int Immunol* (1995) **7**(5):877–82. doi:10.1093/intimm/7.5.877
95. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, et al. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med* (2005) **201**(5):703–11. doi:10.1084/jem.20042251
96. Samuels J, Ng Y, Paget D, Meffre E. Impaired early B-cell tolerance in patients with rheumatoid arthritis. *Arthritis Res Ther* (2005) **7**(Suppl 1):78. doi:10.1186/ar1599
97. Lesley R, Xu Y, Kalled SL, Hess DM, Schwab SR, Shu HB, et al. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* (2004) **20**(4):441–53. doi:10.1016/S1074-7613(04)00079-2
98. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. *J Clin Invest* (2002) **109**(1):59–68. doi:10.1172/JCI0214121
99. Guilbert B, Mahana W, Gilbert M, Mazie JC, Avrameas S. Presence of natural autoantibodies in hyperimmunized mice. *Immunology* (1985) **56**(3):401–8.

100. Naparstek Y, Andre-Schwartz J, Manser T, Wysocki LJ, Breitman L, Stollar BD, et al. A single germline VH gene segment of normal A/J mice encodes autoantibodies characteristic of systemic lupus erythematosus. *J Exp Med* (1986) **164**(2):614–26. doi:10.1084/jem.164.2.614
101. Cunningham MW, Hall NK, Krisher KK, Spanier AM. A study of anti-group A streptococcal monoclonal antibodies cross-reactive with myosin. *J Immunol* (1986) **136**(1):293–8.
102. Lewis M, Botto M. Complement deficiencies in humans and animals: links to autoimmunity. *Autoimmunity* (2006) **39**(5):367–78. doi:10.1080/08916930600739233
103. Li F, Smith P, Ravetch JV. Inhibitory Fcγ receptor is required for the maintenance of tolerance through distinct mechanisms. *J Immunol* (2014) **192**(7):3021–8. doi:10.4049/jimmunol.1302934
104. Baerenwaldt A, Lux A, Danzer H, Spriewald BM, Ullrich E, Heidkamp G, et al. Fcγ receptor IIB (FcγRIIB) maintains humoral tolerance in the human immune system in vivo. *Proc Natl Acad Sci U S A* (2011) **108**(46):18772–7. doi:10.1073/pnas.1111810108
105. Tillman DM, Jou NT, Hill RJ, Marion TN. Both IgM and IgG anti-DNA antibodies are the products of clonally selective B cell stimulation in (NZB x NZW)F1 mice. *J Exp Med* (1992) **176**(3):761–79. doi:10.1084/jem.176.3.761
106. Ikematsu H, Kasaian MT, Schettino EW, Casali P. Structural analysis of the VH-D-JH segments of human polyreactive IgG mAb. Evidence for somatic selection. *J Immunol* (1993) **151**(7):3604–16.
107. Werwitzke S, Trick D, Kamino K, Matthias T, Kniesch K, Schlegelberger B, et al. Inhibition of lupus disease by anti-double-stranded DNA antibodies of the IgM isotype in the (NZB x NZW)F1 mouse. *Arthritis Rheum* (2005) **52**(11):3629–38. doi:10.1002/art.21379
108. Heidenreich F, Jovin T. Synthesis of anti-acetylcholine receptor antibodies by CD5-B cells from peripheral blood of myasthenia gravis patients. *J Neurol* (1996) **243**(1):57–62. doi:10.1007/BF00878532
109. Lubetzki Korn I, Abramsky O. Myasthenia gravis following viral infection. *Eur Neurol* (1981) **20**(6):435–9. doi:10.1159/000115275
110. Hohlfeld R, Kalies I, Kohleisen B, Heininger K, Conti-Tronconi B, Toyka KV. Myasthenia gravis: stimulation of antireceptor autoantibodies by autoreactive T cell lines. *Neurology* (1986) **36**(5):618–21. doi:10.1212/WNL.36.5.618
111. Wang ZY, Okita DK, Howard J Jr, Conti-Fine BM. T-cell recognition of muscle acetylcholine receptor subunits in generalized and ocular myasthenia gravis. *Neurology* (1998) **50**(4):1045–54. doi:10.1212/WNL.50.4.1045
112. Jennette JC, Falk RJ. The rise and fall of horror autotoxicus and forbidden clones. *Kidney Int* (2010) **78**(6):533–5. doi:10.1038/ki.2010.237
113. Jennette JC, Hipp CG. Clq nephropathy: a distinct pathologic entity usually causing nephrotic syndrome. *Am J Kidney Dis* (1985) **6**(2):103–10. doi:10.1016/S0272-6386(85)80150-5
114. Sharman A, Furness P, Feehally J. Distinguishing C1q nephropathy from lupus nephritis. *Nephrol Dial Transplant* (2004) **19**(6):1420–6. doi:10.1093/ndt/gfh139
115. Jacob N, Stohl W. Autoantibody-dependent and autoantibody-independent roles for B cells in systemic lupus erythematosus: past, present, and future. *Autoimmunity* (2010) **43**(1):84–97. doi:10.3109/08916930903374600
116. Waters ST, McDuffie M, Bagavant H, Deshmukh US, Gaskin F, Jiang C, et al. Breaking tolerance to double stranded DNA, nucleosome, and other nuclear antigens is not required for the pathogenesis of lupus glomerulonephritis. *J Exp Med* (2004) **199**(2):255–64. doi:10.1084/jem.20031519
117. Jiang C, Foley J, Clayton N, Kissling G, Jokinen M, Herbert R, et al. Abrogation of lupus nephritis in activation-induced deaminase-deficient MRL/lpr mice. *J Immunol* (2007) **178**(11):7422–31. doi:10.4049/jimmunol.178.11.7422
118. Ma J, Xu J, Madaio MP, Peng Q, Zhang J, Grewal IS, et al. Autoimmune lpr/lpr mice deficient in CD40 ligand: spontaneous ig class switching with dichotomy of autoantibody responses. *J Immunol* (1996) **157**(1):417–26.
119. Ehrenstein MR, Cook HT, Neuberger MS. Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. *J Exp Med* (2000) **191**(7):1253–8. doi:10.1084/jem.191.7.1253
120. Boes M, Schmidt T, Linkemann K, Beaudette BC, Marshak-Rothstein A, Chen J. Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proc Natl Acad Sci U S A* (2000) **97**(3):1184–9. doi:10.1073/pnas.97.3.1184
121. Bolland S, Ravetch JV. Spontaneous autoimmune disease in FcγRIIB-deficient mice results from strain-specific epistasis. *Immunity* (2000) **13**(2):277–85. doi:10.1016/S1074-7613(00)00027-3
122. Côrte-Real J, Rodo J, Almeida P, Garcia J, Coutinho A, Demengeot J, et al. Irf4 is a positional and functional candidate gene for the control of serum IgM levels in the mouse. *Genes Immun* (2009) **10**(1):93–9. doi:10.1038/gene.2008.73
123. Klein-Schneegans A, Kuntz L, Fonteneau P, Loor F. Serum concentrations of IgM, IgG1, IgG2b, IgG3 and IgA in C57BL6 mice and their congenics at the lpr (lymphoproliferation) locus. *J Autoimmun* (1989) **2**(6):869–75. doi:10.1016/0896-8411(89)90013-9
124. Kalpaktsoglou PK, Hong R, Good RA. The five classes of immunoglobulins in normal C3H and BALB/c mice. *Immunology* (1973) **24**(2):303–14.
125. Witte T. IgM antibodies against dsDNA in SLE. *Clin Rev Allergy Immunol* (2008) **34**(3):345–7. doi:10.1007/s12016-007-8046-x
126. Mannoor K, Matejuk A, Xu Y, Beardall M, Chen C. Expression of natural autoantibodies in MRL-lpr mice protects from lupus nephritis and improves survival. *J Immunol* (2012) **188**(8):3628–38. doi:10.4049/jimmunol.1102859
127. Chen Y, Khanna S, Goodyear CS, Park YB, Raz E, Thiel S, et al. Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol* (2009) **183**(2):1346–59. doi:10.4049/jimmunol.0900948
128. Lobo PI, Brayman KL, Okusa MD. Natural IgM anti-leucocyte autoantibodies (IgM-ALA) regulate inflammation induced by innate and adaptive immune mechanisms. *J Clin Immunol* (2014) **34**(Suppl 1):S22–9. doi:10.1007/s10875-014-0027-2
129. Hurez V, Kazatchkine MD, Vassilev T, Ramanathan S, Pashov A, Basuyaux B, et al. Pooled normal human polyspecific IgM contains neutralizing anti-idiotypes to IgG autoantibodies of autoimmune patients and protects from experimental autoimmune disease. *Blood* (1997) **90**(10):4004–13.
130. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* (2009) **182**(10):6031–43. doi:10.4049/jimmunol.0804191
131. Lobo PI. Nature of autolymphocytotoxins present in renal hemodialysis patients: their possible role in controlling alloantibody formation. *Transplantation* (1981) **32**(3):233–7. doi:10.1097/00007890-198109000-00010
132. Honjo K, Kubagawa Y, Jones DM, Dizon B, Zhu Z, Ohno H, et al. Altered Ig levels and antibody responses in mice deficient for the Fc receptor for IgM (FcmuR). *Proc Natl Acad Sci U S A* (2012) **109**(39):15882–7. doi:10.1073/pnas.1206567109
133. Hutchings PR, Varey AM, Cooke A. Immunological defects in SJL mice. *Immunology* (1986) **59**(3):445–50.
134. Li QZ, Xie C, Wu T, Mackay M, Aranow C, Putterman C, et al. Identification of autoantibody clusters that best predict lupus disease activity using glomerular proteome arrays. *J Clin Invest* (2005) **115**(12):3428–39. doi:10.1172/JCI23587
135. Jost SA, Tseng LC, Matthews LA, Vasquez R, Zhang S, Yancey KB, et al. IgG, IgM, and IgA antinuclear antibodies in discoid and systemic lupus erythematosus patients. *ScientificWorldJournal* (2014) **2014**:171028. doi:10.1155/2014/171028
136. Vassilev T, Yamamoto M, Aissaoui A, Bonnin E, Berrih-Aknin S, Kazatchkine MD, et al. Normal human immunoglobulin suppresses experimental myasthenia gravis in SCID mice. *Eur J Immunol* (1999) **29**(8):2436–42. doi:10.1002/(SICI)1521-4141(199908)29:08<2436::AID-IMMU2436>3.0.CO;2-9
137. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* (2003) **349**(16):1526–33. doi:10.1056/NEJMoa021933
138. Lobo PI, Bajwa A, Schlegel KH, Vengal J, Lee SJ, Huang L, et al. Natural IgM anti-leukocyte autoantibodies attenuate excess inflammation mediated by innate and adaptive immune mechanisms involving Th-17. *J Immunol* (2012) **188**(4):1675–85. doi:10.4049/jimmunol.1101762
139. Gobet R, Cerny A, Ruedi E, Hengartner H, Zinkernagel RM. The role of antibodies in natural and acquired resistance of mice to vesicular stomatitis virus. *Exp Cell Biol* (1988) **56**(4):175–80.
140. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* (1999) **286**(5447):2156–9. doi:10.1126/science.286.5447.2156
141. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol* (2010) **10**(11):778–86. doi:10.1038/nri2849

142. Harnett W, Harnett MM. Phosphorylcholine: friend or foe of the immune system? *Immunol Today* (1999) **20**(3):125–9. doi:10.1016/S0167-5699(98)01419-4
143. Tomasz A. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in pneumococcus. *Science* (1967) **157**(3789):694–7. doi:10.1126/science.157.3789.694
144. Lobo PI, Schlegel KH, Yuan W, Townsend GC, White JA. Inhibition of HIV-1 infectivity through an innate mechanism involving naturally occurring IgM anti-leukocyte autoantibodies. *J Immunol* (2008) **180**(3):1769–79. doi:10.4049/jimmunol.180.3.1769
145. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol* (2007) **7**(12):964–74. doi:10.1038/nri2214
146. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* (2000) **105**(12):1731–40. doi:10.1172/JCI8472
147. Gray M, Miles K, Salter D, Gray D, Savill J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* (2007) **104**(35):14080–5. doi:10.1073/pnas.0700326104
148. Kearney JF. Immune recognition of OxLDL in atherosclerosis. *J Clin Invest* (2000) **105**(12):1683–5. doi:10.1172/JCI10426
149. Kim SJ, Gershov D, Ma X, Brot N, Elkon KB. I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. *J Exp Med* (2002) **196**(5):655–65. doi:10.1084/jem.20020542
150. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* (2003) **9**(6):736–43. doi:10.1038/nm876
151. Horkko S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, et al. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *J Clin Invest* (1999) **103**(1):117–28. doi:10.1172/JCI4533
152. Bhatia VK, Yun S, Leung V, Grimsditch DC, Benson GM, Botto MB, et al. Complement C1q reduces early atherosclerosis in low-density lipoprotein receptor-deficient mice. *Am J Pathol* (2007) **170**(1):416–26. doi:10.2353/ajpath.2007.060406
153. Fraser DA, Tenner AJ. Innate immune proteins C1q and mannan-binding lectin enhance clearance of atherogenic lipoproteins by human monocytes and macrophages. *J Immunol* (2010) **185**(7):3932–9. doi:10.4049/jimmunol.1002080
154. Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M, Haskard DO. Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* (2009) **120**(5):417–26. doi:10.1161/CIRCULATIONAHA.109.868158
155. Walport MJ, Davies KA, Botto M. C1q and systemic lupus erythematosus. *Immunobiology* (1998) **199**(2):265–85. doi:10.1016/S0171-2985(98)80032-6
156. Ogden CA, Kowalewski R, Peng Y, Montenegro V, Elkon KB. IGM is required for efficient complement mediated phagocytosis of apoptotic cells in vivo. *Autoimmunity* (2005) **38**(4):259–64. doi:10.1080/08916930500124452
157. Terasaki PI, Mottironi VD, Barnett EV. Cytotoxins in disease. *N Engl J Med* (1970) **283**(14):724–8. doi:10.1056/NEJM197010012831403
158. Lobo PI, Schlegel KH, Vengal J, Okusa MD, Pei H. Naturally occurring IgM anti-leukocyte autoantibodies inhibit T-cell activation and chemotaxis. *J Clin Immunol* (2010) **30**(1):S31–6. doi:10.1007/s10875-010-9412-7
159. Winfield JB, Winchester RJ, Wernet P, Fu SM, Kunkel HG. Nature of cold-reactive antibodies to lymphocyte surface determinants in systemic lupus erythematosus. *Arthritis Rheum* (1975) **18**(1):1–8. doi:10.1002/art.1780180101
160. Ouchida R, Mori H, Hase K, Takatsu H, Kurosaki T, Tokuhisa T, et al. Critical role of the IgM Fc receptor in IgM homeostasis, B-cell survival, and humoral immune responses. *Proc Natl Acad Sci U S A* (2012) **109**(40):E2699–706. doi:10.1073/pnas.1210706109
161. Lobo PI, Sturgill BC, Bolton WK. Cold-reactive alloantibodies and allograft malfunction occurring immediately posttransplant. *Transplantation* (1984) **37**(1):76–80. doi:10.1097/00007890-198401000-00021
162. Sturgill B, Lobo P, Bolton W. Cold-reacting IgM antibody-induced renal allograft failure. *Nephron* (1984) **36**(2):125–7. doi:10.1159/000183131
163. Zhang M, Austen WG Jr, Chiu I, Alicot EM, Hung R, Ma M, et al. Identification of a specific self-reactive IgM antibody that initiates intestinal ischemia/reperfusion injury. *Proc Natl Acad Sci U S A* (2004) **101**(11):3886–91. doi:10.1073/pnas.0400347101
164. Betapudi V. Life without double-headed non-muscle myosin II motor proteins. *Front Chem* (2014) **2**:45. doi:10.3389/fchem.2014.00045
165. Austen WG, Zhang M, Chan R, Friend D, Hechtman HB, Carroll MC, et al. Murine hindlimb reperfusion injury can be initiated by a self-reactive monoclonal IgM. *Surgery* (2004) **136**(2):401–6. doi:10.1016/j.surg.2004.05.016
166. Zhang M, Michael LH, Grosjean SA, Kelly RA, Carroll MC, Entman ML. The role of natural IgM in myocardial ischemia-reperfusion injury. *J Mol Cell Cardiol* (2006) **41**(1):62–7. doi:10.1016/j.yjmcc.2006.02.006
167. Arrondel C, Vodovar N, Knebelmann B, Grunfeld JP, Gubler MC, Antignac C, et al. Expression of the nonmuscle myosin heavy chain IIA in the human kidney and screening for MYH9 mutations in Epstein and Fechtner syndromes. *J Am Soc Nephrol* (2002) **13**(1):65–74.
168. Renner B, Strassheim D, Amura CR, Kulik L, Ljubanovic D, Glogowska MJ, et al. B cell subsets contribute to renal injury and renal protection after ischemia/reperfusion. *J Immunol* (2010) **185**(7):4393–400. doi:10.4049/jimmunol.0903239
169. Li L, Huang L, Sung SS, Lobo PI, Brown MG, Gregg RK, et al. NKT cell activation mediates neutrophil IFN-gamma production and renal ischemia-reperfusion injury. *J Immunol* (2007) **178**(9):5899–911. doi:10.4049/jimmunol.178.9.5899
170. Burne-Taney MJ, Yokota-Ikeda N, Rabb H. Effects of combined T- and B-cell deficiency on murine ischemia reperfusion injury. *Am J Transplant* (2005) **5**(6):1186–93. doi:10.1111/j.1600-6143.2005.00815.x
171. Gigliotti JC, Huang L, Ye H, Bajwa A, Chattrabhuti K, Lee S, et al. Ultrasound prevents renal ischemia-reperfusion injury by stimulating the splenic cholinergic anti-inflammatory pathway. *J Am Soc Nephrol* (2013) **24**(9):1451–60. doi:10.1681/ASN.2013010084
172. Park P, Haas M, Cunningham PN, Bao L, Alexander JJ, Quigg RJ. Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes. *Am J Physiol Renal Physiol* (2002) **282**(2):F352–7. doi:10.1152/ajprenal.00160.2001
173. Gorevic PD. Rheumatoid factor, complement, and mixed cryoglobulinemia. *Clin Dev Immunol* (2012) **2012**:6. doi:10.1155/2012/439018
174. Charles ED, Dustin LB. Hepatitis C virus-induced cryoglobulinemia. *Kidney Int* (2009) **76**(8):818–24. doi:10.1038/ki.2009.247
175. Kubagawa H, Oka S, Kubagawa Y, Torii I, Takayama E, Kang D, et al. Identity of the elusive IgM Fc receptor (FcμR) in humans. *J Exp Med* (2009) **206**(12):2779–93. doi:10.1084/jem.20091107
176. Marchalonis JJ, Schluter SF, Wang E, Dehghanpisheh K, Lake D, Yocum DE, et al. Synthetic autoantigens of immunoglobulins and T-cell receptors: their recognition in aging, infection, and autoimmunity. *Proc Soc Exp Biol Med* (1994) **207**(2):129–47. doi:10.3181/00379727-207-43801
177. Vassilev T, Mihaylova N, Voynova E, Nikolova M, Kazatchkine M, Kaveri S. IgM-enriched human intravenous immunoglobulin suppresses T lymphocyte functions in vitro and delays the activation of T lymphocytes in hu-SCID mice. *Clin Exp Immunol* (2006) **145**(1):108–15. doi:10.1111/j.1365-2249.2006.03098.x
178. Kinsey GR, Sharma R, Huang L, Li L, Vergis AL, Ye H, et al. Regulatory T cells suppress innate immunity in kidney ischemia-reperfusion injury. *J Am Soc Nephrol* (2009) **20**(8):1744–53. doi:10.1681/ASN.2008111160
179. Kendall PL, Woodward EJ, Hulbert C, Thomas JW. Peritoneal B cells govern the outcome of diabetes in non-obese diabetic mice. *Eur J Immunol* (2004) **34**(9):2387–95. doi:10.1002/eji.200324744
180. Ryan GA, Wang CJ, Chamberlain JL, Attridge K, Schmidt EM, Kenefeck R, et al. B1 cells promote pancreas infiltration by autoreactive T cells. *J Immunol* (2010) **185**(5):2800–7. doi:10.4049/jimmunol.1000856
181. Xiu Y, Wong CP, Bouaziz JD, Hamaguchi Y, Wang Y, Pop SM, et al. B lymphocyte depletion by CD20 monoclonal antibody prevents diabetes in nonobese diabetic mice despite isotype-specific differences in Fc gamma R effector functions. *J Immunol* (2008) **180**(5):2863–75. doi:10.4049/jimmunol.180.5.2863
182. Chhabra P, Schlegel K, Okusa MD, Lobo PI, Brayman KL. Naturally occurring immunoglobulin M (nIgM) autoantibodies prevent autoimmune diabetes and mitigate inflammation after transplantation. *Ann Surg* (2012) **256**(4):634–41. doi:10.1097/SLA.0b013e31826b4ba9

183. Andersson Å, Forsgren S, Söderström Å, Holmberg D. Monoclonal, natural antibodies prevent development of diabetes in the non-obese diabetic (NOD) mouse.*J Autoimmun*(1991)**4**(5):733–42.doi:10.1016/0896-8411(91)90169-D
184. Andersson A, Ekstrand-Hammarstrom B, Eriksson B, Overmo C, Holmberg D. Neonatal treatment with monoclonal natural antibodies restores a normal pattern of VH gene utilization in the non-obese diabetic mouse. *Int Immunol* (1994) **6**(4):623–30. doi:10.1093/intimm/6.4.623
185. Symmons DP. Frequency of lupus in people of African origin. *Lupus* (1995) **4**(3):176–8. doi:10.1177/096120339500400303
186. Lobo PI. The first reported case of sarcoidosis in an East African. *East Afr Med J* (1972) **49**(1):37–49.
187. Jacyk WK. Sarcoidosis in the West African. A report of eight Nigerian patients with cutaneous lesions. *Trop Geogr Med* (1984) **36**(3):231–6.
188. Lobo PI, Suratt PM. Studies on the autoantibody to lymphocytes in sarcoidosis. *J Clin Lab Immunol* (1979) **1**(4):283–8.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 14 November 2016
doi: 10.3389/fimmu.2016.00493

Pneumococcal Polysaccharide Vaccination Elicits IgG Anti-A/B Blood Group Antibodies in Healthy Individuals and Patients with Type I Diabetes Mellitus

Wendelin Wolfram[1†], Kai M. T. Sauerwein[2†], Christoph J. Binder[3], Nicole Eibl-Musil[4], Hermann M. Wolf[2,5†] and Michael B. Fischer[1,6†]*

[1] Clinic for Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria, [2] Immunology Outpatient Clinic, Vienna, Austria, [3] Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria, [4] Rudolfstiftung Hospital of the City of Vienna, Vienna, Austria, [5] Sigmund Freud Private University – Medical School, Vienna, Austria, [6] Department for Health Science and Biomedicine, Danube University Krems, Krems, Austria

OPEN ACCESS

Edited by:
Ana María Hernández, Center of Molecular Immunology, Cuba

Reviewed by:
Jean Van Den Elsen, University of Bath, UK
Thomas Vorup-Jensen, Aarhus University, Denmark

***Correspondence:**
Michael B. Fischer
michael.fischer@donau-uni.ac.at

[†]Wendelin Wolfram and Kai M. T. Sauerwein have equally contributed as co-first authors, Hermann M. Wolf and Michael B. Fischer have equally contributed as co-last authors to this manuscript.

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 22 July 2016
Accepted: 25 October 2016
Published: 14 November 2016

Citation:
Wolfram W, Sauerwein KMT, Binder CJ, Eibl-Musil N, Wolf HM and Fischer MB (2016) Pneumococcal Polysaccharide Vaccination Elicits IgG Anti-A/B Blood Group Antibodies in Healthy Individuals and Patients with Type I Diabetes Mellitus. Front. Immunol. 7:493. doi: 10.3389/fimmu.2016.00493

Hypothesis: Blood group antibodies are natural antibodies that develop early in life in response to cross-reactive environmental antigens in the absence of antigen encounter. Even later in life structural similarities in saccharide composition between environmental antigens such as bacterial polysaccharides and blood group A/B antigens could lead to changes in serum levels, IgM/IgG isotype, and affinity maturation of blood group anti-A/B antibodies. We addressed the question whether immunization with pneumococcal polysaccharide (PnP) vaccine Pneumo 23 Vaccine "Pasteur Merieux" (Pn23) could have such an effect in patients with type I diabetes mellitus (DM I), an autoimmune disease where an aberrant immune response to microbial antigens likely plays a role.

Methods: Anti-PnP IgM and IgG responses were determined by ELISA, and the DiaMed-ID Micro Typing System was used to screen anti-A/B antibody titer before and after Pn23 immunization in 28 healthy individuals and 16 patients with DM I. In addition, surface plasmon resonance (SPR) technology using the Biacore® device and a synthetic blood group A/B trisaccharide as the antigen was applied to investigate IgM and IgG anti-A/B antibodies and to measure antibody binding dynamics.

Results: All healthy individuals and DM I patients responded with anti-PnP IgM and IgG antibody production 4–6 weeks after Pn23 immunization, while no increase in blood group anti-A/B antibody titer was observed when measured by the DiaMed-ID Micro Typing System. Interestingly, isotype-specific testing by SPR technology revealed an increase in blood group anti-A/B IgG, but not IgM, following Pn23 immunization in both patients and controls. No change in binding characteristics of blood group anti-A/B antibodies could be detected following Pn23 vaccination, supporting the assumption of an increase in IgG antibody titer with no or very little affinity maturation.

Conclusion: The study provides evidence for epitope sharing between pneumococcal polysaccharides and blood group ABO antigens, which leads to a booster of blood group anti-A/B antibodies of the IgG isotype after Pn23 immunization in healthy individuals. Manifest autoimmunity such as present in DM I patients has no additional effect on the cross-reactive antibody response against pneumococcal polysaccharides and blood group antigens.

Keywords: pneumococcal polysaccharide vaccine, 23-valent pneumococcal polysaccharide vaccine, anti-blood group A/B antibodies, surface plasmon resonance, isoagglutinins, natural antibodies

INTRODUCTION

Natural antibodies are produced in the absence of overt external antigenic stimulation early in life in all healthy individuals with a functional immune system, presumably as a product of germline gene segment assembly (1). Therefore, they show low affinity to many microbial pathogens and certain cross-reactivity, even to some self-antigens (1–4). Antibodies to carbohydrate epitopes share certain characteristics with natural antibodies, e.g., the absence of extensive class switching and affinity maturation due to absent T-cell help (5). Isoagglutinins, i.e., antibodies against carbohydrate epitopes that form the AB0 antigens on red blood cells (RBCs), are considered prototypic natural antibodies that were shown to be compromised in patients with immunodeficiency (1, 4, 6). Their appearance can be explained by inapparent stimulation particularly from intestinal bacteria because antigens cross-reactive with blood group polysaccharides are widely distributed in the environment (1, 7, 8). Natural anti-ABO antibodies have long been considered to be predominantly of the IgM isotype, but antibodies of the IgG isotype are also produced, especially later in life, e.g., after alloimmunization in pregnancy or ABO-incompatible blood transfusions (1). In the case of ABO blood groups, individuals with blood group A develop an immune response to B-like antigens of microorganisms and environmental antigens like plants containing these epitopes (1, 9). B blood group individuals develop anti-A antibodies, O blood group individuals produce both anti-A and anti-B antibodies, while AB blood group subjects have neither anti-A nor anti-B because they express both antigens on their RBCs.

The human ABO blood group antigens are oligosaccharide moieties formed on precursor backbones by glycosyltransferases (4, 9, 10). Blood group O individuals have carbohydrate structures named H-antigen terminated in the sequence alpha-Fuc(1,2)Gal. The blood group A antigen is then formed from the H-antigen by an *N*-acetylgalactosaminyltransferase that uses a UDP-*N*-acetylgalactosamine (GalNAc) donor, and the blood group B antigen is formed by a galactosyltransferase that uses a UDP-galactose (Gal) donor to convert the H-antigen (4). The human A and B blood group antigens differ from each other only in the substitution of an acetamino for a hydroxyl group on the terminal saccharide residue, and this substitution can be differentially recognized by specific antibodies (1, 4, 11, 12). Interindividual variations in the titer of anti-blood group ABO antibodies exist, representing modulation of the production of these natural antibodies in response to cross-reactive environmental antigens, e.g., through immunization (1). B cells could thus recognize shared carbohydrate epitopes due to structural similarities in saccharide composition, e.g., between microbial polysaccharides (PnPs) and blood group A/B antigens, which could also have an effect on blood group anti-A/B specific antibody production after PnP-immunization. Previous findings indicate shared epitopes between blood group oligosaccharides and pneumococcal capsular polysaccharides, as antibodies cross-reacting with human erythrocytes and pneumococcal antigens have been described (7). Furthermore, it was shown that cross-reactive carbohydrate determinants (CCDs) contained in blood group antigens and microbial polysaccharides can be targeted by immunoglobulins of different isotypes, in particular by IgE, but also by IgG antibodies (13).

In addition to their role as a first line of defense against infection, natural antibodies have been shown to play an important role in the initiation of autoimmunity (2, 8, 14–16). Natural antibodies reacting with self-antigens were initially considered to represent breakdown of tolerance, thus functioning as a template for the generation of pathogenic autoantibodies produced through a process of clonal selection, somatic hypermutation, and class switching driven by antigen (14). More recently, these antibodies have been attributed an additional role in the prevention of autoimmune disease, e.g., by inhibiting activation of the adaptive immune system by molecules released from apoptotic cells that could facilitate autoimmune events (15, 16). Formation of natural antibodies with high cross-reactivity could be regulated differently in healthy individuals as compared to individuals with an autoimmune disease such as type 1 diabetes mellitus (DM I) known to present with abnormalities in antibody formation (17). In addition to high-risk HLA genes, environmental factors are thought to play an important role in the pathogenesis of DM I, such as pancreatic virus infection, T cell-mediated and autoantibody-mediated molecular mimicry, and bystander activation of autoreactive lymphocytes by proinflammatory cytokines derived from dendritic cells activated by infection-related stimuli through innate pattern recognition receptors such as TLR (18–21). A single causative infectious agent for DM I autoimmunity has not been found, pointing toward the possibility that different infectious agents are capable of non-specifically enhancing the likelihood of autoimmunity. In addition to virus infection (19, 20), bacterial infection might play a role in the development of DM I, as antibodies against mycobacterial and proinsulin epitopes (22) as well as beta cell antigens (23) show

cross-reactivity in children with new onset DM I. While human IDDM autoantigens are typically T-dependent protein antigens, microbial polysaccharides have been shown to modulate the autoimmune response leading to DM1 (24). Innate signaling *via* TLR and MyD88, pathways critical for T cell-independent (TI) immune defense (25), can reverse anergy in autoreactive B cells, suggesting that environmental factors associated with bacterial infection and inflammation may alter tolerance (26). Concerns that vaccination might lead to the development of autoimmune disease such as DM I have been raised (27), which could also relate to polysaccharide vaccines, as molecular mimicry in bacterial polysaccharide components is capable of inducing autoreactive antibodies, e.g., against some blood group antigens or neuronal gangliosides (28). In the present study, we investigated whether vaccination with Pneumo 23 Vaccine "Pasteur Merieux" (Pn23) has an effect on blood group ABO antibodies in healthy individuals and in patients with DM I. To screen for blood group anti-A/B antibodies, we used the commercially available DiaMed-ID Micro Typing System based on erythrocyte agglutination (6). To measure blood group-specific anti-A/B antibodies in healthy individuals and in patients with DM I in an isotype-specific manner, we used surface plasmon resonance (SPR) technology and synthetic A/B trisaccharides bound *via* amine-coupling to the CM5 chip (6, 11, 12). SPR using blood group-specific A/B synthetic trisaccharides is the ideal technology to investigate a potential IgM to IgG isotype shift and to investigate changes in binding characteristics of the relevant blood group anti-A/B antibodies under near to physiological conditions in real time.

MATERIALS AND METHODS

Vaccination of Healthy Individuals and Patients with Type I DM and Measurement of Anti-PnPs Antibodies

During a clinical study published previously (17), healthy individuals ($n = 39$) and patients with type I DM ($n = 20$) were immunized with the PnPs vaccine Pn23 (a 23-valent vaccine containing pneumococcal capsular polysaccharide serotypes 1, 2, 3, 4, 5, 6B, 7, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F, Pasteur Merieux Connaught, Lyon, France) after informed consent was obtained (17, 29). Type I DM patients were, on average, 16 ± 7.4 years (SD) at the onset of disease, and at that time they tested positive for GAD autoantibodies. Age at initiation of the vaccination study was 31.7 ± 8.8 years (SD). For a detailed description of patients and controls, see Ref. (17). Blood samples were drawn 4–6 weeks after vaccination, the serum was immediately separated from the cellular blood component, dispensed into 500 µl aliquots, and stored until analysis in a freezer at −20°C. For this follow-up study, serum aliquots stored at −20°C were still available from 28 healthy controls and 16 DM I patients; selection of this subset of the previous study was only because no more serum was available from the other controls and DM I patients of the original study. Anti-PnPs antibodies were determined by a home-made ELISA detecting all serotypes as previously described (17, 30).

Determination of Anti-A/B Antibody Titers by the DiaMed-ID Micro Typing System

Titers of blood group anti-A and anti-B antibodies were determined by the DiaMed-ID Micro Typing System (Bio-Rad Lab. Inc., Hercules, CA, USA) used according to the manufacturer's protocol. The ID-Card 50520 (NaCl) was used to measure IgM, while the ID-Card 50531 [LISS/Coombs containing polyspecific anti-human gamma globulin (AHG) serum] was used to determine IgG in addition to IgM. The serum samples were serially twofold diluted with 0.9% saline solution starting with 500 µl serum and 500 µl saline solutions. Also, 25 µl of each dilution were pipetted into a single column of the microtube gel card, and 50 µl of DiaMed-ID-DiaCell ABO/I-II test cell suspensions A_1 or B (Bio-Rad Lab.) was added. ID-Card 50520 (NaCl) cards were incubated at room temperature for 15 min, the ID-Cards (LISS/Coombs) at 37°C, and thereafter centrifuged in a DiaMed-ID-Centrifuge 24 S for 10 min at 910 rpm. Results were read immediately after centrifugation and were subdivided into positive and negative results. The titer that led to visible agglutination of erythrocytes dispersed in the gel was selected positive. In an individual with blood group O, both anti-A and anti-B antibody titers were used for statistical analysis, while in an individual with blood group A, only anti-B antibody titers, and in an individual with blood group B, only anti-A antibody titers were used for statistical analysis; none of the study individuals had blood group AB.

Determination of Anti-A/B Antibodies by Surface Plasmon Resonance

The erythrocyte aggregation assays based on the DiaMed-ID Micro Typing System are semiquantitative assays that cannot determine in an isotype-specific way a subtle increase of anti-A/B IgG and/or IgM antibodies against blood group A/B antigens. Therefore, we used SPR to measure anti-A/B antibodies as previously described (6, 11, 12). Amine-labeled blood group A and B trisaccharides (Dextra Laboratories Ltd., Reading, UK) were immobilized on two-channel sensor chips CM5 (General Electric Health Care, Freiburg, Germany) using standard protocols (31, 32). In brief, the CM5 sensor chip surface was activated with 100 µl 0.05 mol/l *N*-hydroxy-succinimide and 0.2 mol/l *N*-ethyl-*N*′-dimethylamino-propylcarbodiimide injected into the buffer stream of HBS-EP (0.01 mol/l HEPES buffer, pH 7.4, containing 0.15 mol/l NaCl, 3 mmol/l EDTA, and 0.005% surfactant P20) as described previously (6). The buffer stream was passed through FC-1 and FC-2 of the two-channel Biacore® X instrument (GE Health Care) at a flow rate of 5 µl/min for 20 min. Subsequently, 15 µl of a blood group A/B trisaccharide amine derivative solution at a concentration of 1 mg/ml, prepared with borate (pH 8.5), was injected into the buffer stream and passed through FC-1 at a flow rate of 5 µl/min and was halted after injection of 7 µl for 2 h; FC-2 served as a blank control. Residual active ester groups on the sensor surface were then deactivated by injecting 100 µl of 1 mol/l ethanolamine–HCl (pH 8.5) into the buffer stream and passing it through FC-1 and FC-2, and by passing the buffer stream through the cell until a stable baseline was achieved. To measure anti-blood group A/B antibodies, serum was diluted by

half with the HBS-EP, and 100 µl of the diluted plasma samples were passed through FC-1 and FC-2 at a flow rate of 20 µl/min for 5-min duration. When the association phase was finished, binding of antibody was recorded and is expressed as resonance units (RU at "stability" in **Figure 1**, corresponding to RUt0 in **Figure 2**, panel 3). After each measurement, the antibodies bound to the blood group trisaccharides were removed with 50 µl of 50 mmol/l NaOH buffer at a flow rate of 60 µl/min for 50 s and the chip regenerated to reach the same baseline as prior to the measurement. The amounts of anti-A/B antibody that associated with the blood group A/B trisaccharide antigen immobilized on the sensor chip surface were obtained by subtracting the FC-2 value from the FC-1 value.

Alternatively, a four channel Biacore® T200 device (kindly provided by Florian Koelle, GE Health Care) was used, which enabled us to measure both anti-A and anti-B antibodies on one chip surface (CM5-chip) at the same time. The amount of trisaccharides and the coupling procedure remained similar to the two-channel system used previously, except the contact time was adapted to 600 s and the flow rate to 10 µl/min. Flow cell one (FC-1) and three (FC-3) were immobilized without trisaccharides and served as blank during binding analysis. FC-2 was immobilized with 1 mg/ml blood group A trisaccharide and FC-4 with 1 mg/ml blood group B trisaccharide. For binding analysis, serum was diluted by half with HBS-EP and injected in FC-2 and FC-4 for 180 s at a flow rate of 10 µl/min. The flow-path was 2–1 and 4–3. To determine IgM and IgG levels of A- and B-bound antibodies, anti-human IgG Abs [polyclonal aHIgG (γ-chain), Sigma-Aldrich, St. Louis, MO, USA] and anti-human IgM [polyclonal aHIgM (µ-chain), Sigma-Aldrich, St. Louis, MO, USA] were injected for 180 s at a flow rate of 10 µl/min directly after the serum sample. For measurement of anti-A/B antibodies RU at report point "stability" (see **Figure 1**, corresponding to RUt0 in **Figure 2**, panel 3) and for levels of IgM/IgG anti-A/B antibodies levels at report point "enhance_level" (see **Figure 1**) relative to baseline were recorded. After determination of levels of IgM and IgG anti-A/B antibodies, the chip was regenerated twice with 50 mmol NaOH for 30 s at a flow rate of 10 µl/min with a stabilization period of 5 s after the second regeneration to reach the same baseline as prior to the measurement. For kinetic measurement, an association time of 200 s and dissociation time of 800 s was chosen. Dissociation of bound anti-A/B antibodies over time as a semiquantitative correlate for the strength of antibody binding was calculated according to the following formula: % dissociation = 100 − [RU at t1 (800 s)/RU at t0 (200 s)] × 100 (see **Figure 2**, panel 3 as an example for measurement of RU at t0 and at t1). To compute an affinity constant K_D the concentration of IgG antibodies in microgram per

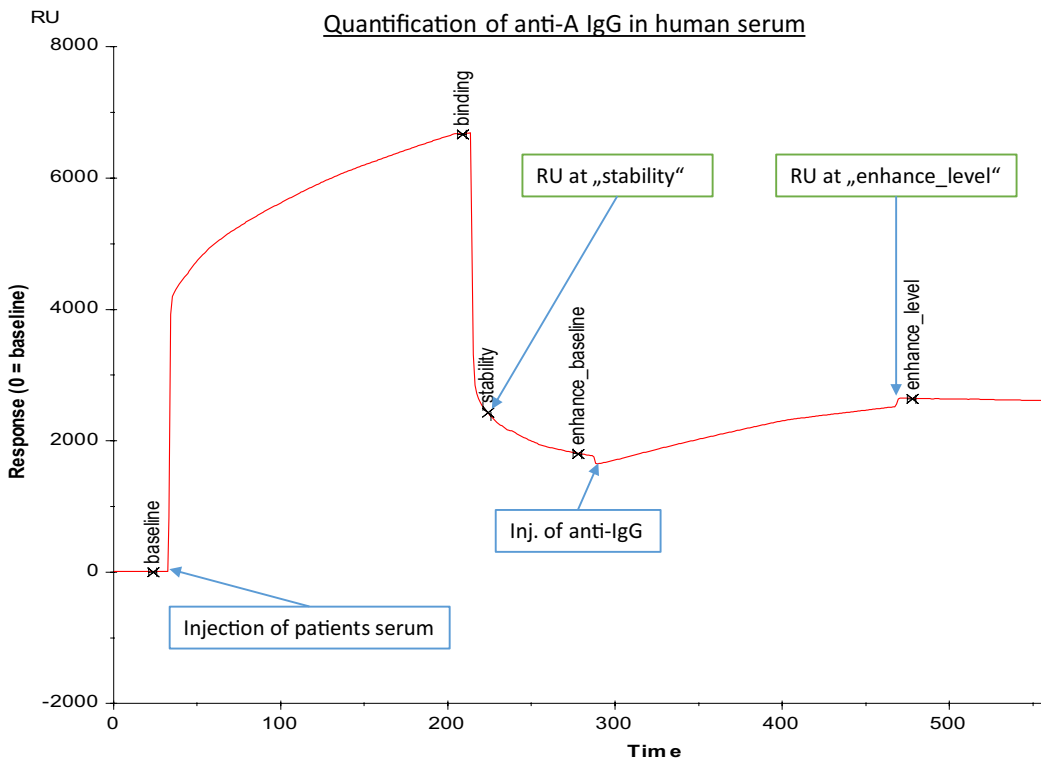


FIGURE 1 | Exemplary binding curve of quantification of anti-A IgG antibodies in human serum, as generated by BIAevaluation® software (GE Healthcare). Injection of human serum was followed by a second injection of anti-IgG; response units (RU) at "stability" were recorded to assess the concentration of anti-A blood group antibodies, while response units at "enhance_level" were representative for anti-A IgG concentration. The *x*-axis gives time in seconds, and the *y*-axis represents response units (RU).

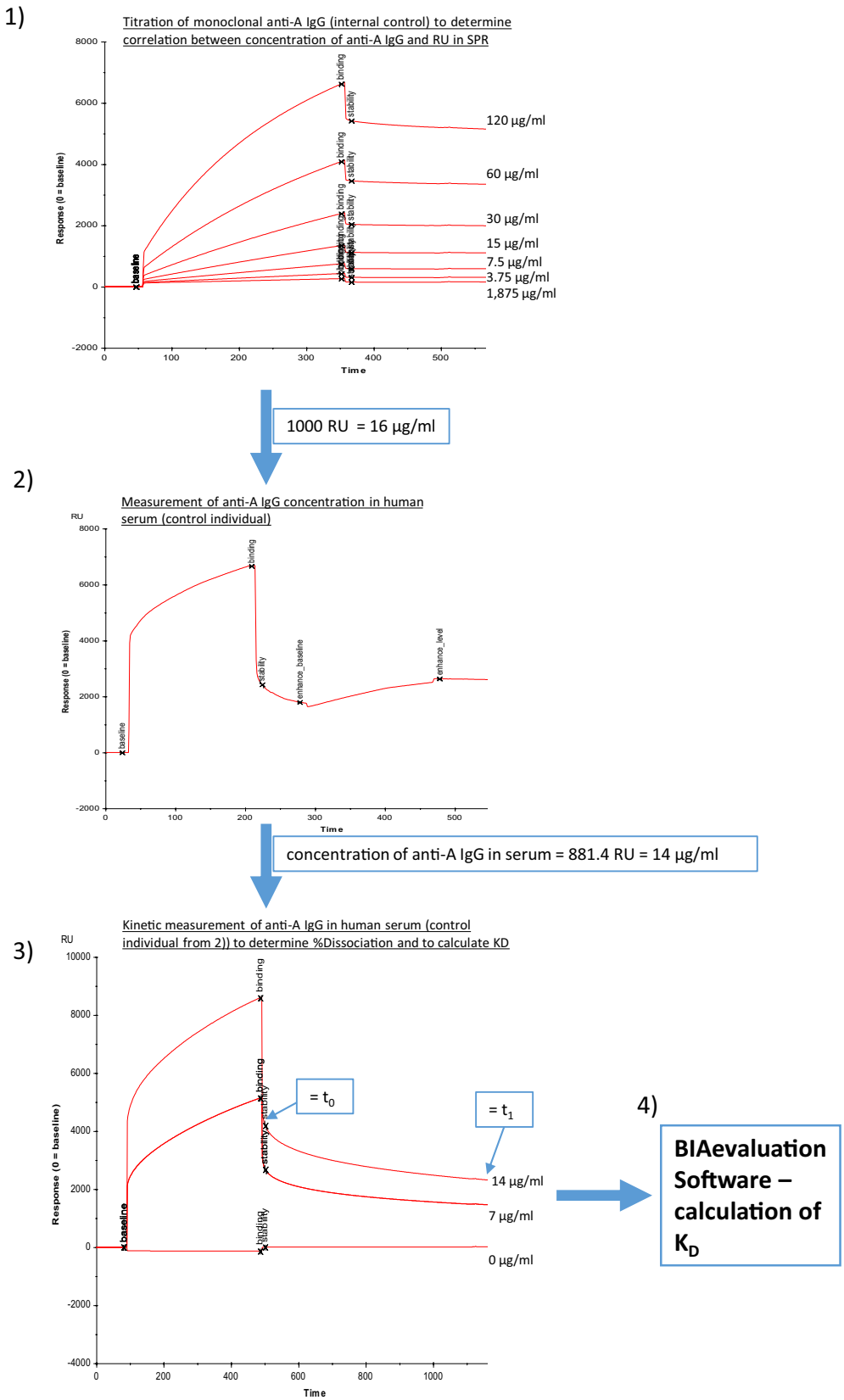


FIGURE 2 | **Determination of binding characteristics of anti-A IgG antibodies in human serum**.

(*Continued*)

FIGURE 2 | Continued

Panel (1) different concentrations of monoclonal anti-A IgG (internal control) were injected to correlate response units to micrograms per milliliter, (2) anti-A IgG levels in human serum were determined utilizing a second injection of anti-IgG (Sigma), (3) different dilutions of human serum were measured without addition of second step reagent, and binding curves were recorded for a total of 1,200 s, (4) an estimate of K_D was calculated using the BIAevaluation Software (GE Healthcare) with input of the concentrations of anti-A IgG determined in steps (2) and (3) as described in Section "Materials and Methods."

milliliter must be known, thus RUs for different concentrations of monoclonal anti-A/B IgG (clone 9A, Abcam, Cambridge, MA, USA; clone NaM87-1F6, BD Pharmingen, San Jose, CA, USA) were measured (**Figure 2**, panel 1). This enabled us to correlate an increase in response units derived from anti-A/B IgG measurements in patient serum with microgram per milliliter of anti-A/B IgG antibody by regression analysis in order to calculate the respective concentration of IgG anti-A/B (**Figure 2**, panel 2). Subsequently two dilutions of patient serum – one of them diluted 1:2 – and a blank were measured (**Figure 2**, panel 3), and the resulting binding curves were used to calculate K_D (computed as $K_D = K_{off}/K_{on}$) with the BIAevaluation® software from GE Healthcare. To fit the association/dissociation curve, a 1:1 binding model was chosen. The molecular weight of the analyte was assumed to be 150,000 Da (IgG). To assess the quality of the calculations, controls within the software were used. No external calculations were performed nor parameters added. Furthermore, as an internal control to test before each measurement was started, anti-blood group A or B IgM mAbs (anti-A murine monoclonal Abs clone MH04 and A3D3, anti-B murine monoclonal Abs clone NB1.19, NB10.5A5, and NB10.3B4, Ortho-Clinical Diagnostics, Neckargemünd, Germany), and anti-blood group A IgG mAb (clone 9A, Abcam, Cambridge, MA, USA; clone NaM87-1F6, BD Pharmingen, San Jose, CA, USA) were used to guarantee optimal chip performance and full recovery. For statistical analysis, both anti-A and anti-B antibody titers (RU) were used in an individual with blood group O, while in an individual with blood group A, only anti-B antibody titers, and in an individual with blood group B, only anti-A antibody titers were used for statistical analysis; none of the individuals in this study had blood group AB.

Statistical Analysis

Statistically significant differences between study groups were calculated using the non-parametric two-tailed Mann–Whitney *U*-test. Results are depicted using box plot diagrams, with the median represented by a cross, the interquartile range (IQR) represented by the box, and 5- and 95-percentile values represented by the whiskers.

RESULTS

Anti-PnPs Response in Healthy Individuals and in Patients with Type I DM after Pneumovax®23 Immunization

The anti-PnPs antibody response prior and 4–6 weeks after immunization with the unconjugated PnPs vaccine Pn23, a 23-valent vaccine, was determined by ELISA in an isotype-specific manner in 28 healthy individuals and 16 patients with DM I. All healthy individuals included in this study showed a significant IgM (**Figure 3A**) and IgG (**Figure 3B**) anti-PnPs antibody response following vaccination. The results confirm previous findings in patients with type I DM (17) showing a normal antibody response to unconjugated pneumococcal polysaccharide, which is likely TI, while primary antibody responses to T-cell-dependent antigens are impaired (17).

Increase in Blood Group IgG Anti-A/B Antibodies in Healthy Individuals and in Patients with Type I DM after Pneumovax®23 Immunization as Determined with the Biacore® Device

To address whether immunization with Pn23 has an effect on blood group anti-A/B antibodies in patients with DM-type I as compared to healthy individuals, we first determined antibody titers in the DiaMed-ID Micro Typing System, using the ID-Card 50520 (NaCl) as well as the ID-Card 50531 (LISS/Coombs containing poly-specific AHG serum) to determine IgG in addition to IgM anti-A/B antibodies. We could observe no difference in blood group anti-A/B antibody titer prior and post immunization with Pn23 in both, healthy individuals and patients with DM-type I when the assay used was based upon erythrocyte aggregation in physiological NaCl (direct agglutination test, **Figure 4A**) or in the presence of anti-IgG Coombs serum (indirect agglutination test, **Figure 4B**). Interestingly, in a considerable percentage of study individuals of both groups (up to approximately 25%) the A/B isoagglutinin titers were lower than 1:4 in both agglutination systems, thus confirming previous findings of a relatively low sensitivity of the agglutination system to detect low isoagglutinin titers (6). To increase sensitivity, SPR technology was applied to further analyze anti-A/B antibodies, thus allowing real-time analysis of molecular interactions between antibodies and the isolated blood group trisaccharide antigen without molecular labeling leaving the structure of both molecules intact (6, 32). With this technology, biologically relevant antibody–antigen interactions can be assessed both quantitatively and qualitatively, as the carbohydrate antigens are presented in a physiological manner (11, 12, 31). Kinetics and affinity were investigated by analyzing the time curve and level of binding and concentration of anti-A/B antibodies in the sample by measuring mass binding, which further allowed the determination of the binding forces. The comparison of the response on the active and control surface allowed for the subtraction of bulk effects associated with buffer changes and for the high serum concentration with its mass binding, thus enabling the calculation of specific binding. Using SPR analysis, we found specific anti-A/B antibody binding to the respective blood group A/B trisaccharides in all individuals tested

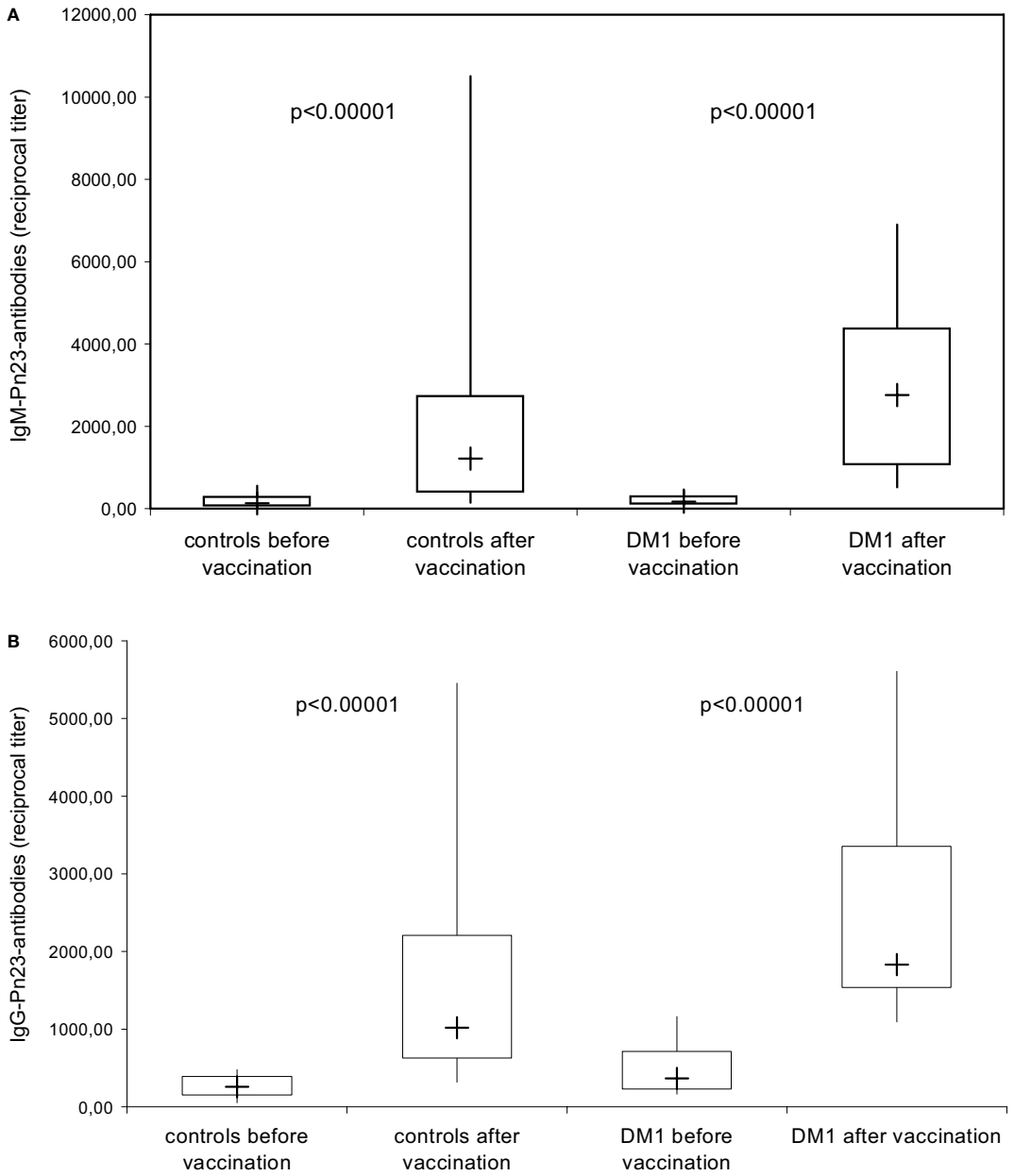


FIGURE 3 | IgM/IgG antibody response to pneumococcal polysaccharides after immunization with Pn23 vaccine. Healthy individuals (n = 28) and patients with type I DM (n = 16) were immunized with the PnPs vaccine Pneumo 23 Vaccine "Pasteur Merieux" (Pn23), and blood samples were drawn 4–6 weeks after vaccination. Antibodies against pneumococcal polysaccharides (anti-PnPs) were determined by ELISA as described in Section "Materials and Methods" and presented as IgM–Pn23-antibody response **(A)** or IgG–Pn23-antibody response **(B)**. Results are depicted using box plot diagrams, with the median represented by a cross, the interquartile range (IQR) represented by the box, and 5- and 95-percentile values represented by the whiskers; statistically significant differences between study groups were calculated using the non-parametric two-tailed Mann–Whitney U-test.

(**Figure 4C**), as has also been described previously for individuals with intact antibody responsiveness (6, 12). For all probes, a high association rate constant for the interaction and the use of a high ligand density on the sensor chip surface was found that promoted the mass transport limitation. As reference analyte with a known concentration, commercially available blood group anti-A or -B antibodies of IgM and blood group anti-A IgG were used (**Figure 2**, panel 1 shows monoclonal anti-A IgG as an example). For the example shown in **Figure 2**, panel 1, a sensor response of 1,000 RU corresponded to a shift of 0.1° in the SPR angle, which in turn correlates to approximately 16 μg/ml of anti-A IgG injected. Since the individuals selected for determination of K_D [one healthy control (Ind1) and one DM I patient (Ind2) as well as a control individual not vaccinated] contained

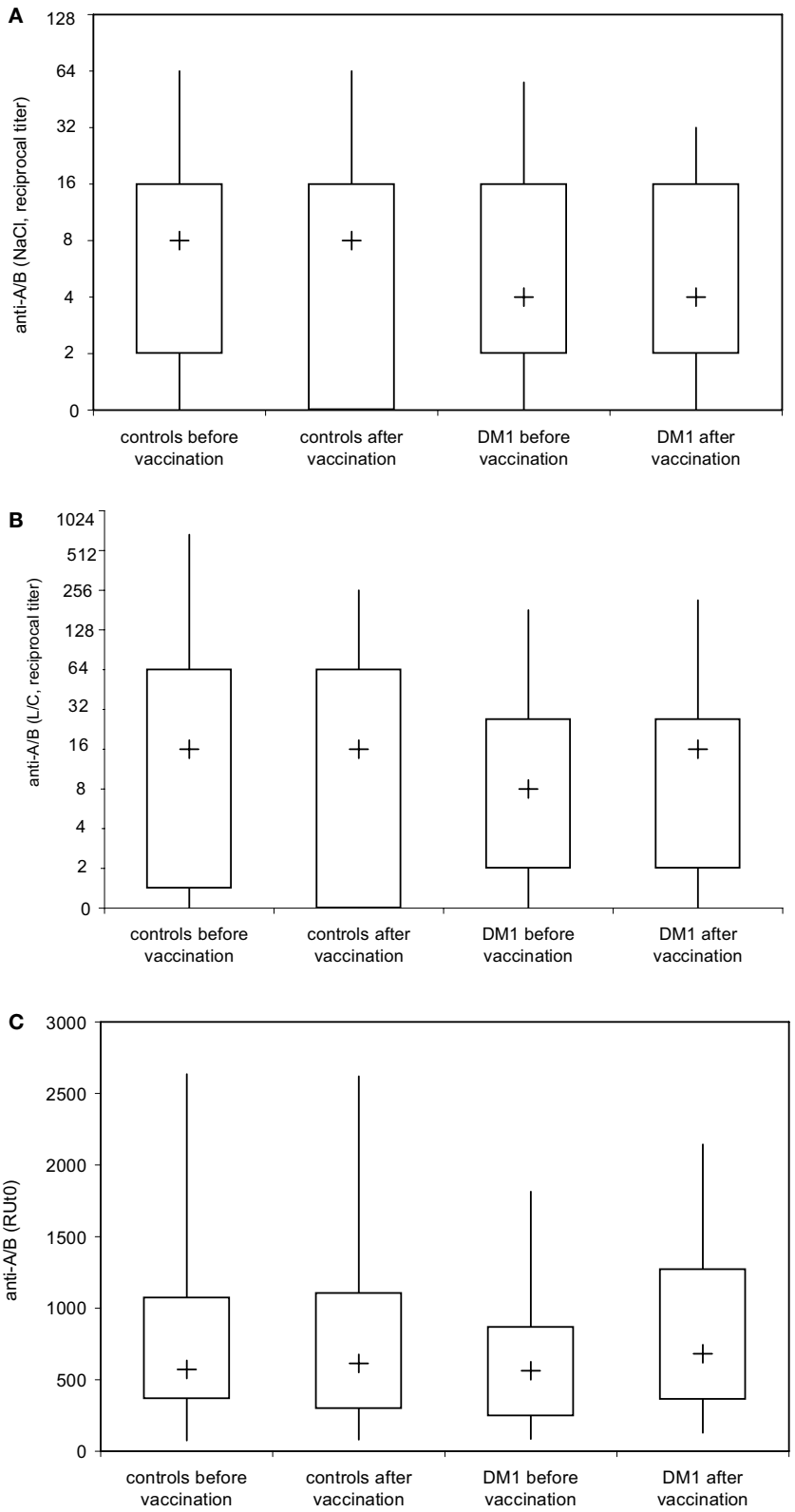


FIGURE 4 | Analysis of blood group anti-A and anti-B isoagglutinin binding to A and B trisaccharide antigen in real time by surface plasmon resonance (SPR) technology.

(*Continued*)

FIGURE 4 | Continued

Serum samples of healthy individuals (n = 28) and patients with type I DM (n = 16) were serially twofold diluted with 0.9% saline solution and blood group anti-A and/or anti-B IgM **(A)** were determined by ID-Card 50520 (NaCl), while the ID-Card 50531 (LISS/Coombs) was used to determine IgG and IgM isoagglutinins **(B)**. The titer that led to visible agglutination of erythrocytes dispersed in the gel was selected positive. Alternatively **(C)**, serum samples diluted in HES-EP buffer (1:2) were injected over either the blood group A or blood group B trisaccharide-coupled CM5 sensor chip. Binding kinetics, i.e., association and dissociation were recorded as sensorgrams in resonance units (RU) against time in FC-I and FC-II, and the amount of anti-A/B antibody that associated with the blood group A/B trisaccharide antigen immobilized on the sensor chip surface was obtained by subtracting the FC-II value from the FC-I value and is given as resonance units (RU). Results are depicted using box plot diagrams, with the median represented by a cross, the interquartile range (IQR) represented by the box, and 5- and 95-percentile values represented by the whiskers; no statistically significant differences were found between study groups as calculated using the non-parametric two-tailed Mann–Whitney U-test.

sufficient amounts of anti-A IgG for affinity analysis with very low to undetectable anti-A IgM, titration was only performed for IgG anti-A/B antibody. Of note, SPR technology is capable to detect anti-A/B antibodies with values of 50–180 RU in serum samples where the microtube column assay showed no erythrocyte aggregation. Even with this sensitive detection system, no change in isoagglutinin titers was found following vaccination with 23-valent PnPs, neither in healthy individuals nor in DM1 patients (**Figure 4C**).

Using the DiaMed-ID Micro Typing System in the direct and indirect agglutination assay as well as SPR technology under close to physiological conditions, both IgM- and IgG-antibodies were measured simultaneously, without discrimination between IgM and IgG response. To examine a possible booster effect of Pn23 vaccination on IgG anti-A/B antibodies only we applied isotype-specific second step antibodies to discriminate between blood group anti-A/B IgM and IgG antibodies. This system enabled us to detect an induction of blood group anti-A/B IgG (**Figure 5B**) but not IgM (**Figure 5A**) antibodies after immunization of healthy individuals with Pn23, an antibody response resembling a secondary/booster response. Interestingly, patients with type I DM showed a comparable induction of IgG isoagglutinins after immunization with Pn23 (**Figure 5B**), in agreement with their intact antibody responsiveness to pneumococcal polysaccharide, a T-independent antigen, while a significant impairment in primary antibody response to vaccination with T-cell-dependent antigens such as hepatitis A virus and diphtheria toxoid was described previously (17).

Determination of Binding Characteristics of Anti-A/B Antibodies after Pneumovax®23 Immunization

To measure whether the induction of IgG-A/B antibodies observed after Pn23 immunization was accompanied by changes in binding characteristics of the blood group anti-A antibodies, as would be the case for a T-dependent IgG-booster response, we analyzed the binding dynamics of the anti-A/B antibodies as described above (**Figure 2**). We found no substantial change in the percentage of dissociation (**Figure 6A**) or the estimate of the affinity constant (K_D, **Figure 6B**) of blood group-specific anti-A antibodies following pneumococcal vaccination, as calculated by BIAevaluation® software from GE Healthcare. For estimation of the affinity constant K_D, healthy individuals and patients with type I DM were selected that had initially high anti-A-antibody titers (>1:256 or >700 RU), associated with high anti-A IgG and low to undetectable anti-A IgM antibody levels. Only three healthy individuals and two patients with type I DM fulfilled this criterion. As shown in **Figure 6** (depicting results from one healthy individual, Ind1 and one DM I patient, Ind2), our study gives evidence that immunization with Pn23 induces an increase in IgG anti-A/B antibody titers but has no effect on binding characteristics of blood group anti-A/B IgG antibodies, neither in the healthy individuals nor in the patients tested.

DISCUSSION

Based on previous findings (7, 13) it was our hypothesis that structural similarities in saccharide composition between pneumococcal polysaccharides and blood group A/B saccharide antigens could lead to production of cross-reactive blood group anti-A/B antibodies after immunization with capsular polysaccharide vaccines, because B cells could recognize shared carbohydrate epitopes (2, 8, 10, 33, 34). In the current study, we indeed show that immunization with Pn23 vaccine is followed by induction of blood group anti-A/B antibodies of the IgG isotype in healthy individuals. Anti-A/B IgG antibodies could only be demonstrated when SPR technology using the Biacore® device was applied, while conventional technology employing erythrocyte aggregation assays using the DiaMed-ID Micro Typing System were unable to detect this side effect of Pn23 vaccination. Apparently, the increased sensitivity of SPR technology as compared to conventional erythrocyte agglutination assays as well as the near to physiological conditions of the molecular interaction between antibodies and the specific blood group trisaccharide antigen enabled us to detect the stimulation of small amounts of cross-reactive IgG antibodies (11, 12, 31, 32). A previous study described an alternative approach to measure isoagglutinins in an isotype-specific and sensitive manner by using synthetic blood group saccharides in ELISA and showed cross-reactivity between alpha-Gal and blood group B oligosaccharide (13). The increase in blood group anti-A/B IgG isotype response observed in our study provides additional evidence for a limited cross-reactivity between PnPs antigens and the carbohydrate moieties of blood group A/B trisaccharides. Whether the observed induction of IgG antibodies cross-reacting with A/B blood group antigens is of clinical relevance in healthy individuals remains to be determined but is unlikely due to the relatively small increase (approximately twofold) observed. Patients with type I DM

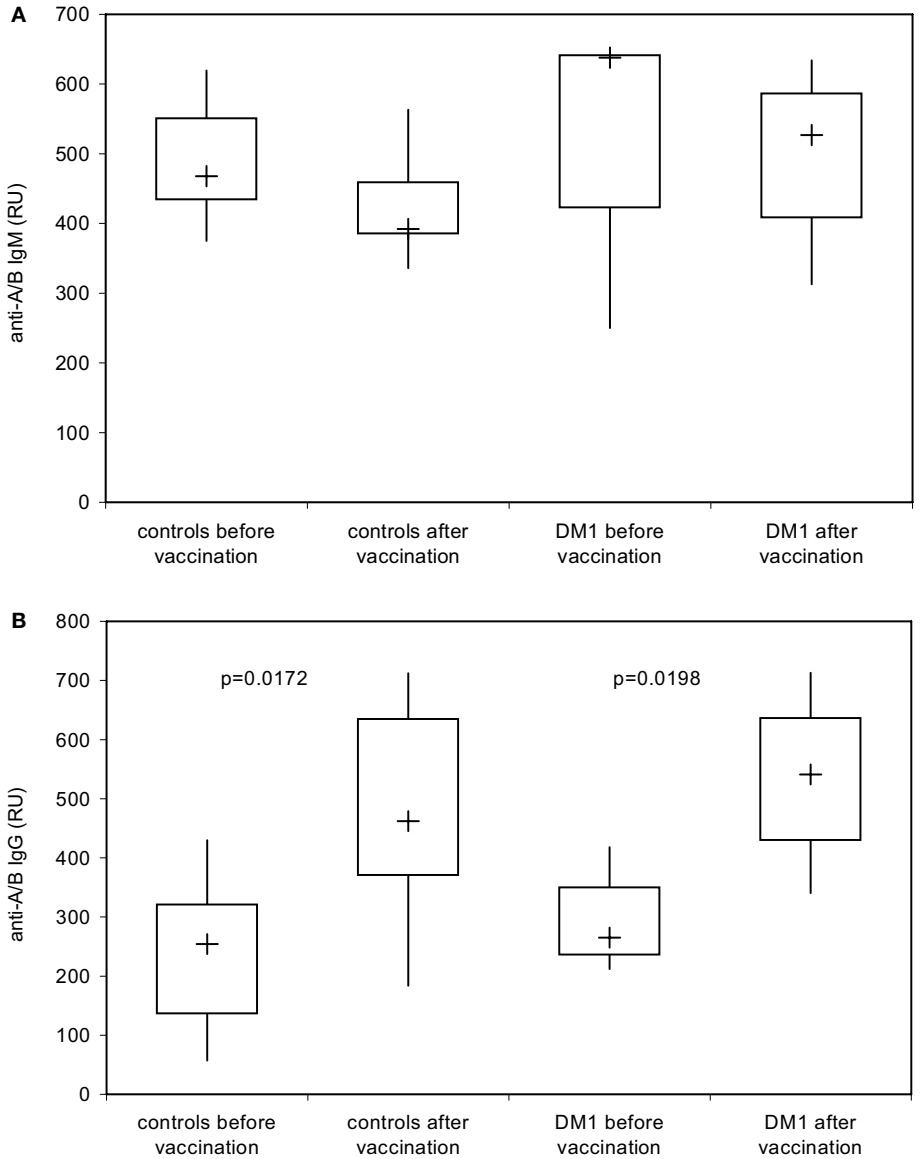


FIGURE 5 | Increase in blood group IgG anti-A/B antibodies in healthy individuals and in patients with type I DM after immunization with Pn23 vaccine as determined with the Biacore® device. To determine IgM **(A)** and IgG **(B)** anti-A/B antibodies in healthy individuals and patients with type I DM prior and post immunization with Pneumo 23 Vaccine "Pasteur Merieux" (Pn23), an anti-human IgM mAb **(A)** or anti-human IgG Abs **(B)** were applied as the second step reagents as described in Section "Materials and Methods." Results are depicted using box plot diagrams, with the median represented by a cross, the interquartile range (IQR) represented by the box, and 5- and 95-percentile values represented by the whiskers; statistically significant differences between study groups were calculated using the non-parametric two-tailed Mann–Whitney *U*-test. For the IgM antibody response, no statistically significant differences were found between study groups.

displayed a comparable cross-reactive IgG antibody response against blood group antigens following Pn23 vaccination, although these patients present an autoimmune disease with profound immunological dysregulation (17, 18). Interestingly, cross-reactive IgM antibodies were not inducible, neither in DM1 patients nor in controls. This is characteristic of a booster response effecting IgG responses only and contrary to the effect of immunization with a conventional polysaccharide antigen such as PnPs inducing both anti-PnPs IgM and IgG antibody responses following booster vaccination (**Figure 3**).

An additional benefit of the SPR-based technology employed in this study is the capability for real-time measurements of affinity of antibody–antigen interactions without the use of molecular labeling, leaving the structure of both interacting molecules intact (11, 12, 32). Calculations of K_D as performed in our study using human serum as a sample can only be an estimate for the true affinity of a given antibody since we are dealing with a variety of polyclonal antibodies of different isotypes contributing to the overall affinity of the blood group antibodies detected in the SPR analysis used. Isotype

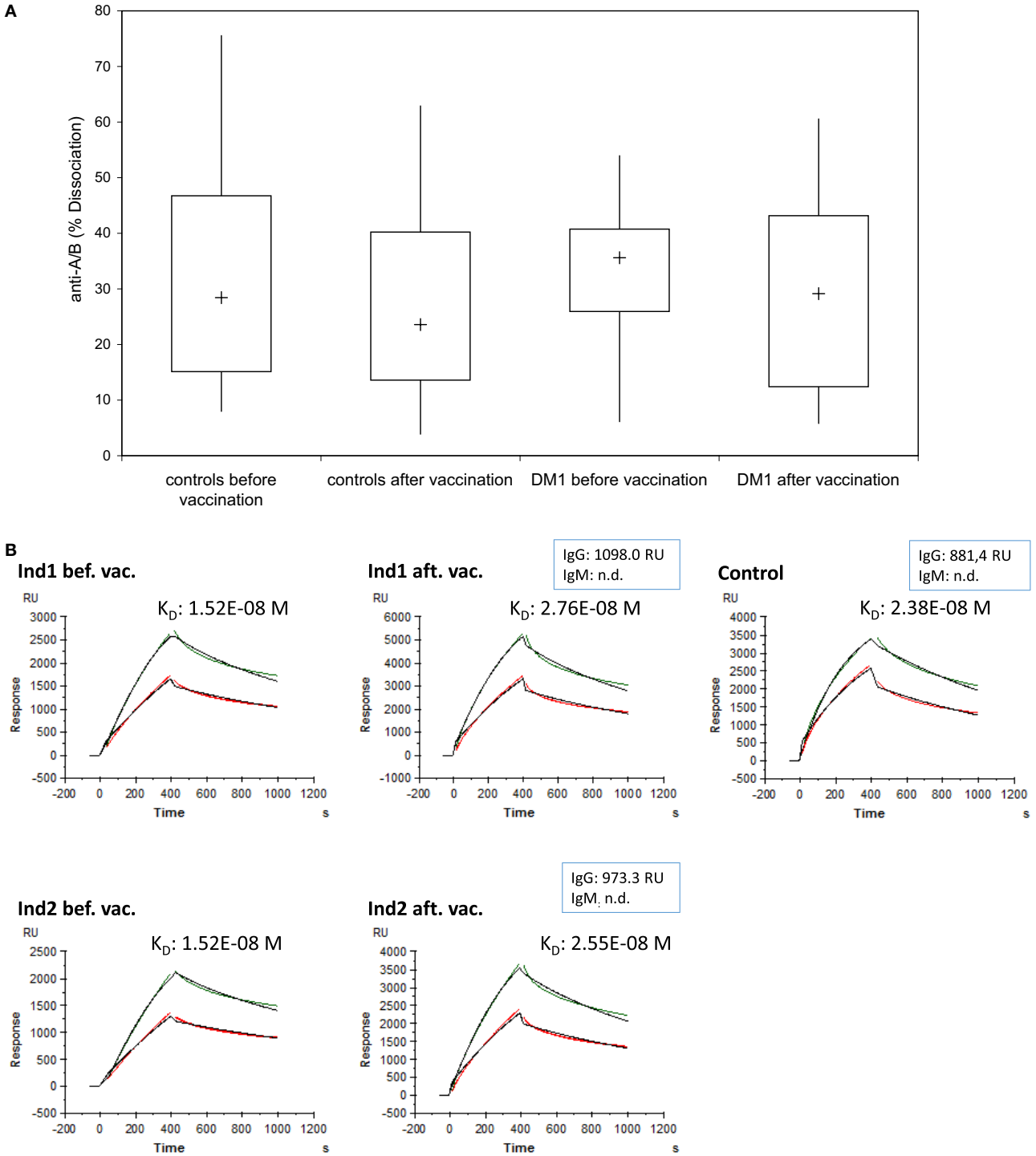


FIGURE 6 | **Binding characteristics of anti-A/B antibodies after immunization with Pn23 vaccine**. The percentage of dissociation **(A)** of blood group anti-A/B antibodies before and after immunization with Pneumo 23 Vaccine "Pasteur Merieux" (Pn23) in healthy individuals (n = 28) and in patients with type I DM (n = 16) was investigated using the Biacore device, the CM5 chip, and the synthetic blood group specific A/B trisaccharides as described in Section "Materials and Methods." Furthermore, the estimated affinity constant K_D **(B)** of blood group specific anti-A antibodies before and after pneumococcal vaccination were analyzed in serum samples of three healthy individuals and two patients with type I DM selected for their initially high anti-A antibody titer (>than 1:256 or >than 700 RU). Kinetic measurements of anti-A antibodies binding to surface bound trisaccharide before and after vaccination in two representative individuals (a healthy individual, Ind1, and a DM I patient, Ind2) who fulfilled these criteria are given; one healthy individual that was not included in the vaccination protocol but showed high titer for blood group anti-A antibodies was measured in parallel (Control). Green curves represent serum sample and red curves serum sample diluted by half; the black curves depict the calculated fit. n.d., not detectable.

bias has been limited since individuals with high blood group A IgG but low to undetectable blood group A IgM antibody were selected to estimate the binding affinity of anti-A IgG antibodies. To determine the affinity (K_D) of serum antibodies of a single specificity, isolation and immobilization of the respective immunoglobulin clone would be required, which is only possible in individuals with monoclonal gammopathy of the desired specificity. Interestingly, we did not find a marked change in binding characteristics of the cross-reactive blood group anti-A/B antibodies, indicating that a relatively small increase in IgG antibody titer occurred without additional affinity maturation. An immune response to carbohydrate antigens such as to pneumococcal capsular polysaccharide serotypes 1, 2, 3, 4, 5, 6B, 7, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F is thought to be generally TI, which can limit the B cell response to the production of IgM antibodies mainly and limited amounts of IgG antibodies, and the absence of affinity maturation of the rearranged antibody genes might lead to the expression of germline sequences of antibodies with limited affinity (5, 34, 35). In previous studies, the affinity of anti-carbohydrate antibodies for their antigens was shown to be 3–5 orders lower in magnitude than affinities of anti-protein or anti-peptide antibodies for their antigens (2, 3, 8, 35). In this study, however, we found no evidence for low affinity binding of blood group A/B antibodies obtained either from healthy individuals or from patients with type I DM. When a mixture of commercially available monoclonal blood group anti-A IgM and IgG antibodies was formulated, simulating the amount and composition of naturally occurring blood group anti-A antibodies, the dissociation rate was comparable to the serum samples analyzed in **Figure 6** (data not shown). Germline antibodies recognizing carbohydrate epitopes might display significant cross-reactivity because the number of potential antigens the immune system must encounter appears to extensively outweigh the recombinational potential of the germline genes (2, 3, 34, 35). The recombinational potential of the germline genes for anti-Gal antibodies specific for blood group B substance in unimmunized individuals was shown to be limited, because anti-Gal antibodies are encoded by a group of 6–8 well-defined and structurally related germline progenitors in humans (36). Anti-Gal was shown to be the most abundant antibody in humans, constituting approximately 1% of immunoglobulins and found to be of IgG, IgM, and IgA isotype (37, 38). Cryptic antigens capable to bind anti-Gal are exposed on senescent human RBCs as well as on RBCs of patients with β-thalassemia and sickle cell anemia, but the amount of cryptic antigens expressed on RBCs was shown to be low (38). The preferential anti-Gal IgG subclass found by ELISA was IgG2 using Gal-specific synthetic saccharides, and these IgG2 antibodies showed cross-reactivity with blood group B antigen (13). ABO blood group antigens were shown to be the ideal targets to explore the concomitant effect of mutation on binding affinity and specificity of anti-carbohydrate antibodies (33). Mutant anti-carbohydrate antibodies with higher affinity achieved by single point mutations of specific residues lining the pocket on binding to the A and B blood group oligosaccharide antigens showed altered polarity, surface complementarity, and side chain aliphatic character, leading to improved binding affinity without loss of specificity (33). Furthermore, the poly-specificity in germline encoded antibodies to carbohydrate epitopes was shown to be due to greater conformational ability in their combining sites, and this flexibility helped to cope with new and changing pathogen surface structures by recognizing distinct highly conserved epitopes on bacterial polysaccharides (4, 33). Structural analysis of the affinity-matured antibody 48G7 and its germline precursor antibody showed that the germline precursor could undergo an induced fit upon binding to its cognate hapten, while the affinity-matured antibody 48G7 displayed lock and key binding. An immunological relationship between specific substances of ABO and type XIV pneumococcus gave further evidence for shared carbohydrate epitopes by showing that adsorption of the hemagglutinins in anti-type XIV horse serum with erythrocytes of each of the A/B blood groups removed the agglutinins not only of that group but also of the other groups (7). In addition to pneumococcus, other germs have been shown to cross-react with carbohydrate structures on the surface of human cells (39–43). The core oligosaccharides of low-molecular-weight LPS of pathogenic *Neisseria* spp. can mimic the carbohydrate moieties of glycosphingolipids (40). The core oligosaccharides of LPS of *Campylobacter jejuni* serotypes, which are associated with the development of Guillian–Barre' syndrome, can exhibit mimicry of gangliosides (41). Finally, the O-chain of a number of *Helicobacter pylori* strains exhibit mimicry of Lewis(x) and Lewis(y) blood group antigens, giving evidence that molecular mimicry can serve to camouflage the bacterial surface from the host (42).

In conclusion, our study provides evidence for a limited carbohydrate epitope sharing between PnPs and blood group sugar epitopes in healthy individuals as well as in patients with type I DM that could be observed after Pn23 immunization. Cross-reactive anti-polysaccharide antibody responses were comparable between patients and controls with respect to the titer and the affinity. Blood group anti-A/B antibodies are considered to be naturally occurring antibodies that occur basically in any individual with an intact immune response also in the absence of antigenic encounter. The clinical relevance of the slight increase in blood group anti-A/B IgG isotype response in healthy individuals after Pn23 immunization is certainly limited; our findings however indicate modulation of anti-A/B IgG antibodies, e.g., a booster response during life not only by encounter with the genuine antigen such as incompatible blood transfusions or alloimmunization during pregnancy but also through molecular mimicry of particular carbohydrate epitopes shared by blood group AB substances and bacterial polysaccharides such as PnPs.

ETHICS STATEMENT

The study was approved by the ethics committee of the Rudolfstiftung Hospital of the City of Vienna. Informed consent was obtained from every study participant.

AUTHOR CONTRIBUTIONS

MF and HW were the principal investigators, and they critically assessed data, performed statistical analysis, wrote the manuscript, and took primary responsibilities for the paper; WW and KS performed the experiments and analyzed the data; CB critically assessed data and was critically involved in the initial draft; NE-M conducted the clinical study and provided clinical data. All the authors critically participated in all revisions of the manuscript.

REFERENCES

1. Milland J, Sandrin MS. ABO blood group and related antigens, natural antibodies and transplantation. *Tissue Antigens* (2006) 68:459–66. doi:10.1111/j.1399-0039.2006.00721.x
2. Nguyen HP, Seto NO, MacKenzie CR, Brade L, Kosma P, Brade H, et al. Germline antibody recognition of distinct carbohydrate epitopes. *Nat Struct Biol* (2003) 10:1019–25. doi:10.1038/nsb1014
3. Brooks CL, Müller-Loennies S, Brade L, Kosma P, Hirama T, MacKenzie CR, et al. Exploration of specificity in germline monoclonal antibody recognition of a range of natural and synthetic epitopes. *J Mol Biol* (2008) 377:450–68. doi:10.1016/j.jmb.2008.01.018
4. Patenaude SI, Seto NOL, Borisova SN, Szpacenko A, Marcus SL, Palcic MM, et al. The structural basis for specificity in human ABO(H) blood group biosynthesis. *Nat Struct Biol* (2002) 9:685–90. doi:10.1038/nsb832
5. Defrance T, Taillardet M, Genestier L. T cell-independent B cell memory. *Curr Opin Immunol* (2011) 23:330–6. doi:10.1016/j.coi.2011.03.004
6. Fischer MB, Wolfram W, Binder CJ, Böhmig GA, Wahrmann M, Eibl MM, et al. Surface plasmon resonance analysis shows an IgG-isotype-specific defect in ABO blood group antibody formation in patients with common variable immunodeficiency. *Front Immunol* (2015) 6:211. doi:10.3389/fimmu.2015.00211
7. Finland M, Curnen EC. Agglutinations for human erythrocytes in type XIV anti-pneumococcic horse serum. *Science* (1938) 87:417–21. doi:10.1126/science.87.2262.417
8. Yuki N. Carbohydrate mimicry: a new paradigm of autoimmune disease. *Curr Opin Immunol* (2005) 17:577–82. doi:10.1016/j.coi.2005.09.004
9. Marcus DM. The ABO and Lewis blood-group system. Immunochemistry, genetics and relation to human disease. *N Engl J Med* (1969) 280:994–1006. doi:10.1056/NEJM196905012801806
10. Yamamoto F. ABO blood group system-ABH oligosaccharide antigens, anti-A and anti-B, A and B glycosyltransferases, and ABO genes. *Immunohematology* (2004) 20:3–22.
11. Kimura S, Yurugi K, Segawa H, Kuroda J, Sato K, Nogawa M, et al. Rapid quantification of immunoglobulin G antibodies specific for blood group antigens A and B by surface plasmon resonance. *Transfusion* (2005) 45:56–62. doi:10.1111/j.1537-2995.2005.04062.x
12. Yurugi K, Kimura S, Ashihara E, Tsuji H, Kawata A, Kamitsuji Y, et al. Rapid and accurate measurement of anti-A/B IgG antibodies in ABO-unmatched living donor liver transplantation by surface plasmon resonance. *Transfus Med* (2007) 17:97–106. doi:10.1111/j.1365-3148.2007.00737.x
13. Rispens T, Derksen NI, Commins SP, Platts-Mills TA, Aalberse RC. IgE production to α-gal is accompanied by elevated levels of specific IgG1 antibodies and low amounts of IgE to blood group B. *PLoS One* (2013) 8(2):e55566. doi:10.1371/journal.pone.0055566
14. Lleo A, Invernizzi P, Gao B, Podda M, Gershwin ME. Definition of human autoimmunity – autoantibodies versus autoimmune disease. *Autoimmun Rev* (2010) 9:A259–66. doi:10.1016/j.autrev.2009.12.002
15. Kubo T, Uchida Y, Watanabe Y, Abe M, Nakamura A, Ono M, et al. Augmented TLR9-induced Btk activation in PIR-B-deficient B-1 cells provokes excessive auto-antibody production and autoimmunity. *J Exp Med* (2009) 206:1971–82. doi:10.1084/jem.20082392
16. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* (2009) 119:1335–49. doi:10.1172/JCI36800
17. Eibl N, Spatz M, Fischer GF, Mayr WR, Samstag A, Wolf HM, et al. Impaired primary immune response in type-1 diabetes: results from a controlled vaccination study. *Clin Immunol* (2002) 103:249–59. doi:10.1006/clim.2002.5220
18. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev* (2011) 91:79–118. doi:10.1152/physrev.00003.2010
19. Christen U, Bender C, von Herrath MG. Infection as a cause of type 1 diabetes? *Curr Opin Rheumatol* (2012) 24:417–23. doi:10.1097/BOR.0b013e3283533719
20. Kerr JR. The role of parvovirus B19 in the pathogenesis of autoimmunity and autoimmune disease. *J Clin Pathol* (2016) 69:279–91. doi:10.1136/jclinpath-2015-203455
21. Pane JA, Coulson BS. Lessons from the mouse: potential contribution of bystander lymphocyte activation by viruses to human type 1 diabetes. *Diabetologia* (2015) 58:1149–59. doi:10.1007/s00125-015-3562-3
22. Masala S, Cossu D, Piccinini S, Rapini N, Mameli G, Manca Bitti ML, et al. Proinsulin and MAP3865c homologous epitopes are a target of antibody response in new-onset type 1 diabetes children from continental Italy. *Pediatr Diabetes* (2015) 16:189–95. doi:10.1111/pedi.12269
23. Masala S, Paccagnini D, Cossu D, Brezar V, Pacifico A, Ahmed N, et al. Antibodies recognizing *Mycobacterium avium* paratuberculosis epitopes cross-react with the beta-cell antigen ZnT8 in Sardinian type 1 diabetic patients. *PLoS One* (2011) 6(10):e26931. doi:10.1371/journal.pone.0026931
24. Williams JM, Bonami RH, Hulbert C, Thomas JW. Reversing tolerance in isotype switch–competent anti-insulin B lymphocytes. *J Immunol* (2015) 195:853–64. doi:10.4049/jimmunol.1403114
25. Maglione PJ, Simchoni N, Black S, Radigan L, Overbey JR, Bagiella E, et al. IRAK-4 and MyD88 deficiencies impair IgM responses against T-independent bacterial antigens. *Blood* (2014) 124:3561–71. doi:10.1182/blood-2014-07-587824
26. Avalos AM, Busconi L, Marshak-Rothstein A. Regulation of autoreactive B cell responses to endogenous TLR ligands. *Autoimmunity* (2010) 43:76–83. doi:10.3109/08916930903374618
27. Chen RT, Pless R, DeStefano F. Epidemiology of autoimmune reactions induced by vaccination. *J Autoimmun* (2001) 16:309–18. doi:10.1006/jaut.2000.0491
28. Moran AP. The role of endotoxin in infection: *Helicobacter pylori* and *Campylobacter jejuni*. *Subcell Biochem* (2010) 53:209–40. doi:10.1007/978-90-481-9078-2_10
29. Bogaert D, Hermans PWM, Adrian PV, Rümke HC, deGroot R. Pneumococcal vaccines: an update on current strategies. *Vaccine* (2004) 22:2209–20. doi:10.1016/j.vaccine.2003.11.038
30. Biagini RE, Schlottmann SA, Sammons DL, Smith JP, Snawder JC, Striley CAF, et al. Method for simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides. *Clin Diagn Lab Immunol* (2003) 10:744–50. doi:10.1128/CDLI.10.5.744-750.2003
31. Canziani GA, Klakamp S, Myszka DG. Kinetic screening of antibodies from crude hybridoma sample using Biacore. *Anal Biochem* (2003) 325:301–7. doi:10.1016/j.ab.2003.11.004
32. Seo HJ, Adachi K, Lee BK, Kang DG, Kim YK, Kim KR, et al. Facile and rapid direct gold surface immobilization with controlled orientation for carbohydrates. *Bioconjug Chem* (2007) 18:2197–201. doi:10.1021/bc700288z
33. Thomas R, Patenaude SI, MacKenzie CR, To R, Hirama T, Young NM, et al. Structure of an anti-blood group A Fv and improvement of its binding affinity without loss of specificity. *J Biol Chem* (2002) 277:2059–64. doi:10.1074/jbc.M104364200
34. Wedemayer GJ, Patten PA, Wang LH, Schultz PG, Stevens RC. Structural insights into the evolution of an antibody combining site. *Science* (1997) 276:1665–9. doi:10.1126/science.276.5319.1665
35. Manimala JC, Roach TA, Li Z, Gildersleeve JC. High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems. *Glycobiology* (2007) 17:17–23. doi:10.1093/glycob/cwm047

36. Wang L, Radic MZ, Galili U. Human anti-Gal heavy chain genes. *J Immunol* (1995) 155:1276–85.
37. Bello-Gil D, Manez R. Exploiting natural anti-carbohydrate antibodies for therapeutic purposes. *Biochemistry (Mosc)* (2015) 80:836–45. doi:10.1134/S0006297915070044
38. Galili U. Anti-Gal: an abundant human natural antibody of multiple pathogeneses and clinical benefits. *Immunology* (2013) 140:1–11. doi:10.1111/imm.12110
39. Gerstenbruch S, Brooks CL, Kosma P, Brade L, MacKenzie CR, Evans SV, et al. Analysis of cross-reactive and specific anti-carbohydrate antibodies against lipopolysaccharide from *Chlamydophila psittaci*. *Glycobiology* (2010) 20:461–72. doi:10.1093/glycob/cwp198
40. Moran AP, Prendergast MM, Appelmelk BJ. Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol* (1996) 16:105–15. doi:10.1111/j.1574-695X.1996.tb00127.x
41. Caudie C, Pinon AQ, Taravel D, Sivadon-Tardy V, Orlikowski D, Rozenberg F, et al. Preceding infections and anti-ganglioside antibody profiles assessed by a dot immunoassay in 306 French Guillain-Barre' syndrome patients. *J Neurol* (2011) 258:1958–64. doi:10.1007/s00415-011-6042-9
42. Moran AP. Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen *Helicobacter pylori*. *Carbohydr Res* (2008) 343:1952–65. doi:10.1016/j.carres.2007.12.012
43. Van Roon AM, Pannu NS, de Vrind JPM, van der Marel GA, van Boom JH, Hokke CH, et al. Structure of an anti-Lewis X Fab fragment in complex with its Lewis X antigen. *Structure* (2004) 12:1227–36. doi:10.1016/j.str.2004.05.008

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 November 2016
doi: 10.3389/fimmu.2016.00512

CD16$^+$ Monocyte Subset Was Enriched and Functionally Exacerbated in Driving T-Cell Activation and B-Cell Response in Systemic Lupus Erythematosus

Huaqun Zhu[1,2,3†], Fanlei Hu[1,2,3†], Xiaolin Sun[1,2,3], Xiaoying Zhang[1,2,3], Lei Zhu[1,2,3], Xu Liu[1,2,3], Xue Li[1,2,3], Liling Xu[1,2,3], Lianjie Shi[4], Yuzhou Gan[1,2,3] and Yin Su[1,2,3]*

[1] Department of Rheumatology and Immunology, Peking University People's Hospital, Beijing, China, [2] Beijing Key Laboratory for Rheumatism Mechanism and Immune Diagnosis (BZ0135), Beijing, China, [3] Peking-Tsinghua Center for Life Sciences, Beijing, China, [4] Peking University International Hospital, Beijing, China

Background: The roles that CD16$^+$ monocyte subset plays in T-cell activation and B-cell response have not been well studied in systemic lupus erythematosus (SLE).

Objective: The present study aimed to investigate the distribution of CD16$^+$ monocyte subsets in SLE and explore their possible roles in T-cell activation and B-cell differentiation.

Methods: The frequencies of monocyte subsets in the peripheral blood of healthy controls (HCs) and patients with SLE were determined by flow cytometry. Monocyte subsets were sorted and cocultured with CD4$^+$ T cells and CD19$^+$ B cells. Then, T and B cells were collected for different subset detection, while the supernatants were collected for immunoglobulin G, IgA, and IgM or interferon-γ and interleukin-17A detection by enzyme-linked immunosorbent assay.

Results: Our results showed that CD16$^+$ monocytes exhibited a proinflammatory phenotype with elevated CD80, CD86, HLA-DR, and CX3CR1 expression on the cell surface. It's further demonstrated that CD16$^+$ monocytes from patients and HCs shared different cell-surface marker profiles. The CD16$^+$ subset was enriched in SLE and had an exacerbated capacity to promote CD4$^+$ T cell polarization into a Th17 phenotype. Also, CD16$^+$ monocytes had enhanced impacts on CD19$^+$ B cells to differentiate into plasma B cells and regulatory B cells with more Ig production.

Conclusion: This study demonstrated that CD16$^+$ monocytes, characterized by different cell-surface marker profiles, were enriched and played a critical role in driving the pathogenic T- and B-cell responses in patients with SLE.

Keywords: systemic lupus erythematosus, CD16$^+$ monocytes, expansion, T cell activation, B cell response

OPEN ACCESS

Edited by:
Ana María Hernández, Center of Molecular Inmunology, Cuba

Reviewed by:
Taruna Madan, National Institute for Research in Reproductive Health, India
Anil Chauhan, Saint Louis University, USA

***Correspondence:**
Yin Su
suyin0921@163.com

†Huaqun Zhu and Fanlei Hu contributed equally to this work.

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *24 August 2016*
Accepted: *02 November 2016*
Published: *21 November 2016*

Citation:
Zhu H, Hu F, Sun X, Zhang X, Zhu L, Liu X, Li X, Xu L, Shi L, Gan Y and Su Y (2016) CD16$^+$ Monocyte Subset Was Enriched and Functionally Exacerbated in Driving T-Cell Activation and B-Cell Response in Systemic Lupus Erythematosus. Front. Immunol. 7:512. doi: 10.3389/fimmu.2016.00512

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with multiorgan damage characterized by immunological abnormalities that include deficient innate immune response and aberrant activation of autoreactive T and B cells, with subsequent production of pathogenic autoantibodies against cell nuclear components and resultant end-organ injury (1–4). Functional

abnormalities in peripheral blood monocytes play a significant role in the pathogenesis of SLE, contributing to both aberrant T-cell activation and B-cell tolerance (2).

CD16 (FcγRIII) is one of the Fc receptors for immunoglobulin G (IgG) (FcγRs) (5). It is an activating FcγR that transmits activation signals through an immune-receptor tyrosine-based activation motif contained in its cytoplasmic region and mediates endocytosis and phagocytosis of immune complexes, including antibody-coated microorganisms (6). In both mice and humans, blood monocyte subsets exhibit differential surface expression of various FcγRs. For the past two decades, CD16 distinguishes human monocytes into two major subsets ($CD16^+$ and $CD16^-$ subsets). Subsequently, the third distinct monocyte subset driven from $CD16^+$ monocytes was described, which was defined as relatively higher levels of CD14 coupled with lower CD16 expression. Therefore, human peripheral monocytes could be categorized into three subsets: the non-classical monocytes (NCM, $CD14^+CD16^{++}$), the intermediate monocytes (IM, $CD14^{++}CD16^+$), and the classical monocytes (CM, $CD14^{++}CD16^-$). NCM and IM are collectively addressed as $CD16^+$ monocytes (7, 8).

$CD16^+$ monocytes exhibit low levels of CCR2, the chemokine receptor of CCL2 also known as monocyte chemoattractant protein 1, and high levels of CX3CR1, known as the fracalkine (CX3CL1) receptor (7, 9). Gene expression reveals that non-classical and intermediate subsets are more closely related (10, 11), which is further supported by separate microarray study performed with rhesus monkeys that possess homologous monocyte subpopulations (12). $CD16^+$ monocytes are considered to be pro-inflammatory, as they are better than $CD16^-$ monocytes at producing the cytokines-tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 in response to microbial-associated molecular patterns (13–15). The results accumulated over the past decades suggest that $CD16^+$ subset is expanded in many different types of diseases, mostly under infection or inflammatory conditions (16–18). Some studies on monocyte subpopulations were performed in patients with SLE. Monocyte subsets exhibited no difference in expression of Mer tyrosine kinase between patients with SLE and normal controls (19), and IM have modulatory effects on CM (20). Mukherjee et al. showed that non-classical inflammatory monocytes increased in patients with SLE (21). Mikołajczyk et al. suggested that $CD14^{dim}CD16^+$ monocytes are associated with subclinical atherosclerosis in SLE (22).

Despite being the main driver of autoimmune diseases, the adaptive immune responses are strongly affected by innate immune cells. On the one hand, human peripheral blood monocytes are shown to act as antigen-processing cells (APCs) to activate T cells and secrete cytokines that shape T-cell differentiation in inflammatory diseases, such as rheumatoid arthritis and thrombocytopenia (23, 24). On the other hand, monocytes, which are generally regarded as precursors of tissue macrophages and dendritic cells (DCs) (25), can functionally promote B-cell differentiation and antibody secretion in patients with SLE (26, 27). A substantial literature is available on rats demonstrating the critical contribution of monocytes to autoreactive T-cell response and B-cell tolerance (28–31). Furthermore, therapies targeting monocyte-derived cytokines, such as TNFα, IL-6, or aberrant B-cell activation, have been successfully put into clinical practice, resulting in dramatically improved clinical outcomes in a number of autoimmune diseases (32–34).

To date, the specific roles of monocyte subsets have not been fully characterized with regards to T-cell activation and B-cell differentiation in SLE. In the present study, the flow cytometry analysis was first performed to compare the distribution of each subset between healthy individuals and patients with SLE. The expression of several cell-surface markers on each monocyte subset was also compared, including costimulatory receptors (CD80 and CD86), major histocompatibility complex (MHC) class II (HLA-DR), scavenger receptor (CD163), and chemokine receptors (CCR5, CX3CR1) from patients and healthy controls (HCs). Also, the role of monocyte subsets in the polarization of Th subsets was explored in patients with SLE. Furthermore, the study also investigated the capacity of SLE monocyte subsets in B-cell activation and differentiation. Understanding of the mechanisms underlying monocyte subset-mediated T-cell activation and B-cell responses in SLE might disclose novel therapeutic targets to treat this disease.

MATERIALS AND METHODS

Patients and Controls

Sixty-two patients with SLE and 35 HCs were enrolled at the Department of Rheumatology, Peking University People's Hospital, China, and blood samples were obtained from these subjects. All of the enrolled patients fulfilled at least four of the 2010 American College of Rheumatology revised criteria for SLE (35). Demographic, clinical, and laboratory data obtained from the medical records of the patients included age, sex, disease duration, blood cell counts, 24-h proteinuria excretion, anti-double-strand DNA (anti-dsDNA) antibody, anti-nucleosome antibody (AnuA), anti-Sm antibody, anti-SSA antibody, anti-SSB antibody, complement component 3 (C3), complement component 4 (C4), IgG, IgM, IgA, and erythrocyte sedimentation rate (ESR). Anti-dsDNA antibody, 24-h proteinuria excretion, C3, and C4 levels were used to predict SLE disease activity. All participants signed the informed consents to donate their blood samples and de-identified clinical information for research. The study was approved by the Institutional Medical Ethics Review Board of Peking University People's Hospital.

Cell Staining and Flow Cytometric Analysis

Monocyte subsets were detected in freshly collected blood from HC and SLE patients by flow cytometry using the following antibodies: fluorescein isothiocyanate (FITC) anti-human CD14 (Biolegend, San Diego, CA, USA, catalog: 325604), allophycocyanin (APC) anti-human CD16 (Biolegend, clone:3G8, catalog: 302012), Brilliant Violet 421 anti-human CD80 (Biolegend, catalog: 305221), Brilliant Violet 510 anti-human CD86 (Biolegend, catalog: 305431), PerCP/Cy5.5 anti-human CD163 (Biolegend, catalog: 333608), phycoerythrin (PE)/Dazzle 594 anti-human HLA-DR (Biolegend, catalog: 307653), AF700 anti-human CCR5

(Biolegend, catalog: 359116), and PE/Cyanine 7 anti-human CX3CR1 (Biolegend, catalog: 347612).

T cell subsets were detected after cocultured with monocytes using the following antibodies: PE-CF594 anti-human CD4 (BD Biosciences, San Diego, CA, USA, catalog: 562281), FITC anti-human interferon (IFN)-γ (Biolegend, catalog: 502506), PE anti-human IL-4 (eBioscience, San Diego, CA, USA, catalog: 12-7049-42), APC anti-human IL-17A (eBioscience, catalog: 17-7179-42), and AF 647 anti-human Foxp 3 (Biolegend, catalog: 320114) Abs.

B cell subsets were detected after cocultured with monocytes using the following antibodies: APC-Cyanine 7 anti-human CD19 (Biolegend, catalog: 302218), FITC anti-human IgD (Biolegend, catalog: 348206), PE anti-human CD24 (eBioscience, catalog: 12-0247-42), PE-Cyanine 7 anti-human CD20 (eBioscience, catalog: 25-0209-42), and APC anti-human CD27 (eBioscience, catalog: 17-0279-42) mAbs were used.

Gating of the single cells in a forward scatter (FSC)-A and FSC-H plot and settings of gates for detecting monocyte subsets were shown in **Figure 1A**. Data were analyzed with FlowJo vX0.7 (Becton Dickinson).

Cell Isolation

Venous blood samples (12 mL) were obtained from all subjects. Human peripheral blood mononuclear cells were isolated from the blood of healthy individuals and patients with SLE using a Ficoll gradient centrifugation protocol. Monocyte subsets were purified by flow cytometry (BD FACSAria II) based on CD14 and CD16 staining. B and T cells were also isolated by flow cytometry. Figure S1 in Supplementary Material demonstrates the gating strategies about the enrichment and purification of monocyte subsets, B cells, and T cells during cell sorting. Sorted cells were further assessed by flow cytometric analysis with purity >95, >95, and >95% for monocyte subsets, B cells, and T cells, respectively (Figure S2 in Supplementary Material).

Cell Coculture

Fresh SLE blood or healthy control buffy coat samples from blood bank were collected for cell coculture assays. The monocytes were incubated in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. To evaluate the effects of monocyte subsets on T-cell activation, each monocyte subset was cocultured with autologous $CD4^+$ T cells (ratio 1:5) in the presence of anti-CD3 (1 μg/mL) (eBioscience, catalog: 16-0037) and anti-CD 28 (1 μg/mL) (eBioscience, catalog: 16-0289) antibodies (36) and macrophage colony-stimulating factor (M-CSF) (50 ng/mL) (Peprotech, Rocky Hill, CT, USA, catalog: 300-35). After 5 days, T cells were stimulated for 5 h with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL), ionomycin (1 μg/mL), and brefeldin A (BFA) (10 μg/mL) (Lian ke, Hanzhou, China, catalog: CS0001, CS0002, and CS0003). Then, T cells were assessed by staining with anti-IFN-γ (Th1), anti-IL-4 (Th2), and anti-17A (Th17) antibodies. Regulatory T cells (Treg, $Foxp3^+CD4^+$) were monitored by intracellular anti-Foxp3 staining. Coculture supernatants were collected for IFN-γ and IL-17A measurement.

To explore the roles of monocyte subsets on B-cell response, each monocyte subset was cocultured with $CD19^+$ B cells from the same donors (ratio 1:2.5) in the presence of anti-CD40 antibody (37) (3 μg/mL) (eBioscience, catalog:16-0409) and M-CSF (50 ng/mL) (Peprotech, catalog: 300-35) for 3 days, after which B cells were assessed for the following subpopulations: memory B cells (MBs, $CD19^+IgD^-CD27^+$), plasma B cells (PBs, $CD19^+CD20^-CD27^+$), and regulatory B cells (Bregs, $CD19^+CD24^+CD27^+$). Coculture supernatants were also harvested for Ig measurement.

T-Cell Proliferation Assay

For labeling isolated T cells, the cells were incubated for 10 min at 37°C in 5 mM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA, catalog: C34554) in phosphate-buffered saline/0.1% bovine serum albumin (BSA/0.1% PBS). Labeling was stopped by adding five volumes of ice-cold RPMI 1640 to the cells. The cells were washed three times before use. Labeled T cells were cocultured with each monocyte subset for 60 h, as described above. T-cell proliferation was defined as CFSE low population indicative of cell division as detected by flow cytometry.

Enzyme-Linked Immunosorbent Assay

Commercially available enzyme-linked immunosorbent assay (ELISA) kits used for measuring IFN-γ, IL-17A, or Ig levels in the supernatants were as follows: IFN-γ ELISA kit from R&D systems (Minneapolis, MN, USA, catalog: DY285); IL-17A ELISA kit from Neobioscience Technology Co., Ltd. (Shenzhen, China, catalog: EHC 170); and IgG, IgA, and IgM ELISA kits from eBioscience (San Diego, CA, USA, catalog: 88-50550, 88-50600, and 88-50620).

Statistical Analysis

All values were expressed as means ± SD. Data were analyzed for significance using the Statistical Package for Social Sciences version 23.0 (SPSS, Chicago, IL, USA) by non-parametric paired *t* test and Mann–Whitney *U* test. Spearman's correlation coefficient (*r*) was applied to determine the correlation between two numerical data. A *P*-value <0.05 was considered statistically significant.

RESULTS

$CD16^+$ Monocyte Subset Demonstrated Different Phenotypes from $CD16^-$ CM

Human peripheral blood monocytes could be categorized into $CD16^+$ monocytes and $CD16^-$ monocytes based on differential CD16 expression. The expression of several surface markers, including costimulatory receptors (CD80 and CD86), MHC class II (HLA-DR), scavenger receptor (CD163), and chemokine receptors (CCR5and CX3CR1), was analyzed to determine the phenotypic difference between $CD16^+$ monocyte subset and $CD16^-$ CM in HC donors.

$CD16^+$ monocyte subset demonstrated higher expression of CD80, CD86, and HLA-DR compared with $CD16^-$ CM in

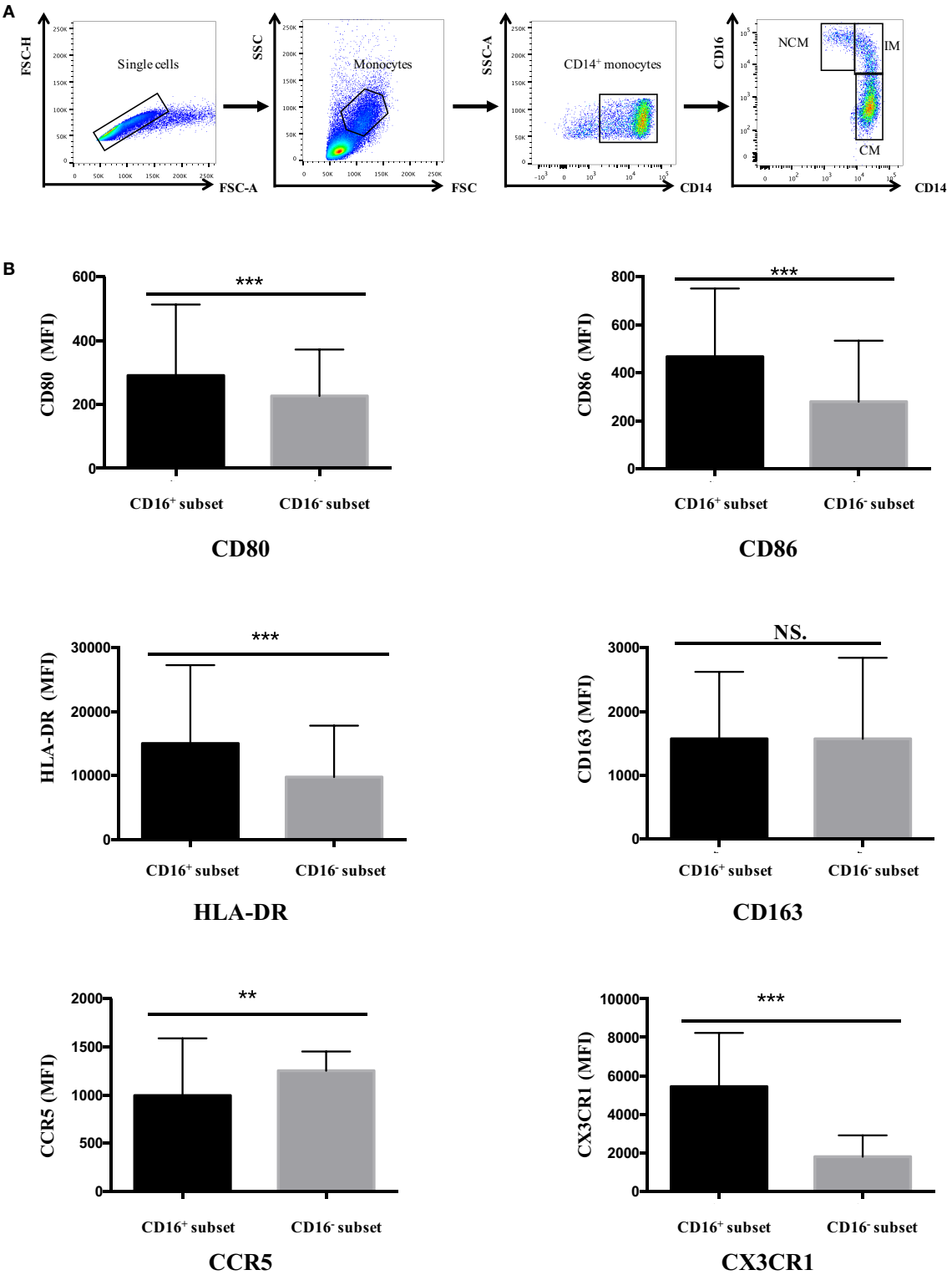


FIGURE 1 | CD16⁺ monocytes exhibited different cell-surface marker expression compared with CD16⁻ monocytes. All samples were collected from fresh blood. **(A)** Representative dot plots in peripheral blood mononuclear cells showed the gating strategy used based on CD14 and CD16 expression. CD16⁺ monocytes could be divided into NCM and IM. **(B)** CD16⁺ and CD16⁻ monocyte subsets showed distinctive patterns of cell-surface receptor expression in HCs. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical monocytes. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$; NS, no significance.

control donors ($P < 0.001$). No difference in CD163 expression was detected between CD16$^+$ and CD16$^-$ monocytes ($P > 0.05$). CCR5 expression was significantly lower in CD16$^+$ subset compared with CD16$^-$ monocytes ($P < 0.01$). Also, the chemokine receptor CX3CR1 was significantly more expressed on CD16$^+$ monocytes than on CD16$^-$ monocytes ($P < 0.001$) (**Figure 1B**).

CD16$^+$ Monocyte Subset Was Enriched in Patients with SLE

Skewed monocyte subsets have been reported in several autoimmune inflammatory diseases such as rheumatoid arthritis (17). The distribution of monocytes was measured in a cohort of 62 patients with SLE and 35 age-matched HCs to investigate whether this change also existed in SLE. The patients with SLE consisted of 57 female and 5 male subjects, and the median disease duration was 60 months with a range of 3–432 months.

The frequencies of CD16$^+$ monocyte subset were significantly elevated in patients with SLE than in healthy individuals (SLE: 33.78 ± 16.19% vs. HC: 19.1 ± 7.49%, $P < 0.001$). Patients with SLE exhibited a significantly higher percentage of NCM (SLE: 14.92 ± 12.51% vs. HC: 11.27 ± 4.83%, $P < 0.05$) and IM (SLE: 18.86 ± 9.44% vs. HC: 7.84 ± 5.09%, $P < 0.001$), with obviously reduced frequencies of CM (SLE: 64.96 ± 16.87% vs. HC: 78.72 ± 8.50%, $P < 0.001$) (**Figure 2**) when compared with control donors.

In patients with SLE, the frequencies of CD16$^+$ monocytes were positively correlated with serum IgA concentrations ($r = 0.267$; $P < 0.05$) and anti-dsDNA antibody levels ($r = 0.349$; $P < 0.01$), which implicated that patients with elevated frequencies of CD16$^+$ monocytes would be at a high risk of Ig production or autoantibody development. On the contrary, the percentage of CD16$^-$ subsets was negatively correlated with serum IgA ($r = -0.26$; $P < 0.05$) and anti-dsDNA antibody levels ($r = -0.345$; $P < 0.01$). The frequencies of NCM subset were correlated with anti-dsDNA antibody levels ($r = 0.302$; $P < 0.05$), while the frequencies of IM subset were correlated with serum IgA levels ($r = 0.287$; $P < 0.05$). Other laboratory data, including IgG, IgM, C3 level, C4 level, AnuA, ESR, and 24-h proteinuria excretion, did not show significant correlation with monocyte subsets (**Table 1**). The frequencies of monocyte subsets were further compared between patients with and without particular antibodies, including AnuA, anti-Sm Ab, anti-SSA Ab, and anti-SSB Ab. Detailed analysis of such autoantibodies in the patient cohort revealed no predictive factors for the expansion of CD16$^+$ monocytes (Table S1 in Supplementary Material). The correlation analysis revealed slightly weak correlation between monocyte subsets and serum anti-dsDNA Abs or IgA levles, indicating that monocyte subset might contribute to B cell response in SLE.

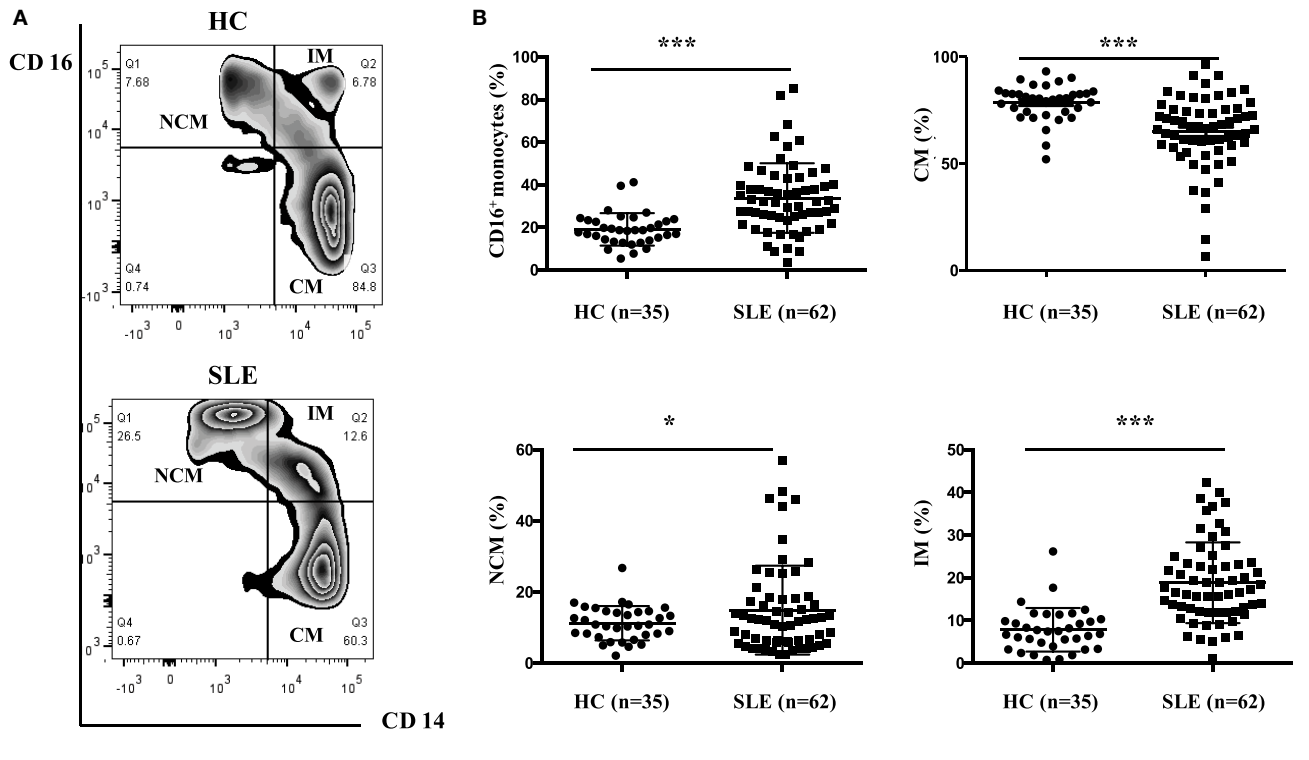


FIGURE 2 | CD16$^+$ monocytes as well as their subsets (NCM and IM) were expanded in patients with SLE. All samples were collected from fresh blood. **(A)** Representative flow charts showed the percentage of NCM, IM, and CM from a patient with SLE and a healthy control (HC). **(B)** Frequencies of NCM, IM, CM, and CD16$^+$ subset were compared between 62 patients with SLE and 35 age-matched HCs. NCM, non-classical monocytes (CD14$^+$CD16^{++}); IM, intermediate monocytes (CD14^{++}CD16$^+$); CM, classical monocytes (CD14^{++}CD16$^-$). Data were expressed as mean ± SD and analyzed by Mann–Whitney *U* test. $^*P < 0.05$, $^{***}P < 0.001$; NS, no significance.

TABLE 1 | Correlations between each monocyte subset frequencies and clinical parameters in SLE.

Clinical parameters	CD16+ subset (%)		NCM (%)		IM (%)		CM (%)	
	Spearman's *r*	*P*	Spearman's *r*	*P*	Spearman's *r*	*P*	Spearman's *r*	*P*
IgA	0.267*	0.038	0.012	0.925	0.287*	0.025	−0.26*	0.043
IgG	0.061	0.642	0.045	0.733	0.095	0.472	−0.055	0.678
IgM	−0.095	0.472	−0.15	0.252	−0.1	0.466	0.09	0.494
C3	0.092	0.481	0.116	0.372	0.06	0.648	−0.071	0.584
C4	0.061	0.642	0.004	0.979	0.185	0.156	−0.031	0.816
Anti-dsDNA Ab	0.349**	0.006	0.302*	0.018	0.129	0.32	−0.345**	0.006
AnuA	0.079	0.533	0.093	0.484	0.055	0.682	−0.098	0.462
ESR	−0.171	0.187	−0.091	0.484	−0.14	0.281	0.164	0.206
24-h proteinuria	0.059	0.681	−0.051	0.722	−0.228	0.107	0.188	0.187

*$P < 0.05$.

**$P < 0.01$.

SLE, systemic lupus erythematosus; Anti-dsDNA Ab, anti-double-strand DNA antibody; AnuA, anti-nucleosome antibody; C3, complement 3; C4, complement 4, ESR, erythrocyte sedimentation rate. Spearman's correlation coefficient (r) was applied to detect correlation between two numerical data.

CD16+ Monocytes in Patients with SLE Shared Different Cell-Surface Marker Profiles from CD16+ Monocytes in HCs

CD16+ monocytes from patients with SLE were characterized by lower expression of HLA-DR ($P < 0.05$) but higher CD163 and CCR5 ($P < 0.001$) expression compared with the monocytes from control donors ($P < 0.001$) (**Figures 3C–E**; Table S2 in Supplementary Material). CD16− monocytes from patients with SLE also expressed less HLA-DR but more CD163 compared with those from HCs ($P < 0.001$) (**Figures 3C,D**; Table S2 in Supplementary Material). Specially, CX3CR1 expression on CD16− monocytes was elevated in patients with SLE ($P < 0.01$) (**Figure 3F**; Table S2 in Supplementary Material). No difference in CD80 and CD86 expression on both CD16+ monocytes and CD16− monocytes was detected between patients with SLE and HCs (**Figures 3A,B**; Table S2 in Supplementary Material). In SLE patients, CD16+ monocytes also shared different phenotypes from CD16− monocytes (**Figure 3**). CD163 is an anti-inflammatory marker, whereas HLA-DR is a proinflammatory marker. These data demonstrated the downregulation of proinflammatory surface markers but the upregulation of anti-inflammatory markers in SLE, which was different from the presumptive results. This discrepancy might be due to a negative feedback existing to maintain monocyte homeostasis in SLE.

CD16+ Monocytes Perpetuated T-Cell-Mediated Inflammation in SLE

Purified monocyte subsets with CD4+ T cells from the same donors were cultured in the presence of anti-CD3 and anti-CD28 antibodies and M-CSF to evaluate the effects of CD16+ and CD16− monocytes on T-cell function. Anti-CD3 and anti-CD28 antibodies were added for supporting T-cell activation, while M-CSF was added for monocyte survival maintenance.

CD16+ Monocytes Were More Functionally Effective on Promoting T-Cell Responses Compared with CD16− Monocytes in HCs

CD4+ T cells demonstrated stronger Th1 (IFN-γ+CD4+) and Th2 (IL-4+CD4+) cell responses in the presence of CD16+ monocytes ($P < 0.05$) (**Figures 4A,B**). CD4+ T cells cocultured with CD16− monocytes also contained higher numbers of Th1 cells than did CD4+ T cells cultured alone ($P < 0.05$) (**Figure 4A**). Nevertheless, CD16− monocytes did not efficiently promote Th2 activation in HCs (**Figure 4B**). No difference in the induction of Th17-cell activation was observed (**Figure 4C**). Compared with the CD16− subset, CD16+ monocytes had no effect on promoting Treg (Foxp3+ CD4+) differentiation in HCs (**Figure 4D**).

CD16+ Monocytes Enhanced Th17-Cell Differentiation in SLE

In patients with SLE, the differentiation of CD4+ T cells into Th1, Th2, and Th17 cells increased in the presence of either CD16+ or CD16− monocytes (**Figures 4A–C**). Specially, CD16+ monocytes induced more Th2 responses compared with CD16− monocytes in patients with SLE ($P < 0.05$) (**Figure 4B**). CD16+ and CD16− monocytes from SLE patients were both potent at promoting CD4+ T cells to differentiate into Treg cells, although CD4+ T cells cocultured with CD16+ monocytes demonstrated weak effects on Treg responses than did CD4+ T cells cocultured with CD16− monocytes ($P < 0.01$) (**Figure 4D**).

The two monocyte subsets, particularly CD16+ monocytes, induced a significant increase in Th17 cells in patients with SLE compared with HCs ($P < 0.05$) (**Figure 4C**). However, no difference was found in the induction of Th1, Th2, and Treg cells by CD16+ monocytes and CD16− monocytes between patients with SLE and HCs, respectively (**Figures 4A,B,D**).

CD16+ Monocytes Induced T Cell Proinflammatory Cytokine Production in SLE

Next, whether CD16+ monocytes could influence the secretion of IFN-γ and IL-17A by T cells was assessed. Both CD16+ monocytes from patients with SLE and HCs upregulated IFN-γ secretion. However, no difference was found in the secretion of IFN-γ induced by CD16+ monocytes and CD16− monocytes between patients with SLE and HCs, respectively (**Figure 5A**). The two monocyte subsets, particularly CD16+ monocytes, induced a significant increase in IL-17A secretion in patients with SLE compared with HCs (**Figure 5B**), which was consistent with the flow cytometry data. Of note, the baseline concentrations of IFN- γ and

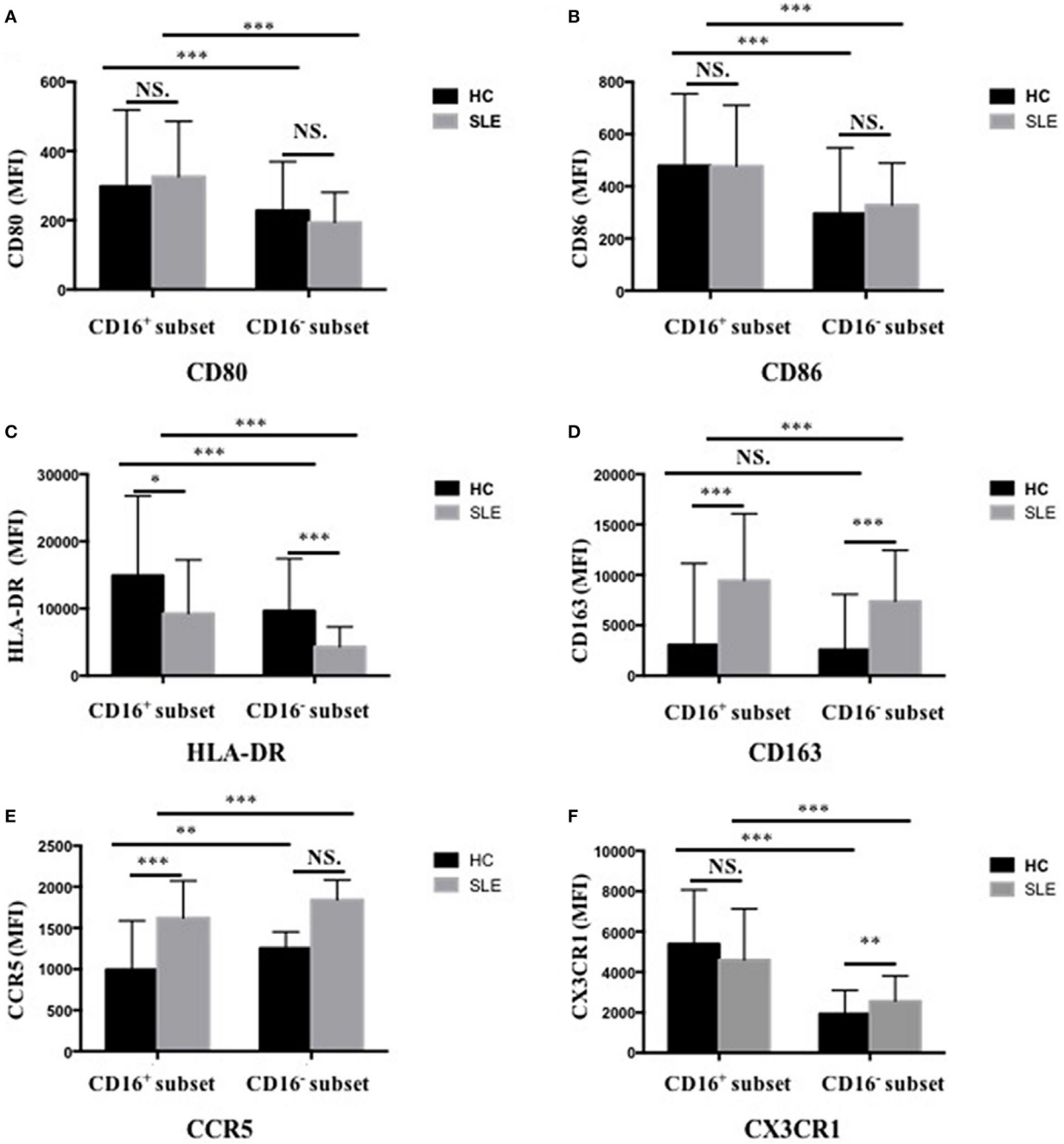


FIGURE 3 | CD16⁺ monocytes as well as CD16⁻ monocytes from patients with SLE and HCs shared different phenotypes regarding cell-surface markers, including CD80 (A), CD86 (B), HLA-DR (C), CD163 (D), CCR5 (E), and CX3CR1 (F). MFI, median fluorescence intensity. All samples were collected from fresh blood. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test and Mann–Whitney *U* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; NS, no significance.

IL-17A in HCs were higher than SLE, probably due to the *in vitro* operation-induced slight activation (collected from buffy coat).

CD16⁺ Monocytes Promoted T-Cell Proliferation in SLE

The percentage of $CFSE^{low}CD4^{+}$ T cells in T cells cocultured with $CD16^{+}$ monocytes was significantly higher compared with those in T cells cocultured with $CD16^{-}$ monocytes or T cells cultured alone ($P < 0.01$ and $P < 0.05$), indicating that $CD16^{+}$ monocytes were the predominant monocyte subset stimulating T-cell proliferation in HCs. Similarly, $CD16^{+}$ monocytes from SLE patients also induced significant $CD4^{+}$ T-cell proliferation ($P < 0.05$). Importantly, $CD16^{-}$ subset from patients with SLE also promoted $CD4^{+}$ T-cell proliferation.

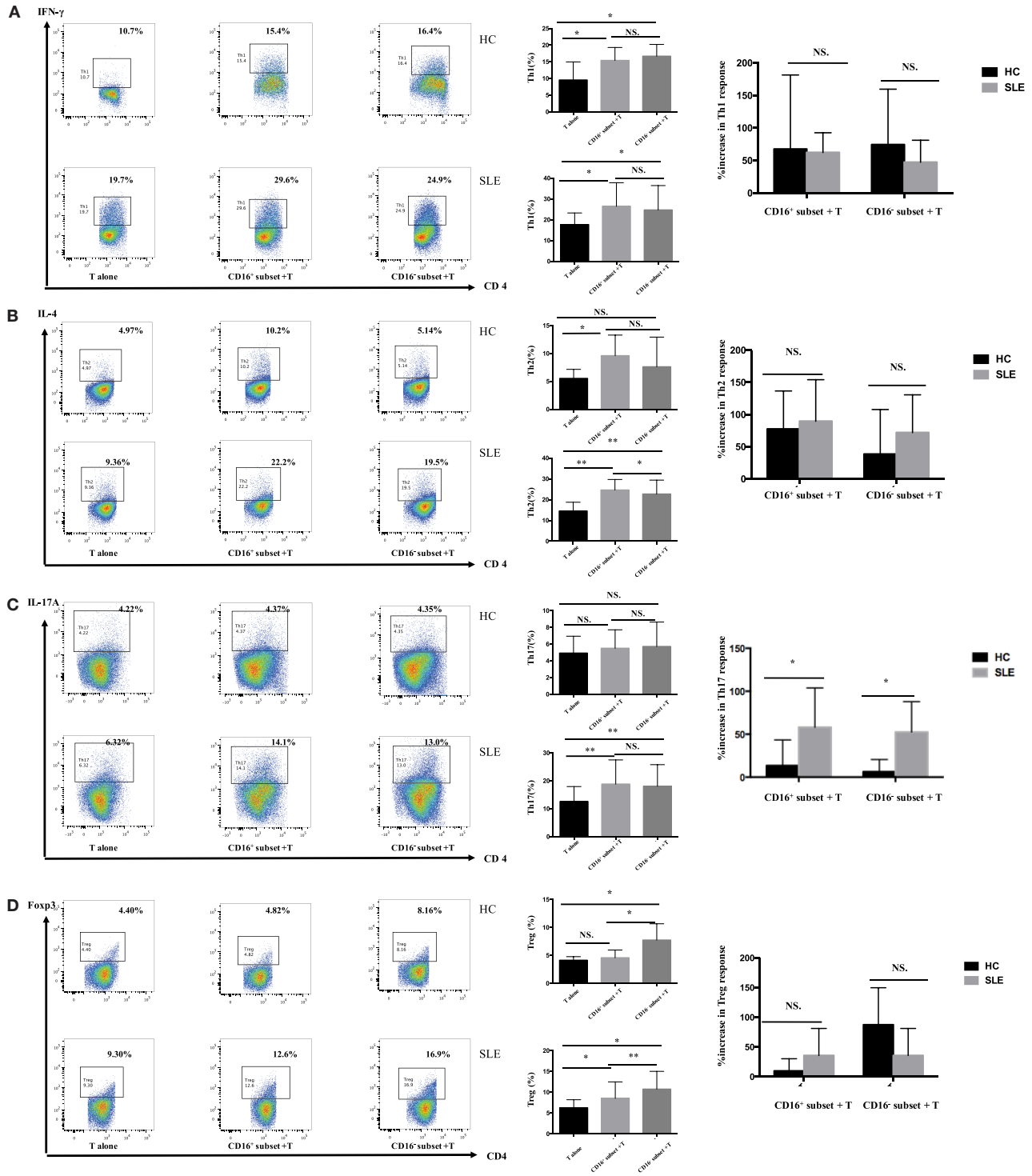


FIGURE 4 | CD16⁺ monocytes promoted T-cell-mediated inflammation in SLE. CD16⁺ or CD16⁻ monocytes were cocultured with CD4⁺ T cells isolated from freshly collected SLE blood or blood bank collected HC blood buffy coat for 5 days in the presence of anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies and M-CSF (50 ng/mL). Intracellular IFN-γ, IL-4, and IL-17A expression was detected by flow cytometry after PMA (50 ng/mL), ionomycin (1 μg/mL), and BFA (10 μg/mL) stimulation for 5 h on day 5. The percentage of Treg ($CD4^+Foxp3^+$) was also analyzed. Representative pseudocolor dots depicted Th1 **(A)**, Th2 **(B)**, Th17 **(C)**, and Treg **(D)** frequencies in CD4⁺ T cells after coculture with each monocyte subset from one patient and one control donor. The proportion of Th1, Th2, Th17, and Treg cells was calculated after coculture of CD4⁺ T cells with each monocyte. The percentage increases in T-cell subsets in cocultures of monocytes and T cells compared with CD4⁺ T cells cultured alone were compared between 7 healthy individuals and 10 patients with SLE. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test and Mann–Whitney *U* test. $^*P < 0.05$, $^{**}P < 0.01$; NS, no significance.

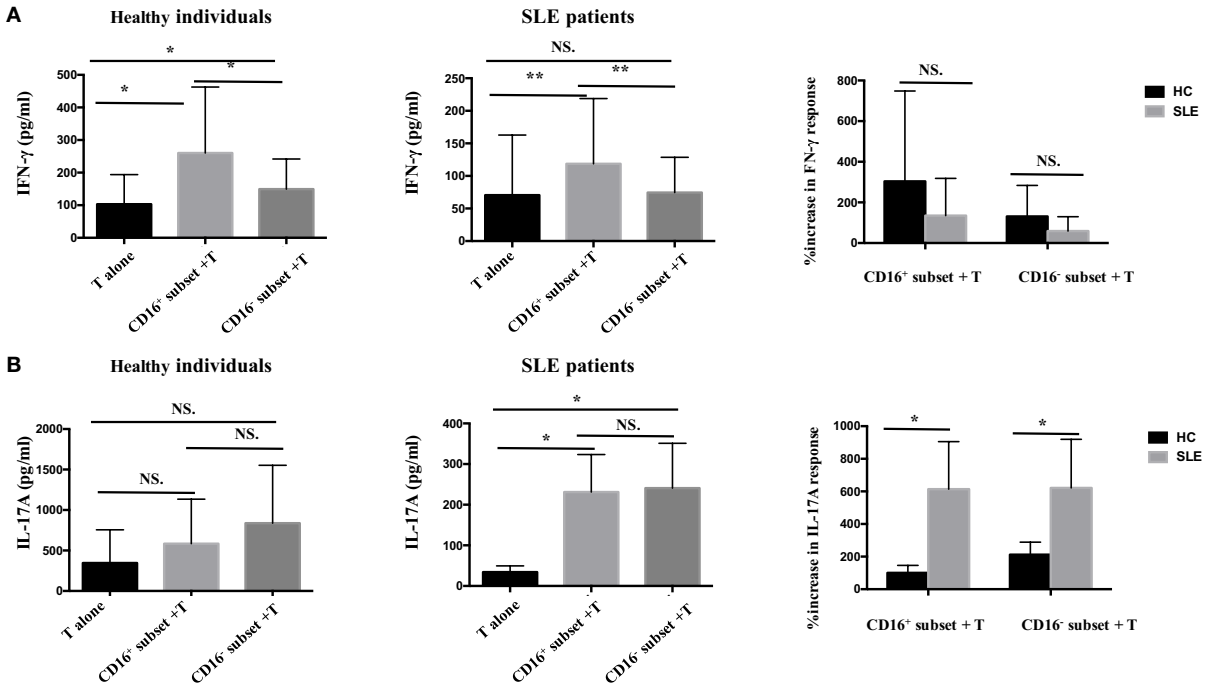


FIGURE 5 | CD16+ monocytes promoted T-cell-mediated cytokine secretion in SLE. CD16+ or CD16− monocytes were cocultured with CD4+ T cells isolated from freshly collected SLE blood or blood bank collected HC blood buffy coat for 5 days in the presence of anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies and M-CSF (50 ng/mL). The concentrations of IFN-γ and IL-17A in the supernatants were measured by ELISA. IFN-γ **(A)** and Th17A **(B)** levels were compared between different groups in HCs and patients with SLE. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test and Mann–Whitney *U* test. **P* < 0.05, ***P* < 0.01; NS, no significance.

However, CD16+ monocyte subset was more efficient than CD16− subset at promoting CD4+ T-cell proliferation in SLE ($P < 0.05$) (**Figures 6A,B**). T-cell proliferation was enhanced when cocultured with both SLE monocyte subsets, particularly CD16+ monocytes, suggesting the potential proinflammatory phenotype of monocyte subsets in SLE when compared with HCs (**Figure 6C**).

CD16+ Monocyte Subset Exacerbated B-Cell Activation in SLE

To test the effects of CD16+ and CD16− monocytes on B-cell activation, purified total B cells (CD19+) were cocultured with or without autologous monocyte subsets in the presence of anti-CD40 antibody and M-CSF. Anti-CD40 antibody and M-CSF supported B-cell and monocyte survival, respectively. Then, the levels of IgG, IgA, and IgM were detected in the coculture supernatants to explore further whether each monocyte subset could impact the total antibody response.

CD16+ Monocytes Induced CD19+ B Cells to Differentiate into MBs and PBs but Inhibited the Generation of Breg Cells in HCs

CD16+ monocytes promoted CD19+ B cells to differentiate into MBs and PBs in HCs (**Figures 7A,B**). However, CD19+ B cells cocultured with CD16− monocytes did not exhibit significantly increased frequencies of MBs and PBs. Both CD16+ and CD16− subsets suppressed CD19+ B cells to differentiate into Breg cells (**Figure 7C**). CD19+ B cells demonstrated more Breg differentiation inhibition, although not significant, in the presence of CD16+ monocytes compared with CD16− subset (**Figure 7C**).

Both PB Activation and Breg Differentiation Induced by CD16+ Monocytes Were Exacerbated in Patients with SLE

Both CD16+ and CD16− monocytes had no effects on MB responses (**Figure 7A**), while both monocyte subsets, particularly CD16+ monocytes, were effective at inducing B cells to differentiate into PBs in SLE (**Figure 7B**). In SLE, both CD16+ and CD16− monocytes slightly promoted Breg differentiation (**Figure 7C**).

MB activation induced by CD16+ monocytes was significantly attenuated in patients with SLE compared with HCs ($P < 0.05$), and MB activation induced by CD16− SLE subset was even inhibited ($P < 0.05$) (**Figure 7A**). In contrast, PB activation induced by CD16+ monocytes and CD16− subset was enhanced in SLE (**Figure 7B**). The phenomenon was also observed in Breg cell differentiation (**Figure 7C**). In patients with SLE, both CD16+ and CD16− subsets promoted Breg cell responses, which were different from the inhibitory roles of CD16+ and CD16− monocytes on Breg differentiation in HCs (**Figure 7C**).

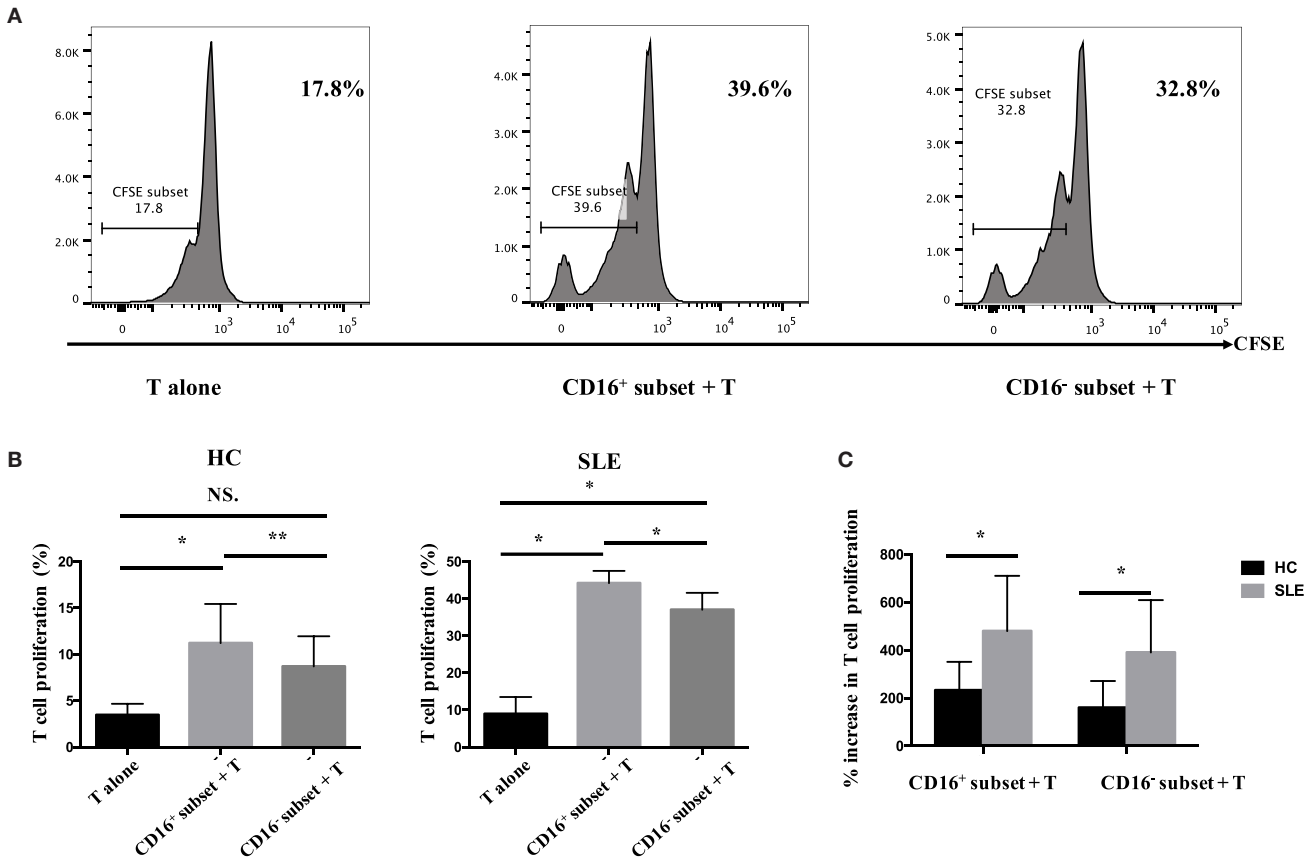


FIGURE 6 | CD16⁺ monocytes promoted T-cell proliferation in SLE. CFSE-labeled CD4⁺ T cells isolated from freshly collected SLE blood or blood bank collected HC blood buffy coat were cocultured with CD16⁺ and CD16⁻ monocytes for 60 h in the presence of anti-CD3 (1 μg/mL) and anti-CD 28 (1 μg/mL) antibodies and M-CSF (50 ng/mL). CFSE histograms depicted the number of events (*y*-axis) and the fluorescence intensity (*x*-axis), with proliferating cells displaying a progressive loss in fluorescence intensity following cell division, indicative of proliferating cells. **(A)** Representative histogram plots from a patient with SLE displayed the frequencies of $CFSE^{low}$ T cells in monocyte/T-cell cocultures and T cells cultured alone. **(B)** Percentages of $CFSE^{low}$ T cells in CD4⁺ T cells after coculture with each monocyte subset from five healthy donors and six patients with SLE. **(C)** The percentage increase in $CFSE^{low}$ T cells in cocultures of monocytes and T cells compared with CD4⁺ T cells cultured alone was compared between five healthy individuals and six patients with SLE. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test and Mann–Whitney *U* test. $^{*}P < 0.05$, $^{**}P < 0.01$; NS, no significance.

CD16⁺ Monocytes Were More Effective in Stimulating B-Cell IgG Secretion in SLE

Both CD16⁺ monocytes and CD16⁻ monocytes efficiently induced CD19⁺ B cells to secrete IgG and IgA in HCs. In particular, CD16⁺ subset was less efficient than CD16⁻ monocytes at promoting IgA production. Both CD16⁺ and CD16⁻ subsets had no effect on IgM response (**Figure 8A**).

Both CD16⁺ and CD16⁻ monocytes were potent at inducing CD19⁺ B cells to produce IgG in SLE. CD19⁺ B cells cocultured with CD16⁺ monocytes from SLE patients secreted elevated levels of IgA. On the contrary, CD19⁺ B cells cocultured with CD16⁻ monocytes exhibited increased production of IgM in SLE (**Figure 8B**). Compared with HCs, IgG responses induced by CD19⁺ B cells in the presence of CD16⁺ ($P < 0.05$) and CD16⁻ ($P < 0.01$) subsets were significantly exacerbated in patients with SLE. CD16⁻ monocytes from patients with SLE induced less IgA secretion compared with CD16⁻ monocytes from HCs ($P < 0.05$). No difference in IgM response induced by CD16⁺ and CD16⁻ subsets between patients with SLE and HCs was observed (**Figure 8C**). Of note, the baseline concentrations of Ig levels in HCs were a bit higher than SLE, probably due to the *in vitro* operation-induced slight activation (collected from buffy coat).

DISCUSSION

This study showed that an enrichment of CD16⁺ monocytes in the peripheral blood of patients with SLE is associated with serum autoantibody production and that CD16⁺ monocytes exhibited a proinflammatory phenotype with high CD80, CD86, HLA-DR, and CX3CR1 expression. In SLE, CD16⁺ monocyte subset induced both Th1/Th2 cell expansion and promoted Treg development and had an enhanced capacity to promote T-cell proliferation and differentiation into a Th17 phenotype. The study demonstrated for the first time that CD16⁺ monocytes from patients with SLE could efficiently drive B-cell responses, with

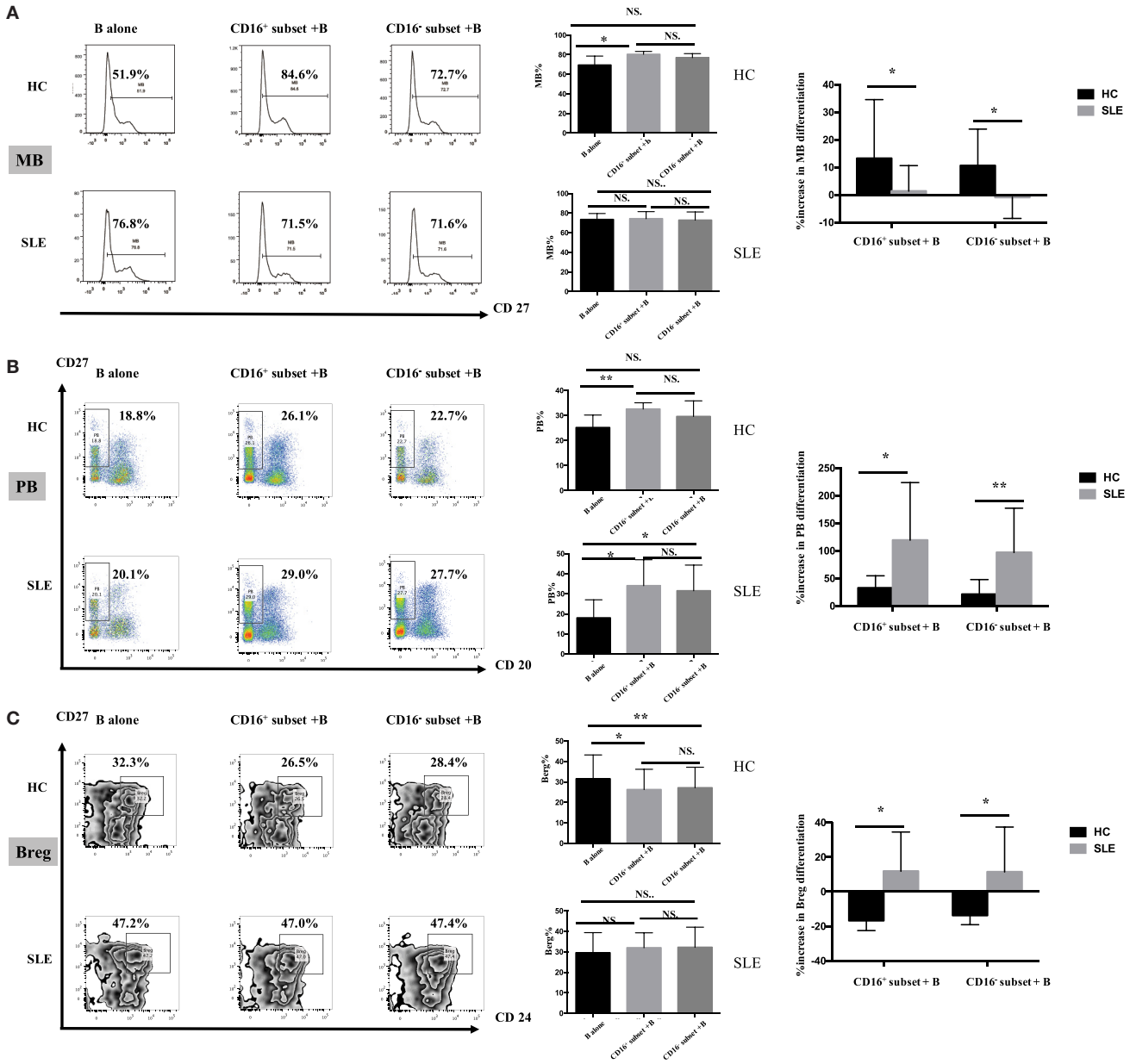


FIGURE 7 | **CD16⁺ monocyte subset exacerbated B-cell activation in SLE**. SLE CD16⁺ monocytes had an enhanced capacity on PB and Breg differentiation but attenuated MB development. Monocyte subsets were cocultured with CD19⁺ B cells isolated from freshly collected SLE blood or blood bank collected HC blood buffy coat for 3 days in the presence anti-CD40 antibody (3 μg/mL) and M-CSF (50 ng/mL). The expression of CD20 and CD27 as well as CD24 was assessed. **(A)** Representative histogram plots from HCs and patients with SLE displayed the frequencies of MB ($CD27^+CD19^+$) cells in monocyte/B-cell cocultures. Graphs showed the cumulative MB frequencies in cocultures of monocytes/B cells and $CD19^+$ B cells cultured alone from eight healthy individuals and seven patients with SLE. The difference in the percentage increase in MB cells in cocultures of monocytes and B cells compared with $CD19^+$ B cells cultured alone was compared between HCs and SLEs. **(B)** Representative dot plots from HCs and patients with SLE demonstrated the frequencies of PB ($CD20^-CD27^+CD19^+$) cells in monocyte/B-cell cocultures. Graphs showed the cumulative frequencies of PBs in cocultures of monocytes/B cells and $CD19^+$ B cells cultured alone from eight healthy individuals and seven patients. Percentage increase in PBs in cocultures of monocytes and B cells compared with $CD19^+$ B cells cultured alone was identified between HCs and patients with SLE. **(C)** Representative contour plots from HCs and patients with SLE showed the frequencies of Breg ($CD24^+CD27^+CD19^+$) cells in monocyte/B-cell cocultures. Graphs displayed the cumulative frequency of Bregs in cocultures of monocytes/B cells and $CD19^+$ B cells cultured alone from eight healthy individuals and five patients with SLE. Percentage increase in Bregs in cocultures of monocytes and B cells compared with $CD19^+$ B cells cultured alone was compared between HCs and patients with SLE. MBs, memory B cells; PBs, plasma B cells; Bregs, regulatory B cells. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test and Mann–Whitney *U* test. $^*P < 0.05$, $^{**}P < 0.01$; NS, no significance.

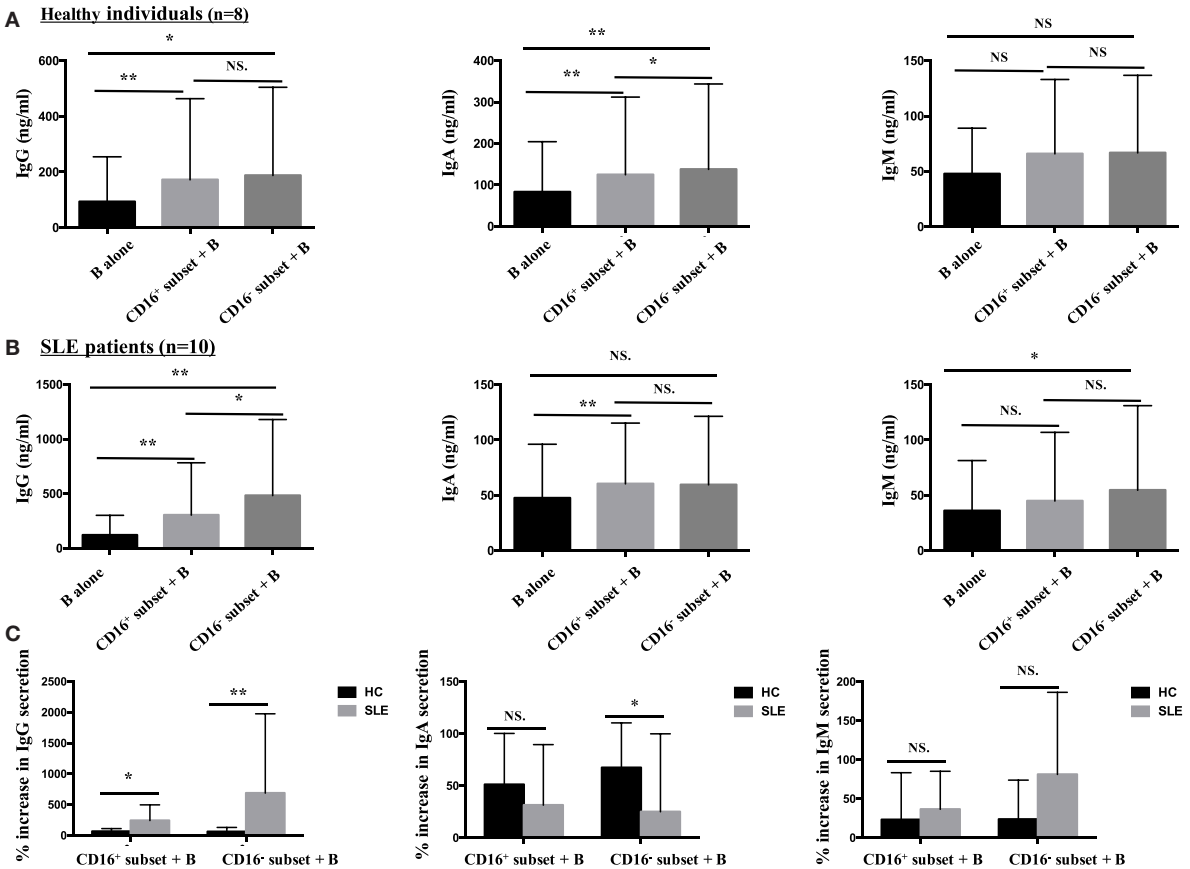


FIGURE 8 | CD16⁺ monocytes were more effective in stimulating B-cell IgG secretion in patients with SLE. CD16⁺ or CD16⁻ monocytes were cocultured with CD19⁺ B cells isolated from freshly collected SLE blood or blood bank collected HC blood buffy coat for 3 days in the presence of anti-CD40 antibody (3 μg/mL) and M-CSF (50 ng/mL). The total Ig was assayed by ELISA. **(A)** Bar graphs showed the Ig (IgG, IgA, and IgM) levels in the supernatant of monocyte/B-cell cocultures and CD19⁺ B cells cultured alone from eight healthy individuals. **(B)** Bar graphs demonstrated the Ig (IgG, IgA, and IgM) levels in the supernatant of monocyte/B-cell cocultures and CD19⁺ B cells cultured alone from 10 patients with SLE. **(C)** The Ig increase in cocultures of monocytes and B cells compared with CD19⁺ B cells cultured alone was compared between eight healthy individuals and 10 patients with SLE. Data were expressed as mean ± SD and analyzed by non-parametric paired *t* test and Mann–Whitney *U* test. $^{*}P < 0.05$, $^{**}P < 0.01$; NS, no significance.

exacerbated impacts on PB and Breg differentiation as well as IgG production but attenuated effects on the generation of MB cells.

This study showed that the frequencies of CD16⁺ subset increased, while CD16⁻ monocytes decreased in patients with SLE. Further analysis showed that the proportions of non-classical and IM were higher in SLE than their healthy counterparts, which was consistent with the findings of Mukherjee (21). This observation was also consistent with the data showing that CD16⁺ monocyte subsets are enriched in some autoimmune diseases and may be involved in the induction of inflammatory immune response (38–41). The possible explanation of monocyte alteration is that the *in vivo* cytokine and hormone environments in SLE may lead to the conversion of CD16⁻ monocytes into CD16⁺ monocytes (20). It was shown that CD16⁺ monocytes were the producers of proinflammatory cytokines, including TNFα, IL-1, and IL-6 (13–16, 42). Mikołajczyk et al. demonstrated that CD14dimCD16⁺ monocytes might be an important subpopulation of proinflammatory monocytes related to increased development of atherosclerosis in SLE (22). The elevated surface expression of CD80, CD86, HLA-DR, and CX3CR1 (43) on CD16⁺ monocytes further indicated their involvement in inflammatory immune response. The chemokine receptor CCR5 plays an important role in recruiting these cells into inflamed organs and consumes its own ligands to restrain local chemokine levels, thereby limiting inflammatory cell influx (44). The CCR5 downregulation on CD16⁺ intermediate and non-classical subsets may explain their anti-inflammatory features during the disease course. Both CD16⁺ subsets and CD16⁻ monocytes from SLE patients exhibited a widely changes on cell-surface marker expression, which may be explained by immunosuppressive therapy in patients with SLE (45), but it remains unknown whether treatment with SLE agents can change the monocyte phenotypes and further study was necessary for reasonable explanation in the future.

Disturbed T-cell signaling and Th17/Treg imbalance are documented to play an important role in developing SLE

and could be responsible for an increased proinflammatory response, especially in the active form of the disease (4, 46, 47). Human monocytes are well known to influence $CD4^+$ cells to differentiate into discrete Th cell subsets. The results presented in this work investigated the effects of each monocyte subset on the control of Th and Treg development in patients with SLE and their healthy counterparts. In contrast to $CD16^-$ monocytes, $CD16^+$ subset significantly promoted the expansion of IL-4-producing T cells in HCs. Also, $CD16^+$ monocyte subset had no effects on Treg induction, supporting its defective inflammation regulatory roles. $CD16^+$ subset seemed to be equivalent to $CD16^-$ subset at inducing the activation of IFN-γ-producing T cells. Interestingly, Th17 cell development was not influenced by both $CD16^+$ inflammatory monocytes and classical $CD16^-$ monocytes in HCs. These data suggested the predominant role of circulating $CD16^+$ monocyte subset in controlling the balance in Th-polarized immune response. $CD16^+$ blood monocytes as well as $CD16^-$ subsets can modulate Th1, Th2, and Treg development in patients with SLE. The critical finding of the present study was the identification of each monocyte subset that could modulate Th17 expansion in patients with SLE, to a lesser extent, than in HCs. In recent years, IL-17-producing $CD4^+$ T cells have emerged as a major pathogenic T-cell population that is present at increased frequencies in SLE and that correlates with disease severity (47). Recovery of the immune balance between Th17 and regulatory T cells may serve as a treatment for SLE (48). The underlying mechanisms of this expansion of the Th17-cell population are not fully understood, but the present results demonstrated that SLE monocyte subsets were able to drive remarkable Th17-cell expansion *in vitro*, indicating a central role of abnormal monocyte distribution in this process.

Autoantibodies are a hallmark of SLE, and B-cell deletion remains one of the main effective therapies in this disease (49). The present study demonstrated for the first time that both $CD16^+$ and $CD16^-$ monocytes from patients with SLE exhibited exacerbated capacity for B-cell response, leading to abnormal antibody secretion. In particular, $CD16^+$ and $CD16^-$ monocytes from patients with SLE could efficiently induce more PBs compared with $CD16^+$ and $CD16^-$ monocyte subset from HCs. Although HC monocytes were able to induce IgG secretion, $CD16^+$, and $CD16^-$ monocytes from patients with SLE were more efficient than the corresponding HC monocyte subsets at inducing IgG response. The present results also indicated that the expansion of $CD16^+$ monocytes was positively correlated with autoantibody production in patients with SLE, which further confirmed that abnormal monocyte subset was responsible for aberrant B-cell activation. All these suggested that a potential heightened activation state of each monocyte subset in SLE might result in skewed B-cell responses, leading to augment autoantibody production, which was consistent with the finding that B cells were overactivated in SLE (1, 50, 51). Specially, MB activation induced by $CD16^+$ monocytes was significantly attenuated, and MB differentiation induced by $CD16^-$ monocytes was even inhibited in patients with SLE. The underlying mechanism of this discrepancy need to be further studied.

In summary, the present study demonstrated the expansion of pathogenic $CD16^+$ monocytes in SLE, revealing their important roles in stimulating Th cell subsets and the differentiation of B-cell response in diseased condition. These data highlighted the importance of the innate immune system in eliciting pathogenic T- and B-cell responses in SLE and suggested that specific monocyte subsets might be critical targets to control the inflammation.

AUTHOR CONTRIBUTIONS

HZ performed most of the experiments. FH, XS, and YS conceived the study and participated in the design and interpretation of results. XZ, XLiu, and XLi helped to collect samples. LZ, LX, LS, and YG participated in the experiments and drafting the manuscript. All the authors read and approved the final manuscript.

FUNDING

This work was supported by grants from National Natural Science Foundation of China (81172844, 81471600, and 81671609 to Dr. YS; 81671604 and 81302554 to Dr. FH).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00512/full#supplementary-material.

REFERENCES

1. Crispín JC, Liossis SN, Kis-Toth K, Lieberman LA, Kyttaris VC, Juang YT, et al. Pathogenesis of human systemic lupus erythematosus: recent advances. *Trends Mol Med* (2010) 16(2):47–57. doi:10.1016/j.molmed.2009.12.005
2. Choi J, Kim ST, Craft J. The pathogenesis of systemic lupus erythematosus – an update. *Curr Opin Immunol* (2012) 24:651–7. doi:10.1016/j.coi.2012.10.004
3. Lisnevskaia L, Murphy G, Isenberg D. Systemic lupus erythematosus. *Lancet* (2014) 384(9957):1878–88. doi:10.1016/S0140-6736(14)60128-8
4. Rother N, van der Vlag J. Disturbed T cell signaling and altered Th17 and regulatory T cell subsets in the pathogenesis of systemic lupus erythematosus. *Front Immunol* (2015) 6:610. doi:10.3389/fimmu.2015.00610
5. Hargreaves CE, Rose-Zerilli MJ, Machado LR, Iriyama C, Hollox EJ, Cragg MS, et al. Fcγ receptors: genetic variation, function, and disease. *Immunol Rev* (2015) 268(1):6–24. doi:10.1111/imr.12341
6. Guilliams M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fcγ receptors in dendritic cells and macrophages. *Nat Rev Immunol* (2014) 14(2):94–108. doi:10.1038/nri3582
7. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* (2010) 116(16):e74–80. doi:10.1182/blood-2010-02-258558
8. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. *Immunol Res* (2012) 53(1–3):41–57. doi:10.1007/s12026-012-8297-3
9. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science* (2010) 327(5966):656–61. doi:10.1126/science.1178331
10. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and non-classical human monocyte subsets. *Blood* (2011) 118(5):e16–31. doi:10.1182/blood-2010-12-326355

11. Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, et al. SuperSAGE evidence for CD14 CD16 monocytes as a third monocyte subset. *Blood* (2011) 118(12):e50–61. doi:10.1182/blood-2011-01-326827
12. Kim WK, Sun Y, Do H, Autissier P, Halpern EF, Piatak M Jr, et al. Monocyte heterogeneity underlying phenotypic changes in monocytes according to SIV disease stage. *J Leukoc Biol* (2010) 87(4):557–67. doi:10.1189/jlb.0209082
13. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, et al. The proinflammatory CD14+CD16+DR++monocytes are a major source of TNF. *J Immunol* (2002) 168:3536–42. doi:10.4049/jimmunol.168.7.3536
14. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. *Immunobiology* (2006) 211(6–8):609–18. doi:10.1016/j.imbio.2006.05.025
15. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* (2007) 81(3):584–92. doi:10.1189/jlb.0806510
16. Fingerle G, Pforte A, Passlick B, Blumenstein M, Ströbel M, Ziegler-Heitbrock HW. The novel subset of CD14+/CD16+blood monocytes is expanded in sepsis patients. *Blood* (1993) 82:3170–6.
17. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, et al. CD14+ CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum* (2002) 46:2578–86. doi:10.1002/art.10545
18. Castano D, Garcia LF, Rojas M. Increased frequency and cell death of CD16+ monocytes with Mycobacterium tuberculosis infection. *Tuberculosis (Edinb)* (2011) 91:348–60. doi:10.1016/j.tube.2011.04.002
19. Hilliard BA, Zizzo G, Ulas M, Linan MK, Schreiter J, Cohen PL. Increased expression of Mer tyrosine kinase in circulating dendritic cells and monocytes of lupus patients: correlations with plasma interferon activity and steroid therapy. *Arthritis Res Ther* (2014) 16(2):R76. doi:10.1186/ar4517
20. Burbano C, Vasquez G, Rojas M. Modulatory effects of CD14+CD16++ monocytes on CD14++CD16- monocytes: a possible explanation of monocyte alterations in Systemic Lupus Erythematosus. *Arthritis Rheumatol* (2014) 66(12):3371–81. doi:10.1002/art.38860
21. Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B. Non-Classical monocytes display inflammatory features: validation in Sepsis and Systemic Lupus Erythematous. *Sci Rep* (2015) 5:13886. doi:10.1038/srep13886
22. Mikołajczyk TP, Osmenda G, Batko B, Wilk G, Krezelok M, Skiba D, et al. Heterogeneity of peripheral blood monocytes, endothelial dysfunction and subclinical atherosclerosis in patients with systemic lupus erythematosus. *Lupus* (2016) 25(1):18–27. doi:10.1177/0961203315598014
23. Zhong H, Bao W, Li X, Miller A, Seery C, Haq N, et al. CD16+ monocytes control T-cell subset development in immune thrombocytopenia. *Blood* (2012) 120(16):3326–35. doi:10.1182/blood-2012-06-434605
24. Roberts CA, Dickinson AK, Taams LS. The interplay between monocytes/macrophages and CD4(+) T cell subsets in rheumatoid arthritis. *Front Immunol* (2015) 6:571. doi:10.3389/fimmu.2015.00571
25. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* (2009) 27:669–92. doi:10.1146/annurev.immunol.021908.132557
26. Joo H, Coquery C, Xue Y, Gayet I, Dillon SR, Punaro M, et al. Serum from patients with SLE instructs monocytes to promote IgG and IgA plasma-blast differentiation. *J Exp Med* (2012) 209(7):1335–48. doi:10.1084/jem.20111644
27. Xu W, Joo H, Clayton S, Dullaers M, Herve MC, Blankenship D, et al. Macrophages induce differentiation of plasma cells through CXCL10/IP-10. *J Exp Med* (2012) 209(10):S1–2. doi:10.1084/jem.20112142
28. Dubois B, Vanbervliet B, Fayette J, Massacrier C, Van Kooten C, Brière F, et al. Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J Exp Med* (1997) 185(5):941–51. doi:10.1084/jem.185.5.941
29. Teichmann LL, Ols ML, Kashgarian M, Reizis B, Kaplan DH, Shlomchik MJ. Dendritic cells in lupus are not required for activation of T and B cells but promote their expansion, resulting in tissue damage. *Immunity* (2010) 33(6):967–78. doi:10.1016/j.immuni.2010.11.025
30. Mohr E, Serre K, Manz RA, Cunningham AF, Khan M, Hardie DL, et al. Dendritic cells and monocyte/macrophages that create the IL-6/APRIL-rich lymph node microenvironments where plasmablasts mature. *J Immunol* (2009) 182(4):2113–23. doi:10.4049/jimmunol.0802771
31. Xu W, Banchereau J. The antigen presenting cells instruct plasma cell differentiation. *Front Immunol* (2014) 4:504. doi:10.3389/fimmu.2013.00504
32. Wijbrandts CA, Dijkgraaf MG, Kraan MC, Vinkenoog M, Smeets TJ, Dinant H, et al. The clinical response to infliximab in rheumatoid arthritis is in part dependent on pretreatment tumor necrosis factor alpha expression in the synovium. *Ann Rheum Dis* (2008) 67(8):1139–44. doi:10.1136/ard.2007.080440
33. Ding C, Jones G. Anti-interleukin-6 receptor antibody treatment in inflammatory autoimmune diseases. *Rev Recent Clin Trials* (2006) 1(3):193–200. doi:10.2174/157488706778250168
34. Kamal A, Khamashta M. The efficacy of novel B cell biologics as the future of SLE treatment: a review. *Autoimmun Rev* (2014) 13(11):1094–101. doi:10.1016/j.autrev.2014.08.020
35. Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* (2012) 64(8):2677–86. doi:10.1002/art.34473
36. Hu F, Mu R, Zhu J, Shi L, Li Y, Liu X, et al. Hypoxia and hypoxia-inducible factor-1α provoke toll-like receptor signalling-induced inflammation in rheumatoid arthritis. *Ann Rheum Dis* (2014) 73(5):928–36. doi:10.1136/annrheumdis-2012-202444
37. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* (2013) 5(173):173ra23. doi:10.1126/scitranslmed.3005407
38. Katayama K, Matsubara T, Fujiwara M, Koga M, Furukawa S. CD14+CD16+ monocyte subpopulation in Kawasaki disease. *Clin Exp Immunol* (2000) 121(3):566–70. doi:10.1046/j.1365-2249.2000.01321.x
39. Okamoto H, Mizuno K, Horio T. Circulating CD14+ CD16+ monocytes are expanded in sarcoidosis patients. *J Dermatol* (2003) 30(7):503–9. doi:10.1111/j.1346-8138.2003.tb00424.x
40. Hanai H, Iida T, Takeuchi K, Watanabe F, Yamada M, Kikuyama M, et al. Adsorptive depletion of elevated pro-inflammatory CD14+CD16+DR++ monocytes in patients with inflammatory bowel disease. *Am J Gastroenterol* (2008) 103(5):1210–6. doi:10.1111/j.1572-0241.2007.01714.x
41. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14brightCD16 monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheum* (2012) 64(3):671–7. doi:10.1002/art.33418
42. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* (2010) 33(3):375–86. doi:10.1016/j.immuni.2010.08.012
43. Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW, et al. Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J Exp Med* (2003) 197(12):1701–7. doi:10.1084/jem.20022156
44. Turner JE, Paust HJ, Bennstein SB, Bramke P, Krebs C, Steinmetz OM, et al. Protective role for CCR5 in murine lupus nephritis. *Am J Physiol Renal Physiol* (2012) 302(11):F1503–15. doi:10.1152/ajprenal.00382.2011
45. Varga G, Ehrchen J, Brockhausen A, Weinhage T, Nippe N, Belz M, et al. Immune suppression via glucocorticoid-stimulated monocytes: a novel mechanism to cope with inflammation. *J Immunol* (2014) 193(3):1090–9. doi:10.4049/jimmunol.1300891
46. Shin MS, Lee N, Kang I. Effector T-cell subsets in systemic lupus erythematosus: update focusing on Th17 cells. *Curr Opin Rheumatol* (2011) 23(5):444–8. doi:10.1097/BOR.0b013e328349a255
47. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: correlation with disease activity. *Cytokine* (2015) 72(2):146–53. doi:10.1016/j.cyto.2014.12.027
48. Yang J, Yang X, Zou H, Chu Y, Li M. Recovery of the immune balance between Th17 and regulatory T cells as a treatment for systemic lupus erythematosus.

Rheumatology (Oxford) (2011) 50(8):1366–72. doi:10.1093/rheumatology/ker116

49. Sanz I, Lee FE. B cells as therapeutic targets in SLE. *Nat Rev Rheumatol* (2010) 6(6):326–37. doi:10.1038/nrrheum.2010.68
50. Bird AK, Meednu N, Anolik JH. New insights into B cell biology in systemic lupus erythematosus and Sjögren's syndrome. *Curr Opin Rheumatol* (2015) 27(5):461–7. doi:10.1097/BOR.0000000000000201
51. Jacob N, Stohl W. Autoantibody-dependent and autoantibody-independent roles for B cells in systemic lupus erythematosus: past, present, and future. *Autoimmunity* (2010) 43(1):84–97. doi:10.3109/08916930903374600

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

PERSPECTIVE
published: 26 July 2017
doi: 10.3389/fimmu.2017.00872

Defining Natural Antibodies

Nichol E. Holodick[1], Nely Rodríguez-Zhurbenko[2] and Ana María Hernández[2]**

[1] Department of Biomedical Sciences, Center for Immunobiology, Western Michigan University Homer Stryker M.D. School of Medicine, Kalamazoo, MI, United States, [2] Natural Antibodies Group, Tumor Immunology Division, Center of Molecular Immunology, Havana, Cuba

The traditional definition of natural antibodies (NAbs) states that these antibodies are present prior to the body encountering cognate antigen, providing a first line of defense against infection thereby, allowing time for a specific antibody response to be mounted. The literature has a seemingly common definition of NAbs; however, as our knowledge of antibodies and B cells is refined, re-evaluation of the common definition of NAbs may be required. Defining NAbs becomes important as the function of NAb production is used to define B cell subsets (1) and as these important molecules are shown to play numerous roles in the immune system (**Figure 1**). Herein, we aim to briefly summarize our current knowledge of NAbs in the context of initiating a discussion within the field of how such an important and multifaceted group of molecules should be defined.

Keywords: natural antibody, antibodies, natural antibody repertoire, B-1 cells, B cell subsets, B cells

OPEN ACCESS

Edited by:
Harry W. Schroeder, University of Alabama at Birmingham, United States

Reviewed by:
Andre M. Vale, Federal University of Rio de Janeiro, Brazil
Michael Zemlin, Universitätsklinikum des Saarlandes, Germany

****Correspondence:***
Nichol E. Holodick
nichol.holodick@med.wmich.edu;
Ana María Hernández
anita@cim.sld.cu

Specialty section:
This article was submitted to B Cell Biology, a section of the journal Frontiers in Immunology

Received: *27 April 2017*
Accepted: *10 July 2017*
Published: *26 July 2017*

Citation:
Holodick NE, Rodríguez-Zhurbenko N and Hernández AM (2017) Defining Natural Antibodies. Front. Immunol. 8:872. doi: 10.3389/fimmu.2017.00872

NATURAL ANTIBODY (NAb) PRODUCING CELLS

Both murine and human NAbs have been discussed in detail since the late 1960s (2, 3); however, cells producing NAbs were not identified until 1983 in the murine system (4, 5). These cells, named B-1 cells, were originally identified by their expression of CD5 and were further characterized by surface expression of IgM^{high}, IgD^{low}, $CD19^{high}$, $B220^{low}$, $CD23^{-}$, and $CD43^{+}$ (6), which contrasts with the surface phenotype of follicular B-2 cells: $CD5^{-}$, IgM^{low}, IgD^{high}, $CD19^{+}$, $B220^{+}$, $CD23^{+}$, and $CD43^{-}$. Later, an additional population of B-1 cells was identified, which shared the characteristics of $CD5^{+}$ B-1 but lacked CD5 expression (7). These two populations of B-1 cells are termed B-1a ($CD5^{+}$) and B-1b ($CD5^{-}$) cells. B-1 cells also express CD11b; however, this expression is limited to B-1 cells residing in the body cavities and is lost upon migration to the spleen (8, 9). Furthermore, the B-1 cell population can be divided not only phenotypically but also functionally into natural or antigen-induced antibody secreting cells (10).

B-1 cells are found in various tissues of adult mice, which include the peritoneal cavity, pleural cavity, spleen, bone marrow, lymph nodes, and blood [reviewed in Ref. (11)]. The tissue location may influence the functional role of B-1 cells. The peritoneal and pleural cavities have been shown to be an important reservoir for B-1 cells that respond to various stimuli (12–16) and subsequently migrate to the spleen/mesenteric or mediastinal lymph nodes, respectively, where they begin to secrete antibody (17). In mice depleted of B cells, peritoneal B-1 cells have the ability to fully reconstitute natural serum immunoglobulin (Ig) M as well as B-1 cells in all tissue locations (18); yet, in normal healthy mice, peritoneal B-1a cells do not directly contribute to natural serum IgM (19). Instead, the direct sources of natural serum IgM are B-1a cells located in the spleen and bone marrow (19). It has been shown that peritoneal B-1a cells recirculate from the peritoneum to the blood in a CXCL13-dependent manner (20). Interestingly, in the absence of CXCL13, mice are devoid of peritoneal B-1 cells but still have splenic B-1 cells; yet, despite having normal levels of serum IgM these mice have significantly less natural IgM specific for phosphorylcholine (20).

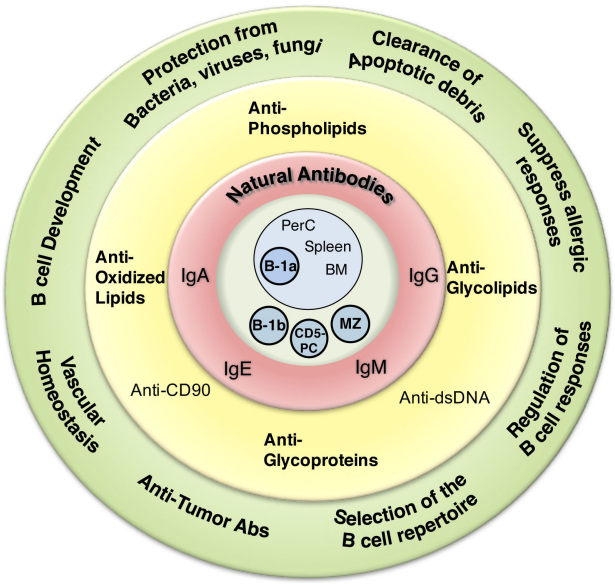


FIGURE 1 | Overview of natural antibodies' (NAbs) attributes. Graphical representation of the various NAb functions (outside green circle), epitope recognition (inside yellow circle), isotype (inside red circle), and cells shown to produce NAbs (inside blue circle).

This study suggests that it is possible for peritoneal B-1 cells to contribute to the splenic B-1 cell population and this recirculation might be particularly important for certain NAb reactivites. To date, the exact developmental relationship between the NAb secreting splenic/bone marrow B-1a cells and peritoneal B-1a cells is still unknown.

Beyond heterogeneity at different tissue sites, various subpopulations of B-1a cells have been defined based on surface marker expression. In the peritoneal cavity, B-1a subpopulations include PD-L2 (PD-L2$^{+/-}$) (21, 22), CD25 (CD25$^{+/-}$) (23), CD73 (CD73$^{hi/lo}$) (24), and PC-1 (PC-1$^{hi/lo}$). The PD-L2, CD25, and CD73 subsets showed no difference in the amount of natural IgM secretion between positive and negative subsets (21–24). Conversely, PC-1 B-1a cell subsets differed in the level of natural IgM secretion. PC-1^{lo} B-1a cells were shown to produce the large majority of natural IgM (25). PC-1^{hi} B-1a cells produced a significantly lower level of natural IgM and contained B-1a cells producing the antiphosphatidylcholine (anti-PtC) specificity (25, 26). B-1a cells have also been shown to produce IL-10 in the absence of stimulation (27); however, the relationship between regulatory (B10) cells (28) and B-1a cells is still unknown. In the bone marrow, a fetal-derived B cell subset was recently identified, which phenotypes as a plasmablast/plasma cell (CD5$^-$IgM$^+$IgD$^-$CD138$^+$B220$^{lo/-}$ FSC^{hi}CD43$^+$) (29); it is unknown whether this population is a terminally differentiated B-1, B-2, or novel population of cells (19). In the spleen, a population of CD138 + B-1a cells is present in unimmunized mice, which rapidly respond to stimulation prior to immigration of peritoneal B-1 cells to the spleen (30). The spleen is also home to marginal zone B cells, which also produce NAbs (31); however, it has been demonstrated that greater than 90% of NAb is produced by B-1 cells (18).

Given the evidence described previously, it is clear in mice, more than one B cell population is responsible for NAb production and not all subsets of B-1 cells spontaneously secrete NAbs that accumulate in serum. Thus, the generalization that all B-1 cells secrete NAbs should be avoided. This point has important implications when comparing the molecular repertoire of a certain B-1 cell subset as it relates to the total natural serum IgM repertoire, which would include the molecular repertoire of numerous B-1 cell subsets from various locations (10).

Natural antibody secreting cells in humans were first identified as CD5$^+$ peripheral B cells (32–35). Later, it was demonstrated that CD5$^-$CD45RA^{lo} peripheral B cells could also produce natural IgM (36). Much of the early work in humans focused on characterization and comparison of polyreactive antibodies, which were shown to utilize VH4 more frequently than monoreactive antibodies (37). More recently, strides have been made to refine the phenotypic characterization of NAb producing cells in the human system by starting with functional characteristics such as natural/spontaneous antibody secretion. This approach yielded a new phenotypic definition, CD20$^+$CD27$^+$CD43$^+$CD70$^-$CD38^{mod}, of NAb secreting cells, the majority of which express CD5 (1, 38). Nevertheless, the phenotype of antibody secreting cells in the peripheral blood of humans is still evolving. Further investigation of NAb secreting cells in the human system is needed to elucidate the specific types of cells that are capable of producing NAbs, as well as the location of these cells beyond peripheral blood.

NAb REACTIVITY

Although NAbs are known for their broad reactivity against self-antigens, some have the ability to recognize evolutionarily fixed epitopes present in foreign antigens. Whether or not NAb recognition of foreign structures is always the result of cross-reactivity against self-antigens is still a matter of debate. Generally, the most well-characterized epitopes to date include phospholipids, oxidized lipids, glycolipids, and glycoproteins. The best characterized B-1 cell-derived NAb binds the phospholipid phosphorylcholine and utilizes VHS107.1 (39). Phosphorylcholine is found within the bacterial cell wall of *Streptococcus pneumoniae* (40) and is also exposed on apoptotic cells and oxidized lipids (41–45). In normal healthy cells, phosphorylcholine is hidden within the head group of another well-characterized NAb epitope, PtC. PtC is a normal constituent of cell membranes, which is exposed upon treatment with the protease, bromelain (46–49). Early studies revealed NAb binding to red blood cells treated with bromelain were B-1 cell derived and utilized VH11 (50, 51), VH12 (52), and Q52 (53).

Antibodies that recognize glycan epitopes are also highly abundant in both mice and humans (54, 55). Glycan epitopes are observed on both glycoproteins and glycolipids and can be present in autologous or pathogen-associated exogenous structures. In mice, the specificities of such antibodies are thoroughly reviewed by New et al., which include alpha-1,3-glucan, *N*-acetyl-D-glucosamine, and alpha-1,3-galactose epitopes (56). In humans, the best known antiglycan antibodies react with blood group antigens A and B (57), the xenoantigen Gal-alpha-1,

3Gal-beta-1,4GlcNAc (58, 59), Forssman glycolipid antigen, and gangliosides such as the tumor-associated antigen Neu-5GcGM3 (60).

NAb FUNCTIONS

Natural antibodies provide various essential functions within the immune system. The most prevalently studied function is the ability to provide protection against bacterial, viral, and fungal infections. Such protection is afforded by NAbs' epitope recognition. In particular, NAbs have been shown to provide protection against *S. pneumoniae* (61–63), sepsis (64), *Borrelia hermsii* (65), influenza virus (66), *Listeria monocytogenes* (67), vesicular stomatitis virus (67), lymphocytic choriomeningitis virus (67), *Cryptococcus neoformans* (68), and *Pneumocystis murina* (69). In addition to NAbs to the aforementioned organisms, B-1 cells produce "induced" antibody responses against *S. pneumoniae* (61), *B. hermsii* (65, 70, 71), influenza virus (12, 66, 72), and *Francisella tularensis* (13, 73).

Beyond protection against various infections, NAbs serve a number of other essential functions in the immune system. These functions have been reviewed extensively elsewhere (56) and include regulation of B cell development (10, 74, 75), selection of the B cell repertoire (74, 76), regulation of B cell responses (77), clearance of apoptotic debris (45), vascular homeostasis/protection against atherosclerosis (78–81), allergic suppression (82, 83), and protection from cancer (84, 85) (**Figure 1**). Despite this broad range of identified NAb functions, the role of NAbs in the immune system continues to expand.

NAb CHARACTERISTICS

In mice, typical characteristics of NAbs include germline-like nucleotide structure, repertoire skewing, IgM, IgA, or IgE (86) isotype, and T cell independence. Classically, NAbs are defined as being germline like as evidenced by these antibodies lacking non-templated nucleotides (N-additions) and having little to no somatic hypermutation (39, 87, 88). Antigen receptor diversity is increased during VDJ recombination when the enzyme TdT is present, which adds N-additions to the V-D and D-J junctions (89). Such germline characteristics have been shown to be essential in NAbs' ability to protect against infection. The prototypical B-1a anti-phosphorylcholine antibody, T15, has no N-addition (90, 91). In mice with forced expression of TdT, all anti-PC antibodies generated after vaccination with heat killed *S. pneumoniae* contain N-additions; however, these anti-phosphorylcholine antibodies containing N-additions were shown to provide no protection against *S. pneumoniae* infection (92). This study highlights the importance of germline structure in the protection provided by evolutionarily conserved NAb. In addition, NAbs derived from murine B-1a cells have a restricted repertoire. On average 5–15% of peritoneal B-1a cells recognize PtC and utilize VH11 and VH12 (93).

Other studies have shown that these "classical" characteristics of NAbs do not always apply. For instance, B-1a cells from 6- to 24-month-old mice produce Igs with significantly more N-additions (94, 95). Furthermore, it was demonstrated that B-1a cells accumulate somatic hypermutations with increasing age, which is AID dependent (96). In this same study, isotype switching was also increased in B-1a cells with age (96). Nonetheless, throughout the decades of NAb investigation, IgG and IgA have been shown to be present within the NAb pool (97–99); however, natural IgG and IgA levels decrease significantly in germ-free mice, whereas IgM levels remain unaffected (100). This suggests the amount of natural serum IgG and IgA are dependent upon exogenous antigen stimulation, whereas the level of natural serum IgM is not.

In humans, studying NAbs in the absence of antigen exposure is a challenge; however, studies performed during early human life provide a period of limited exogenous antigen exposure in the presence of undistributed, strictly controlled intrauterine antigen milieu (101). It was demonstrated that inside the fetal B cell population at 12–14 weeks of human gestation, only IgM and IgD transcripts were detected (101). Yet, after 26 weeks of gestation, B cell clones encoding IgG start to appear in a frequency similar to a frequency observed in healthy infants, which suggests IgM is not the only isotype present in the prenatal repertoire of human B cells. Furthermore, somatic hypermutations occur during human fetal B cell development even in a T cell-independent fashion (101). As described in mice, early human NAbs are also diverse in isotype and structure.

Non-templated nucleotides (junctional diversity) are also an important mechanism of generating Ig structural diversity, which along with combinatorial diversity and somatic mutation results in numerous Ig specificities (102–104). In mice, natural B-1a cell-derived IgM is characterized by a low number of N-additions (105). Interestingly, TdT expression is restricted to adult life in mice (89), which is after the majority of fetal derived B-1a cell development has occurred (105, 106). Therefore, in mice, fetal-derived B-1a cells lack N-additions (106), whereas adult bone marrow-derived B-1a cells display a high level of N-additions (95, 107–109). In contrast, TdT is expressed during both fetal and adult life in humans, and as a result, both fetal and adult derived human B cells express Ig with numerous N-additions (110). Yet, it has been shown human and mouse fetal sequences share both similarities and differences in their repertories (111). For example, even though TdT is present throughout early human life, it has been demonstrated that the number of N-additions/CDR-H3 length in B cells from preterm and term infants are shorter than that of adults (112).

DEFINING NAbs

As one reads through the body of NAb literature from the early 1960s to the present day, it becomes increasingly difficult to find a common concrete definition. The most frequently used definition describes NAbs as preimmune antibodies generated in the absence of exogenous antigenic stimulation, which are non-specific, broadly cross-reactive, low affinity, germline-like antibodies. As summarized in **Figure 1**, NAbs have many attributes, although NAbs cannot be defined by several of these

characteristics. Furthermore, NAbs cannot be defined based on a single B cell subset or location. Different subsets of B cells in different locations are capable of secreting NAbs. Neither a specific isotype nor a specific function can define NAbs. Therefore, the characteristics left to define NAbs include how they are generated (presence or absence of endogenous and/or exogenous antigen) and their structural composition (germline-like or diverse).

In terms of specific reactivity to exogenous antigens, studies have indicated that B-1a cells in the peritoneal cavity serve as a long-term reservoir of "natural" antibody-producing cells after first exposure to the antigen (17). However, if these B-1 cells have previously seen their cognate antigen it might be more appropriate to term these as memory B-1 cells. In fact, some subsets of peritoneal B-1a cells share similarities to memory B cells such as PD-L2 and CD73 expression (21, 24). Thus, it has been suggested that within the B-1 cell population, those residing in the bone marrow and the spleen are the true NAb-secreting cells (17), whereas body cavity B-1 cells constitute a population of responder (memory type) lymphocytes, which after stimulation migrate and differentiate to IgM-secreting cells. As such, it is possible body cavity B-1 cells should not be considered NAb secretors since intentional stimulation is required to upregulate the secreting process.

Other studies indicate exogenous antigens are required for selection of the overall B cell repertoire (76). In addition, altering antigenic exposure during neonatal life has been shown to significantly change the repertoire of adult B cells (82). B-1a cells are generated mainly during the fetal/neonatal period; therefore, any antigen exposure during neonatal life would be expected to significantly influence the development of B-1a cell-derived NAbs. Interestingly, it has been suggested that the neonatal period is subject to increased intestinal permeability and this access point for antigen exposure could direct the development of NAbs (56). Nonetheless, no significant difference was observed between the B-1a cell derived IgM repertoire in germ-free mice when compared with specific pathogen-free mice (96, 98, 113, 114). In adult humans, the issue of antigen exposure is more of a problem as the antigenic exposure of humans cannot be controlled; therefore, studying a pre-immune repertoire is nearly impossible.

Schroeder and colleagues demonstrated the importance of both endogenous self-antigens and germline structure of antibodies in shaping the NAb repertoire. They showed the ability of the T15 NAb to clear endogenous antigen (oxidized low-density lipoprotein) is only dependent upon selection driven by self-antigens regardless of germline antibody structure, whereas the effectiveness of T15 to protect against exogenous antigen (phosphorylcholine/*S. pneumoniae*) is dependent upon both germline conservation and selection by self-antigen (115, 116). This is in line with studies by Kearney et al. demonstrating the influence of exogenous antigen upon the effectiveness of anti-phosphorylcholine antibody against *S. pneumonia* versus allergy (82). Together, these studies demonstrate how endogenous antigen, exogenous antigen, and germline composition create and alter the NAb repertoire.

Overall, these NAb studies call into question how NAbs can/should be accurately defined. Recently, this point has been plainly

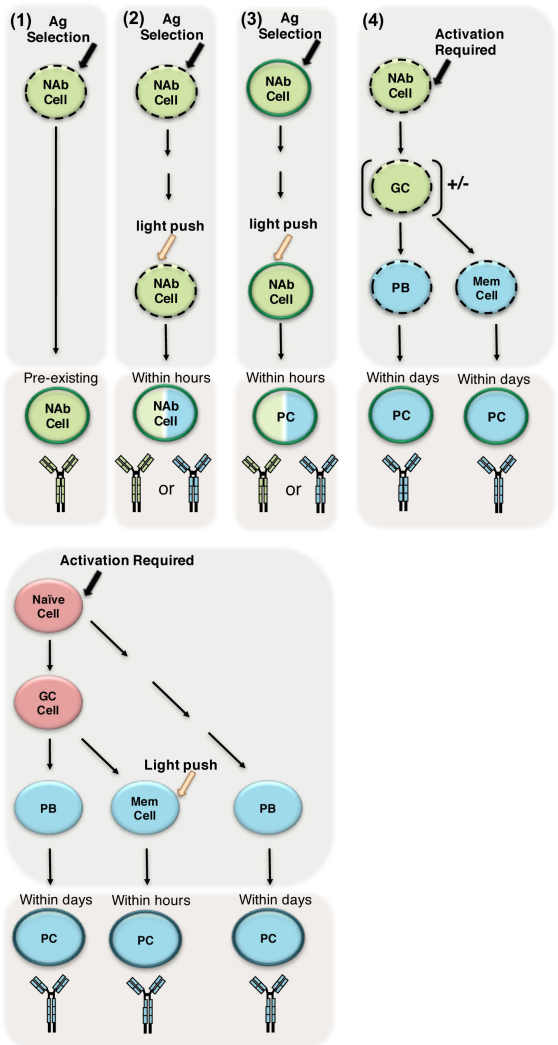


FIGURE 2 | Determining how to define natural antibodies (NAbs). (1) Most frequently used definition of NAbs: preimmune antibodies generated in the absence of exogenous antigenic stimulation, which are broadly reactive, low affinity, germline-like antibodies selected in the presence of endogenous antigen (depicted in green). These antibodies are pre-existing/always present in the serum. The dotted outline represents the intrinsic properties the cell might have (e.g., increased levels of IgM and/or CD86/CD80, lower activation threshold). The dark outline indicates the cell is secreting Ig. (2–4) In panels 2–4, we suggest possible antigenic experiences of NAbs; however, further investigation is required to determine whether antibodies produced after such antigenic experiences are the same as preimmune, pre-existing NAbs in terms of germline structure and/or repertoire. (2) Here, we suggest a NAb producing cell may require an extra antigen experience (light push[(1)]) to start immediately (within hours) secreting. This antigen experience contrasts the strong activation required for naive B-2 cells (depicted in red) to differentiate into plasma cells (PC), which results in highly specific non-germline antibody (depicted in blue). However, it is unknown what affect this light push might have upon the antibody produced by the NAb producing cell (this is depicted by giving the cell both blue and green antibody colors). (3) We suggest a NAb producing cell that is already secreting NAbs experiences antigen, which induces differentiation into a plasma cell (PC). Again, it is unknown what affect this differentiation might have upon the antibody produced (this is depicted by giving the cell both blue and green antibody colors). (4) Finally, we depict NAb producing cells experiencing strong/specific activation and subsequently following the traditional pathway leading to memory and plasma cell differentiation.

discussed in two separate reviews. The first by Baumgarth et al. (17), suggests an explicit definition: "we suggest the term *natural IgM production* be restricted to the truly antigen-independent elaboration of IgM in the spleen and bone marrow and not be extended to antigen-induced responses by B-1 cells." The second by New et al. (56) states: "Thus, the generalization often made that the NAb repertoire develops independently of exogenous is not universal for all NAb specificities, and further research focusing on the factors contributing to the development and the composition of the NAb repertoire is warranted." Herein, we suggest that these seemingly separate points of view can find common ground with further investigation.

It is clear a NAb repertoire can be created in the absence of exogenous antigens and/or germinal center maturation, and perhaps this is the definition of NAbs in its purest form. Yet, it has also been demonstrated that NAbs are affected by the presence of exogenous antigen, which is encountered in normal functioning systems. As an attempt to incorporate the role of antigen in the NAb repertoire, we propose the following starting point for investigation. To be a NAb, two requirements are necessary: (1) the ability to exert a protective, regulatory, or other biological function and (2) pre-existing/immediately responsive antibody. In the first requirement, the biological function might be protective, regulatory, or provide a function yet to be elucidated. In the second requirement, the antibody must already be present and secreted, or the NAb encoding cell would need only a light push[1] for the NAb to be secreted.[2] The role of antigen comes into play when considering the light push that some NAb secreting cells might need to immediately produce antibody. Furthermore, the ability of the NAb encoding cell to respond to the light push would be dependent upon its intrinsic properties such as status of surface phenotype or activation threshold. Further experimentation is required to determine whether the NAb produced by NAb secreting cells needing antigen exposure to immediately produce antibody differs from antibodies produced by other cells capable of immediate production of antibody (i.e., memory cells) (**Figure 2**). It is these authors perspective, as a field studying this clearly essential part of the immune system, we need to further investigate all contexts in which NAbs are produced and regulated (**Figure 2**).

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and development of the perspective put forth. All authors worked together to edit and revise the manuscript.

[1] We are suggesting a light push to mean exposure to antigen or other stimuli leading to a differentiation pathway operating outside of the strong stimuli provided by the germinal center environment and thus, different from the naive B-2 cell requirement for stimulation.

[2] The subset of NAb-producing cells needing a light push might be poised to secrete but are not detected as such by the methods currently available.

REFERENCES

1. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* (2011) 208:67–80. doi:10.1084/jem.201014992084c
2. Boyden SV. Natural antibodies and the immune response. *Adv Immunol* (1966) 5:1–28. doi:10.1016/S0065-2776(08)60271-0
3. Michael JG. Natural antibodies. *Curr Top Microbiol Immunol* (1969) 48:43–62.
4. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med* (1983) 157:202–18. doi:10.1084/jem.157.1.202
5. Hayakawa K, Hardy RR, Honda M, Herzenberg LA, Steinberg AD, Herzenberg LA. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc Natl Acad Sci U S A* (1984) 81:2494–8. doi:10.1073/pnas.81.8.2494
6. Baumgarth N. B-cell immunophenotyping. *Methods Cell Biol* (2004) 75:643–62. doi:10.1016/S0091-679X(04)75027-X
7. Kantor AB, Stall AM, Adams S, Herzenberg LA, Herzenberg LA. Differential development of progenitor activity for three B-cell lineages. *Proc Natl Acad Sci U S A* (1992) 89:3320–4. doi:10.1073/pnas.89.8.3320
8. Ghosn EE, Yang Y, Tung J, Herzenberg LA, Herzenberg LA. CD11b expression distinguishes sequential stages of peritoneal B-1 development. *Proc Natl Acad Sci U S A* (2008) 105:5195–200. doi:10.1073/pnas.0712350105
9. Hastings WD, Gurdak SM, Tumang JR, Rothstein TL. CD5+/Mac-1- peritoneal B cells: a novel B cell subset that exhibits characteristics of B-1 cells. *Immunol Lett* (2006) 105:90–6. doi:10.1016/j.imlet.2006.01.002
10. Baumgarth N. B-1 cell heterogeneity and the regulation of natural and antigen-induced IgM production. *Front Immunol* (2016) 7:324. doi:10.3389/fimmu.2016.00324
11. Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* (2011) 11:34–46. doi:10.1038/nri2901
12. Choi YS, Baumgarth N. Dual role for B-1a cells in immunity to influenza virus infection. *J Exp Med* (2008) 205:3053–64. doi:10.1084/jem.20080979
13. Cole LE, Yang Y, Elkins KL, Fernandez ET, Qureshi N, Shlomchik MJ, et al. Antigen-specific B-1a antibodies induced by *Francisella tularensis* LPS provide long-term protection against *F. tularensis* LVS challenge. *Proc Natl Acad Sci U S A* (2009) 106:4343–8. doi:10.1073/pnas.0813411106
14. Kawahara T, Ohdan H, Zhao G, Yang YG, Sykes M. Peritoneal cavity B cells are precursors of splenic IgM natural antibody-producing cells. *J Immunol* (2003) 171:5406–14. doi:10.4049/jimmunol.171.10.5406
15. Watanabe N, Ikuta K, Fagarasan S, Yazumi S, Chiba T, Honjo T. Migration and differentiation of autoreactive B-1 cells induced by activated gamma/delta T cells in antierythrocyte immunoglobulin transgenic mice. *J Exp Med* (2000) 192:1577–86. doi:10.1084/jem.192.11.1577
16. Waffarn EE, Hastey CJ, Dixit N, Soo Choi Y, Cherry S, Kalinke U, et al. Infection-induced type I interferons activate CD11b on B-1 cells for subsequent lymph node accumulation. *Nat Commun* (2015) 6:8991. doi:10.1038/ncomms9991
17. Baumgarth N, Waffarn EE, Nguyen TT. Natural and induced B-1 cell immunity to infections raises questions of nature versus nurture. *Ann N Y Acad Sci* (2015) 1362:188–99. doi:10.1111/nyas.12804
18. Lalor PA, Herzenberg LA, Adams S, Stall AM. Feedback regulation of murine Ly-1 B cell development. *Eur J Immunol* (1989) 19:507–13. doi:10.1002/eji.1830190315
19. Savage HP, Baumgarth N. Characteristics of natural antibody-secreting cells. *Ann N Y Acad Sci* (2015) 1362:132–42. doi:10.1111/nyas.12799
20. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* (2002) 16:67–76. doi:10.1016/S1074-7613(01)00257-6
21. Zhong X, Tumang JR, Gao W, Bai C, Rothstein TL. PD-L2 expression extends beyond dendritic cells/macrophages to B1 cells enriched for V(H)11/V(H)12 and phosphatidylcholine binding. *Eur J Immunol* (2007) 37:2405–10. doi:10.1002/eji.200737461
22. Zhong X, Lau S, Bai C, Degauque N, Holodick NE, Steven SJ, et al. A novel subpopulation of B-1 cells is enriched with autoreactivity in normal and lupus-prone mice. *Arthritis Rheum* (2009) 60:3734–43. doi:10.1002/art.25015
23. Tumang JR, Holodick NE, Vizconde TC, Kaku H, Frances R, Rothstein TL. A CD25(−) positive population of activated B1 cells expresses LIFR and responds to LIF. *Front Immunol* (2011) 2:6. doi:10.3389/fimmu.2011.00006

24. Kaku H, Cheng KF, Al-Abed Y, Rothstein TL. A novel mechanism of B cell-mediated immune suppression through CD73 expression and adenosine production. *J Immunol* (2014) 193:5904–13. doi:10.4049/jimmunol.1400336
25. Wang H, Shin DM, Abbasi S, Jain S, Kovalchuk AL, Beaty N, et al. Expression of plasma cell alloantigen 1 defines layered development of B-1a B-cell subsets with distinct innate-like functions. *Proc Natl Acad Sci U S A* (2012) 109:20077–82. doi:10.1073/pnas.1212428109
26. Wang H, Lin JX, Li P, Skinner J, Leonard WJ, Morse HC III. New insights into heterogeneity of peritoneal B-1a cells. *Ann N Y Acad Sci* (2015) 1362:68–76. doi:10.1111/nyas.12791
27. O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* (1992) 22:711–7. doi:10.1002/eji.1830220314
28. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol Rev* (2008) 224:201–14. doi:10.1111/j.1600-065X.2008.00661.x
29. Reynolds AE, Kuraoka M, Kelsoe G. Natural IgM is produced by CD5− plasma cells that occupy a distinct survival niche in bone marrow. *J Immunol* (2015) 194:231–42. doi:10.4049/jimmunol.1401203
30. Yang Y, Tung JW, Ghosn EE, Herzenberg LA, Herzenberg LA. Division and differentiation of natural antibody-producing cells in mouse spleen. *Proc Natl Acad Sci U S A* (2007) 104:4542–6. doi:10.1073/pnas.0700001104
31. Ichikawa D, Asano M, Shinton SA, Brill-Dashoff J, Formica AM, Velcich A, et al. Natural anti-intestinal goblet cell autoantibody production from marginal zone B cells. *J Immunol* (2015) 194:606–14. doi:10.4049/jimmunol.1402383
32. Casali P, Notkins AL. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. *Immunol Today* (1989) 10:364–8. doi:10.1016/0167-5699(89)90268-5
33. Antin JH, Emerson SG, Martin P, Gadol N, Ault KA. Leu-1+ (CD5+) B cells. A major lymphoid subpopulation in human fetal spleen: phenotypic and functional studies. *J Immunol* (1986) 136:505–10.
34. Kipps TJ, Fong S, Tomhave E, Chen PP, Goldfien RD, Carson DA. High-frequency expression of a conserved kappa light-chain variable-region gene in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* (1987) 84:2916–20. doi:10.1073/pnas.84.9.2916
35. Casali P, Burastero SE, Nakamura M, Inghirami G, Notkins AL. Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. *Science* (1987) 236:77–81. doi:10.1126/science.3105056
36. Kasaian MT, Ikematsu H, Casali P. Identification and analysis of a novel human surface CD5− B lymphocyte subset producing natural antibodies. *J Immunol* (1992) 148:2690–702.
37. Casali P, Schettino EW. Structure and function of natural antibodies. *Curr Top Microbiol Immunol* (1996) 210:167–79.
38. Quach TD, Rodriguez-Zhurbenko N, Hopkins TJ, Guo X, Hernandez AM, Li W, et al. Distinctions among circulating antibody-secreting cell populations, including B-1 cells, in human adult peripheral blood. *J Immunol* (2016) 196:1060–9. doi:10.4049/jimmunol.1501843
39. Feeney AJ. Predominance of the prototypic T15 anti-phosphorylcholine junctional sequence in neonatal pre-B cells. *J Immunol* (1991) 147:4343–50.
40. Brundish DE, Baddiley J. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. *Biochem J* (1968) 110:573–82. doi:10.1042/bj1100573
41. Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* (2000) 105:1731–40. doi:10.1172/JCI8472
42. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* (2003) 9:736–43. doi:10.1038/nm876
43. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *J Immunol* (2009) 182:6031–43. doi:10.4049/jimmunol.0804191
44. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, et al. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* (2009) 119:1335–49. doi:10.1172/JCI36800
45. Gronwall C, Vas J, Silverman GJ. Protective roles of natural IgM antibodies. *Front Immunol* (2012) 3:66. doi:10.3389/fimmu.2012.00066
46. Cunningham AJ. Large numbers of cells in normal mice produce antibody components of isologous erythrocytes. *Nature* (1974) 252:749–51. doi:10.1038/252749a0
47. Lord EM, Dutton RW. The properties of plaque-forming cells from autoimmune and normal strains of mice with specificity for autologous erythrocyte antigens. *J Immunol* (1975) 115:1199–205.
48. Cox KO, Hardy SJ. Autoantibodies against mouse bromelain-modified RBC are specifically inhibited by a common membrane phospholipid, phosphatidylcholine. *Immunology* (1985) 55:263–9.
49. Mercolino TJ, Arnold LW, Haughton G. Phosphatidyl choline is recognized by a series of Ly-1+ murine B cell lymphomas specific for erythrocyte membranes. *J Exp Med* (1986) 163:155–65. doi:10.1084/jem.163.1.155
50. Reininger L, Ollier P, Poncet P, Kaushik A, Jaton JC. Novel V genes encode virtually identical variable regions of six murine monoclonal anti-bromelain-treated red blood cell autoantibodies. *J Immunol* (1987) 138:316–23.
51. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K. A single VH gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the VH11 family. *J Immunol* (1989) 142:3643–51.
52. Pennell CA, Sheehan KM, Brodeur PH, Clarke SH. Organization and expression of VH gene families preferentially expressed by Ly-1+ (CD5) B cells. *Eur J Immunol* (1989) 19:2115–21. doi:10.1002/eji.1830191122
53. Seidl KJ, MacKenzie JD, Wang D, Kantor AB, Kabat EA, Herzenberg LA, et al. Frequent occurrence of identical heavy and light chain Ig rearrangements. *Int Immunol* (1997) 9:689–702. doi:10.1093/intimm/9.5.689
54. Dai H, Zhang Y, Lv P, Gao XM. A study on the glycan specificity of natural antibody repertoires in rodents. *Cell Mol Immunol* (2009) 6:453–9. doi:10.1038/cmi.2009.57
55. Huflejt ME, Vuskovic M, Vasiliu D, Xu H, Obukhova P, Shilova N, et al. Anti-carbohydrate antibodies of normal sera: findings, surprises and challenges. *Mol Immunol* (2009) 46:3037–49. doi:10.1016/j.molimm.2009.06.010
56. New JS, King RG, Kearney JF. Manipulation of the glycan-specific natural antibody repertoire for immunotherapy. *Immunol Rev* (2016) 270:32–50. doi:10.1111/imr.12397
57. Shilova N, Huflejt ME, Vuskovic M, Obukhova P, Navakouski M, Khasbiullina N, et al. Natural antibodies against sialoglycans. *Top Curr Chem* (2015) 366:169–81. doi:10.1007/128_2013_469
58. McKane W, Lee J, Preston R, Hacking A, Simpson P, Lynds S, et al. Polymorphism in the human anti-pig natural antibody repertoire: implications for antigen-specific immunoadsorption. *Transplantation* (1998) 66:626–33. doi:10.1097/00007890-199809150-00014
59. Galili U. Anti-Gal: an abundant human natural antibody of multiple pathogeneses and clinical benefits. *Immunology* (2013) 140:1–11. doi:10.1111/imm.12110
60. Rodriguez-Zhurbenko N, Martinez D, Blanco R, Rondon T, Grinan T, Hernandez AM. Human antibodies reactive to NeuGcGM3 ganglioside have cytotoxic antitumor properties. *Eur J Immunol* (2013) 43:826–37. doi:10.1002/eji.201242693
61. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity* (2005) 23:7–18. doi:10.1016/j.immuni.2005.04.011
62. Briles DE, Nahm M, Schroer K, Davie J, Baker P, Kearney J, et al. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *J Exp Med* (1981) 153:694–705. doi:10.1084/jem.153.3.694
63. Mi QS, Zhou L, Schulze DH, Fischer RT, Lustig A, Rezanka LJ, et al. Highly reduced protection against *Streptococcus pneumoniae* after deletion of a single heavy chain gene in mouse. *Proc Natl Acad Sci U S A* (2000) 97:6031–6. doi:10.1073/pnas.110039497
64. Boes M, Prodeus AP, Schmidt T, Carroll MC, Chen J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *J Exp Med* (1998) 188:2381–6. doi:10.1084/jem.188.12.2381

65. Alugupalli KR, Gerstein RM, Chen J, Szomolanyi-Tsuda E, Woodland RT, Leong JM. The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *J Immunol* (2003) 170:3819–27. doi:10.4049/jimmunol.170.7.3819
66. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A* (1999) 96:2250–5. doi:10.1073/pnas.96.5.2250
67. Ochsenbein AF, Fehr T, Lutz C, Suter M, Brombacher F, Hengartner H, et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* (1999) 286:2156–9. doi:10.1126/science.286.5447.2156
68. Subramaniam KS, Datta K, Quintero E, Manix C, Marks MS, Pirofski LA. The absence of serum IgM enhances the susceptibility of mice to pulmonary challenge with *Cryptococcus neoformans*. *J Immunol* (2010) 184:5755–67. doi:10.4049/jimmunol.0901638
69. Rapaka RR, Ricks DM, Alcorn JF, Chen K, Khader SA, Zheng M, et al. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus *Pneumocystis murina*. *J Exp Med* (2010) 207:2907–19. doi:10.1084/jem.20100034
70. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* (2004) 21:379–90. doi:10.1016/j.immuni.2004.06.019
71. Colombo MJ, Alugupalli KR. Complement factor H-binding protein, a putative virulence determinant of *Borrelia hermsii*, is an antigenic target for protective B1b lymphocytes. *J Immunol* (2008) 180:4858–64. doi:10.4049/jimmunol.180.7.4858
72. Baumgarth N, Herman OC, Jager GC, Brown LE, Herzenberg LA, Chen J. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J Exp Med* (2000) 192:271–80. doi:10.1084/jem.192.2.271
73. Yang Y, Ghosn EE, Cole LE, Obukhanych TV, Sadate-Ngatchou P, Vogel SN, et al. Antigen-specific antibody responses in B-1a and their relationship to natural immunity. *Proc Natl Acad Sci U S A* (2012) 109:5382–7. doi:10.1073/pnas.1121631109
74. Nguyen TT, Elsner RA, Baumgarth N. Natural IgM prevents autoimmunity by enforcing B cell central tolerance induction. *J Immunol* (2015) 194:1489–502. doi:10.4049/jimmunol.1401880
75. Nguyen TT, Klasener K, Zurn C, Castillo PA, Brust-Mascher I, Imai DM, et al. The IgM receptor FcmuR limits tonic BCR signaling by regulating expression of the IgM BCR. *Nat Immunol* (2017) 18:321–33. doi:10.1038/ni.3677
76. Freitas AA, Viale AC, Sundblad A, Heusser C, Coutinho A. Normal serum immunoglobulins participate in the selection of peripheral B-cell repertoires. *Proc Natl Acad Sci U S A* (1991) 88:5640–4. doi:10.1073/pnas.88.13.5640
77. Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol* (1998) 160:4776–87.
78. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B cells and humoral immunity in atherosclerosis. *Circ Res* (2014) 114:1743–56. doi:10.1161/CIRCRESAHA.113.301145
79. Zhang M, Alicot EM, Chiu I, Li J, Verna N, Vorup-Jensen T, et al. Identification of the target self-antigens in reperfusion injury. *J Exp Med* (2006) 203:141–52. doi:10.1084/jem.20050390
80. Binder CJ, Shaw PX, Chang MK, Boullier A, Hartvigsen K, Horkko S, et al. The role of natural antibodies in atherogenesis. *J Lipid Res* (2005) 46:1353–63. doi:10.1194/jlr.R500005-JLR200
81. Binder CJ, Silverman GJ. Natural antibodies and the autoimmunity of atherosclerosis. *Springer Semin Immunopathol* (2005) 26:385–404. doi:10.1007/s00281-004-0185-z
82. Kearney JF, Patel P, Stefanov EK, King RG. Natural antibody repertoires: development and functional role in inhibiting allergic airway disease. *Annu Rev Immunol* (2015) 33:475–504. doi:10.1146/annurev-immunol-032713-120140
83. Patel PS, Kearney JF. Neonatal exposure to pneumococcal phosphorylcholine modulates the development of house dust mite allergy during adult life. *J Immunol* (2015) 194:5838–50. doi:10.4049/jimmunol.1500251
84. Vollmers HP, Brandlein S. Natural antibodies and cancer. *N Biotechnol* (2009) 25:294–8. doi:10.1016/j.nbt.2009.03.016
85. Madi A, Bransburg-Zabary S, Maayan-Metzger A, Dar G, Ben-Jacob E, Cohen IR. Tumor-associated and disease-associated autoantibody repertoires in healthy colostrum and maternal and newborn cord sera. *J Immunol* (2015) 194:5272–81. doi:10.4049/jimmunol.1402771
86. McCoy KD, Harris NL, Diener P, Hatak S, Odermatt B, Hangartner L, et al. Natural IgE production in the absence of MHC class II cognate help. *Immunity* (2006) 24:329–39. doi:10.1016/j.immuni.2006.01.013
87. Tornberg UC, Holmberg D. B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. *EMBO J* (1995) 14:1680–9.
88. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* (1997) 158:1175–86.
89. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, et al. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* (1984) 311:752–5. doi:10.1038/311752a0
90. Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against *Streptococcus pneumoniae*. *J Exp Med* (1982) 156:1177–85. doi:10.1084/jem.156.4.1177
91. Benedict CL, Gilfillan S, Thai TH, Kearney JF. Terminal deoxynucleotidyl transferase and repertoire development. *Immunol Rev* (2000) 175:150–7. doi:10.1111/j.1600-065X.2000.imr017518.x
92. Benedict CL, Kearney JF. Increased junctional diversity in fetal B cells results in a loss of protective anti-phosphorylcholine antibodies in adult mice. *Immunity* (1999) 10:607–17. doi:10.1016/S1074-7613(00)80060-6
93. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G. Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J Exp Med* (1988) 168:687–98. doi:10.1084/jem.168.2.687
94. Forster I, Gu H, Rajewsky K. Germline antibody V regions as determinants of clonal persistence and malignant growth in the B cell compartment. *EMBO J* (1988) 7:3693–703.
95. Holodick NE, Vizconde T, Hopkins TJ, Rothstein TL. Age-related decline in natural IgM function: diversification and selection of the B-1a cell pool with age. *J Immunol* (2016) 196:4348–57. doi:10.4049/jimmunol.1600073
96. Yang Y, Wang C, Yang Q, Kantor AB, Chu H, Ghosn EE, et al. Distinct mechanisms define murine B cell lineage immunoglobulin heavy chain (IgH) repertoires. *Elife* (2015) 4:e09083. doi:10.7554/eLife.09083
97. Avrameas S. Natural autoantibodies: from 'horror autotoxicus' to 'gnothi seauton'. *Immunol Today* (1991) 12:154–9. doi:10.1016/0167-5699(91)90080-D
98. Coutinho A, Kazatchkine MD, Avrameas S. Natural autoantibodies. *Curr Opin Immunol* (1995) 7:812–8. doi:10.1016/0952-7915(95)80053-0
99. Ikematsu H, Schettino EW, Casali P. Structure of the VH-D-JH segments of human natural polyreactive IgM and IgG antibodies. Use of germline or somatically mutated forms of commonly expressed VH genes. *Ann N Y Acad Sci* (1995) 764:362–9. doi:10.1111/j.1749-6632.1995.tb55848.x
100. Hooijkaas H, Benner R, Pleasants JR, Wostmann BS. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. *Eur J Immunol* (1984) 14:1127–30. doi:10.1002/eji.1830141212
101. Rechavi E, Lev A, Lee YN, Simon AJ, Yinon Y, Lipitz S, et al. Timely and spatially regulated maturation of B and T cell repertoire during human fetal development. *Sci Transl Med* (2015) 7:276ra225. doi:10.1126/scitranslmed.aaa0072
102. Tonegawa S. Somatic generation of antibody diversity. *Nature* (1983) 302:575–81. doi:10.1038/302575a0
103. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. *Proc Natl Acad Sci U S A* (1982) 79:4118–22. doi:10.1073/pnas.79.13.4118
104. Xu JL, Davis MM. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* (2000) 13:37–45. doi:10.1016/S1074-7613(00)00006-6
105. Feeney AJ. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J Exp Med* (1990) 172:1377–90. doi:10.1084/jem.172.5.1377

106. Kantor AB. V-gene usage and N-region insertions in B-1a, B-1b and conventional B cells. *Semin Immunol* (1996) 8:29–35. doi:10.1006/smim.1996.0005
107. Holodick NE, Repetny K, Zhong X, Rothstein TL. Adult BM generates CD5+ B1 cells containing abundant N-region additions. *Eur J Immunol* (2009) 39:2383–94. doi:10.1002/eji.200838920
108. Duber S, Hafner M, Krey M, Lienenklaus S, Roy B, Hobeika E, et al. Induction of B-cell development in adult mice reveals the ability of bone marrow to produce B-1a cells. *Blood* (2009) 114:4960–7. doi:10.1182/blood-2009-04-218156
109. Esplin BL, Welner RS, Zhang Q, Borghesi LA, Kincade PW. A differentiation pathway for B1 cells in adult bone marrow. *Proc Natl Acad Sci U S A* (2009) 106:5773–8. doi:10.1073/pnas.0811632106
110. Asma GE, van den Bergh RL, Vossen JM. Characterization of early lymphoid precursor cells in the human fetus using monoclonal antibodies and anti-terminal deoxynucleotidyl transferase. *Clin Exp Immunol* (1986) 64:356–63.
111. Schroeder HW Jr. Similarity and divergence in the development and expression of the mouse and human antibody repertoires. *Dev Comp Immunol* (2006) 30:119–35. doi:10.1016/j.dci.2005.06.006
112. Zemlin M, Bauer K, Hummel M, Pfeiffer S, Devers S, Zemlin C, et al. The diversity of rearranged immunoglobulin heavy chain variable region genes in peripheral blood B cells of preterm infants is restricted by short third complementarity-determining regions but not by limited gene segment usage. *Blood* (2001) 97:1511–3. doi:10.1182/blood.V97.5.1511
113. Hayakawa K, Asano M, Shinton SA, Gui M, Allman D, Stewart CL, et al. Positive selection of natural autoreactive B cells. *Science* (1999) 285:113–6. doi:10.1126/science.285.5424.113
114. Hayakawa K, Carmack CE, Hyman R, Hardy RR. Natural autoantibodies to thymocytes: origin, VH genes, fine specificities, and the role of Thy-1 glycoprotein. *J Exp Med* (1990) 172:869–78. doi:10.1084/jem.172.3.869
115. Vale AM, Cavazzoni CB, Nobrega A, Schroeder HW Jr. The global self-reactivity profile of the natural antibody repertoire is largely independent of germline DH sequence. *Front Immunol* (2016) 7:296. doi:10.3389/fimmu.2016.00296
116. Vale AM, Kapoor P, Skibinski GA, Elgavish A, Mahmoud TI, Zemlin C, et al. The link between antibodies to OxLDL and natural protection against pneumococci depends on D(H) gene conservation. *J Exp Med* (2013) 210:875–90. doi:10.1084/jem.20121861

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on