frontiers in Immunology

GENERAL COMMENTARY
published: 16 July 2015
doi: 10.3389/fimmu.2015.00351

Commentary: "There's been a flaw in our thinking"

E. Sally Ward[1,2,3]* *and Raimund J. Ober*[1,4]

[1] Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, College Station, TX, USA, [2] Department of Microbial Pathogenesis and Immunology, Texas A&M Health Science Center, Bryan, TX, USA, [3] Department of Immunology, University of Texas Southwestern Medical Center, Dallas, TX, USA, [4] Department of Biomedical Engineering, Texas A&M University, College Station, TX, USA

Keywords: FcRn, IgG, subcellular trafficking, pinocytosis, transport

A commentary on

There's been a flaw in our thinking
by Anderson CL. Front Immunol (2014) 5:540. doi: 10.3389/fimmu.2014.00540

We thank the editors for the opportunity to address the overstatements in the recent opinion article (1). Over the past two decades, the role of FcRn in regulating the levels and transport of IgG in the body has been established (2–5), validating the insightful prediction of Brambell in the 1960s that IgG is salvaged from catabolism by receptors located within cellular compartments and/or on the surface of cells (6, 7). Remarkably, this hypothesis was made in the absence of knowledge of the molecular details of IgG–FcRn interactions. It is now well known that FcRn binds to IgG at acidic pH (~6.0) with very low or negligible affinity at pH 7–7.4 (8–11), providing an elegant biological solution to achieve exocytic release of recycled IgG. Further, the negligible binding of IgG to FcRn at pH 7–7.4 supports the concept that fluid-phase, pinocytic uptake is the primary mediator of ligand entry into cells exposed to this pH range. However, in the absence of information concerning the pH dependence of FcRn–IgG interactions around 50 years ago, it was not possible to postulate the mechanism of IgG uptake into cells bathed at acidic pH. Notably, in the light of the pH dependence of complex formation, receptor-mediated internalization of IgG for cells at acidic pH is expected to represent a major pathway, although this does not preclude the occurrence of concomitant fluid-phase processes. Consequently, the relative contributions of fluid-phase vs. receptor-mediated pathways for IgG internalization are highly dependent on the pH of the extracellular environment. Further, FcRn biology has been enriched over the past decade by the recognition of its much broader expression pattern and the elucidation of its role in multiple diverse processes, including antigen presentation and mucosal immunity (12–15). Collectively, these developments have motivated multiple *in vitro* cellular studies under conditions designed to emulate the physiological environment of interest.

Numerous analyses of FcRn/IgG trafficking have been performed using cells bathed in medium containing relatively high concentrations (~1–17 μM) of wild type IgG at pH 7.0–7.4 to enable fluid-phase, pinocytic uptake (16–22). Importantly, IgGs that bind with negligible affinity to FcRn accumulate in cells under these conditions (18, 21). Reciprocally, the use of low concentrations (~130 nM) of IgGs that bind to FcRn with the typical pH dependence results in almost background levels of internalization (23). The endosomal sorting of fluorescently labeled wild type IgG in FcRn-expressing endothelial cells has been analyzed at near neutral pH using IgG concentrations (~3–7 μM) that favor fluid-phase uptake (18). These studies demonstrated that IgG is quantitatively routed within sorting (or early) endosomes in association with FcRn into tubulovesicular transport carriers, supporting the concept that sorting endosomes are major sites of FcRn-mediated recycling of IgG following pinocytosis. By contrast, an engineered IgG (H435A mutant) that does not bind to FcRn accumulates in the vacuole of the sorting endosomes and is subsequently delivered to

OPEN ACCESS

Edited by:
Inger Sandlie,
University of Oslo, Norway

Reviewed by:
Richard S. Blumberg,
Brigham and Women's Hospital, USA
Derry Charles Roopenian,
The Jackson Laboratory, USA

***Correspondence:**
E. Sally Ward
sally.ward@medicine.tamhsc.edu

Specialty section:
This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology

Received: *11 March 2015*
Accepted: *28 June 2015*
Published: *16 July 2015*

Citation:
Ward ES and Ober RJ (2015) Commentary: "There's been a flaw in our thinking". Front. Immunol. 6:351. doi: 10.3389/fimmu.2015.00351

lysosomes. In a related study, exocytic processes involving FcRn and wild type IgG have been characterized at the single molecule level following exposure of cells to relatively high IgG concentrations at pH 7.4 (19). Further, IgG recycling and saturation of FcRn recycling pathways (21, 23, 24) were quantitated under similar conditions. Analyses of the transport of wild type IgG within endothelial, trophoblast and renal epithelial cells have also been performed analogously (16, 17, 20). In light of these studies, the statement advanced by the author of the recent opinion article that "it proved virtually impossible to perform *in vitro* studies of IgG uptake by cultured cells unless the medium was acidic" is perplexing.

In any studies of receptor/ligand trafficking, it is essential to distinguish the behavior of ligand from that of receptor. Considering the negligible affinity of most naturally occurring IgGs for FcRn at near neutral pH, these ligands are unsuitable for use in labeled form as FcRn tracers under these conditions. Consequently, engineered IgG ligands with increased affinity for FcRn at pH ~7 have been used at low concentrations (10–30 nM) that result in negligible fluid-phase pinocytosis (23) to track receptor during endocytosis and trafficking to sorting endosomes (25, 26). Parenthetically, these engineered antibodies compete very effectively with wild type IgG for FcRn binding and therefore have utility as IgG depleting agents in therapy and diagnosis (27, 28). The potential applications of antibodies of this class ("Abdegs") have motivated analyses of their subcellular trafficking behavior using conditions where receptor-mediated uptake predominates (23, 29).

By contrast with analyses at near neutral pH, experiments have been conducted using acidic pH to mimick the *in vivo* environment corresponding to biological systems for which this is appropriate, such as the apical surface of gut epithelium. These cells are exposed to an acidic microenvironment due to the activity of Na+/H+ exchangers (30). These conditions enable receptor-mediated endocytosis of IgGs at low concentrations that limit fluid-phase accumulation (23, 31, 32). This experimental design results in FcRn-mediated transcytosis and/or recycling [e.g., Ref. (3, 5, 33–35)], and multiple studies including electron tomographic analyses validate the physiological relevance of this approach [e.g., Ref. (31, 36)]. Anderson questions the validity of bathing cells at acidic pH, substantiating his argument with "Gut pH had been measured only once, with litmus paper, and the observation was never repeated." This statement is surprising, as publications can readily be found in which different techniques demonstrate that the pH of the proximal portion of the intestinal lumen is mildly acidic [pH 6–7 (37, 38)]. For instance, this is well illustrated clinically with the post-pyloric feeding tube placement pH testing in neonates and children (39, 40).

Further, the argument of the author of the recent opinion article that there is a minimal receptor-mediated internalization by (epithelial) cells at acidic pH due to the low proportion of FcRn present on the cell surface relative to intracellular levels neglects consideration of receptor dynamics. Specifically, the low steady state levels of FcRn on the plasma membrane do not exclude the possibility of rapid receptor endocytosis following exocytic events. Indeed, the observation that engineered antibodies with high affinity for FcRn at near neutral pH efficiently accumulate to relatively high levels within cells of multiple different lineages, but only if the cells express FcRn, is consistent with such dynamic cycling behavior (32).

In summary, the primary conclusion that the subcellular pathways taken by IgG following fluid-phase, pinocytic uptake into cells have been ignored for two decades is unfortunately premised on a highly selective review of the literature. To the contrary, a cursory survey of the relevant publications clearly demonstrates that Brambell's model for regulating IgG homeostasis and transport by receptor-mediated salvage has formed the conceptual foundation to investigate these processes using modern experimental tools. Beyond Brambell's predictions, the discovery of new and unexpected roles for FcRn has also prompted experiments tailored to specifically investigate the biological questions at hand.

Acknowledgments

We thank Dr. Richard Blumberg for helpful discussions concerning FcRn and intestinal epithelial cells. This commentary was supported in part by a grant from the NIH to E.S.W. (R01 AR056478).

References

1. Anderson CL. There's been a flaw in our thinking. *Front Immunol* (2014) **5**:540. doi:10.3389/fimmu.2014.00540
2. Ghetie V, Hubbard JG, Kim JK, Tsen MF, Lee Y, Ward ES. Abnormally short serum half-lives of IgG in β2-microglobulin-deficient mice. *Eur J Immunol* (1996) **26**(3):690–6. doi:10.1002/eji.1830260327
3. Dickinson BL, Badizadegan K, Wu Z, Ahouse JC, Zhu X, Simister NE, et al. Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. *J Clin Invest* (1999) **104**(7):903–11. doi:10.1172/JCI6968
4. Spiekermann GM, Finn PW, Ward ES, Dumont J, Dickinson BL, Blumberg RS, et al. Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung. *J Exp Med* (2002) **196**(3):303–10. doi:10.1084/jem.20020400
5. McCarthy KM, Yoong Y, Simister NE. Bidirectional transcytosis of IgG by the rat neonatal Fc receptor expressed in a rat kidney cell line: a system to study protein transport across epithelia. *J Cell Sci* (2000) **113**(Pt 7): 1277–85. http://jcs.biologists.org/content/113/7/1277.abstract
6. Brambell FWR, Hemmings WA, Morris IG. A theoretical model of γ-globulin catabolism. *Nature* (1964) **203**:1352–5. doi:10.1038/2031352a0
7. Brambell FWR. *The Transmission of Passive Immunity from Mother to Young*. Amsterdam: North Holland Publ Corp (1970).
8. Rodewald R. pH-dependent binding of immunoglobulins to intestinal cells of the neonatal rat. *J Cell Biol* (1976) **71**(2):666–9. doi:10.1083/jcb.71.2.666
9. Simister NE, Rees AR. Isolation and characterization of an Fc receptor from neonatal rat small intestine. *Eur J Immunol* (1985) **15**(7):733–8. doi:10.1002/eji.1830150718
10. Kim JK, Tsen MF, Ghetie V, Ward ES. Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor. *Eur J Immunol* (1994) **24**(10):2429–34. doi:10.1002/eji.1830241025
11. Raghavan M, Chen MY, Gastinel LN, Bjorkman PJ. Investigation of the interaction between the class I MHC-related Fc receptor and its immunoglobulin G ligand. *Immunity* (1994) **1**(4):303–15. doi:10.1016/1074-7613(94)90082-5
12. Ward ES, Ober RJ. Multitasking by exploitation of intracellular transport functions: the many faces of FcRn. *Adv Immunol* (2009) **103**:77–115. doi:10.1016/S0065-2776(09)03004-1
13. Baker K, Rath T, Pyzik M, Blumberg RS. The role of FcRn in antigen presentation. *Front Immunol* (2014) **5**:408. doi:10.3389/fimmu.2014.00408
14. Yoshida M, Kobayashi K, Kuo TT, Bry L, Glickman JN, Claypool SM, et al. Neonatal Fc receptor for IgG regulates mucosal immune

responses to luminal bacteria. *J Clin Invest* (2006) **116**(8):2142–51. doi:10.1172/JCI27821
15. Vidarsson G, Stemerding AM, Stapleton NM, Spliethoff SE, Janssen H, Rebers FE, et al. FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis. *Blood* (2006) **108**(10):3573–9. doi:10.1182/blood-2006-05-024539
16. Ellinger I, Rothe A, Grill M, Fuchs R. Apical to basolateral transcytosis and apical recycling of immunoglobulin G in trophoblast-derived BeWo cells: effects of low temperature, nocodazole, and cytochalasin D. *Exp Cell Res* (2001) **269**(2):322–31. doi:10.1006/excr.2001.5330
17. Kobayashi N, Suzuki Y, Tsuge T, Okumura K, Ra C, Tomino Y. FcRn-mediated transcytosis of immunoglobulin G in human renal proximal tubular epithelial cells. *Am J Physiol Renal Physiol* (2002) **282**(2):F358–65. doi:10.1152/ajprenal.0164.2001
18. Ober RJ, Martinez C, Vaccaro C, Zhou J, Ward ES. Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. *J Immunol* (2004) **172**(4):2021–9. doi:10.4049/jimmunol.172.4.2021
19. Ober RJ, Martinez C, Lai X, Zhou J, Ward ES. Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. *Proc Natl Acad Sci U S A* (2004) **101**:11076–81. doi:10.1073/pnas.0402970101
20. Goebl NA, Babbey CM, Datta-Mannan A, Witcher DR, Wroblewski VJ, Dunn KW. Neonatal Fc receptor mediates internalization of Fc in transfected human endothelial cells. *Mol Biol Cell* (2008) **19**(12):5490–505. doi:10.1091/mbc.E07-02-0101
21. Ward ES, Zhou J, Ghetie V, Ober RJ. Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. *Int Immunol* (2003) **15**(2):187–95. doi:10.1093/intimm/dxg018
22. Leitner K, Ellinger I, Grill M, Brabec M, Fuchs R. Efficient apical IgG recycling and apical-to-basolateral transcytosis in polarized BeWo cells overexpressing hFcRn. *Placenta* (2006) **27**(8):799–811. doi:10.1016/j.placenta.2005.08.008
23. Vaccaro C, Zhou J, Ober RJ, Ward ES. Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. *Nat Biotechnol* (2005) **23**(10):1283–8. doi:10.1038/nbt1143
24. Vaccaro C, Bawdon R, Wanjie S, Ober RJ, Ward ES. Divergent activities of an engineered antibody in murine and human systems have implications for therapeutic antibodies. *Proc Natl Acad Sci U S A* (2006) **103**(49):18709–14. doi:10.1073/pnas.0606304103
25. Ram S, Prabhat P, Chao J, Ward ES, Ober RJ. High accuracy 3D quantum dot tracking with multifocal plane microscopy for the study of fast intracellular dynamics in live cells. *Biophys J* (2008) **95**(12):6025–43. doi:10.1529/biophysj.108.140392
26. Gan Z, Ram S, Ober RJ, Ward ES. Using multifocal plane microscopy to reveal novel trafficking processes in the recycling pathway. *J Cell Sci* (2013) **126**(Pt 5):1176–88. doi:10.1242/jcs.116327
27. Patel DA, Puig-Canto A, Challa DK, Perez Montoyo H, Ober RJ, Ward ES. Neonatal Fc receptor blockade by Fc engineering ameliorates arthritis in a murine model. *J Immunol* (2011) **187**(2):1015–22. doi:10.4049/jimmunol.1003780
28. Swiercz R, Chiguru S, Tahmasbi A, Ramezani SM, Hao GY, Challa DK, et al. Use of Fc-engineered antibodies as clearing agents to increase contrast during PET. *J Nucl Med* (2014) **55**(7):1204–7. doi:10.2967/jnumed.113.136481
29. Gan Z, Ram S, Vaccaro C, Ober RJ, Ward ES. Analyses of the recycling receptor, FcRn, in live cells reveal novel pathways for lysosomal delivery. *Traffic* (2009) **10**(5):600–14. doi:10.1111/j.1600-0854.2009.00887.x
30. Hoogerwerf WA, Tsao SC, Devuyst O, Levine SA, Yun CH, Yip JW, et al. NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. *Am J Physiol* (1996) **270**(1 Pt 1):G29–41.
31. He W, Ladinsky MS, Huey-Tubman KE, Jensen GJ, McIntosh JR, Bjorkman PJ. FcRn-mediated antibody transport across epithelial cells revealed by electron tomography. *Nature* (2008) **455**(7212):542–6. doi:10.1038/nature07255
32. Perez-Montoyo H, Vaccaro C, Hafner M, Ober RJ, Mueller W, Ward ES. Conditional deletion of the MHC Class I-related receptor, FcRn, reveals the sites of IgG homeostasis in mice. *Proc Natl Acad Sci U S A* (2009) **106**(8):2788–93. doi:10.1073/pnas.0810796106
33. Claypool SM, Dickinson BL, Wagner JS, Johansen FE, Venu N, Borawski JA, et al. Bidirectional transepithelial IgG transport by a strongly polarized basolateral membrane Fc-γ receptor. *Mol Biol Cell* (2004) **15**:1746–59. doi:10.1091/mbc.E03-11-0832
34. Tzaban S, Massol RH, Yen E, Hamman W, Frank SR, Lapierre LA, et al. The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity. *J Cell Biol* (2009) **185**(4):673–84. doi:10.1083/jcb.200809122
35. Jerdeva GV, Tesar DB, Huey-Tubman KE, Ladinsky MS, Fraser SE, Bjorkman PJ. Comparison of FcRn- and pIgR-mediated transport in MDCK cells by fluorescence confocal microscopy. *Traffic* (2010) **11**(9):1205–20. doi:10.1111/j.1600-0854.2010.01083.x
36. Cooper PR, Kliwinski CM, Perkinson RA, Ragwan E, Mabus JR, Powers GD, et al. The contribution of cell surface FcRn in monoclonal antibody serum uptake from the intestine in suckling rat pups. *Front Pharmacol* (2014) **5**:225. doi:10.3389/fphar.2014.00225
37. Zarate N, Mohammed SD, O'Shaughnessy E, Newell M, Yazaki E, Williams NS, et al. Accurate localization of a fall in pH within the ileocecal region: validation using a dual-scintigraphic technique. *Am J Physiol Gastrointest Liver Physiol* (2010) **299**(6):G1276–86. doi:10.1152/ajpgi.00127.2010
38. Koziolek M, Grimm M, Becker D, Iordanov V, Zou H, Shimizu J, et al. Investigation of pH and temperature profiles in the GI tract of fasted human subjects using the intellicap system. *J Pharm Sci* (2014). doi:10.1002/jps.24274
39. Gharpure V, Meert KL, Sarnaik AP, Metheny NA. Indicators of postpyloric feeding tube placement in children. *Crit Care Med* (2000) **28**(8):2962–6. doi:10.1097/00003246-200008000-00046
40. Irving SY, Lyman B, Northington L, Bartlett JA, Kemper C, Group NPW. Nasogastric tube placement and verification in children: review of the current literature. *Nutr Clin Pract* (2014) **29**(3):267–76. doi:10.1177/0884533614531456

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.