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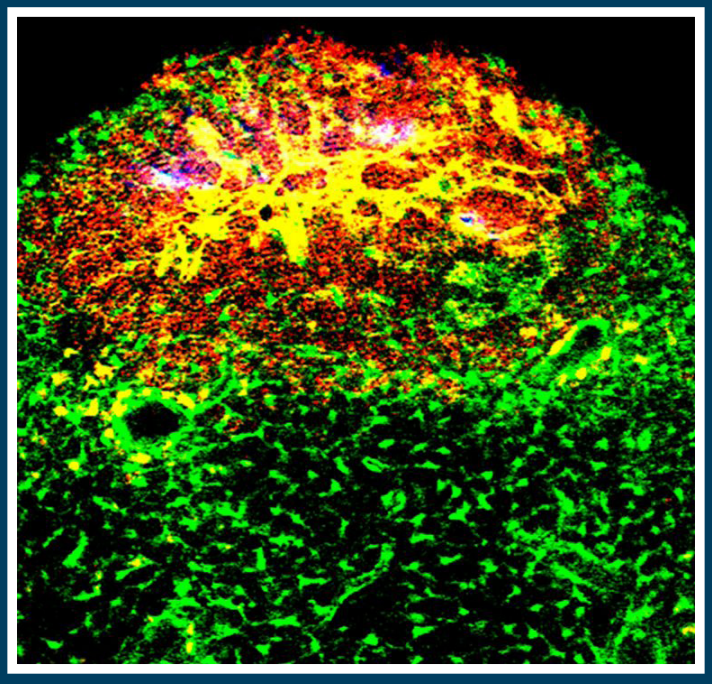

EMERGING IMMUNE FUNCTIONS OF NON-HEMATOPOIETIC STROMAL CELLS

Topic Editors
Christopher G. Mueller, Paul Kaye,
Mark C. Coles and Burkhard Ludewig

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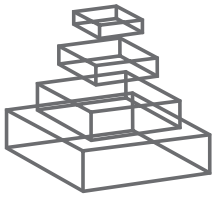

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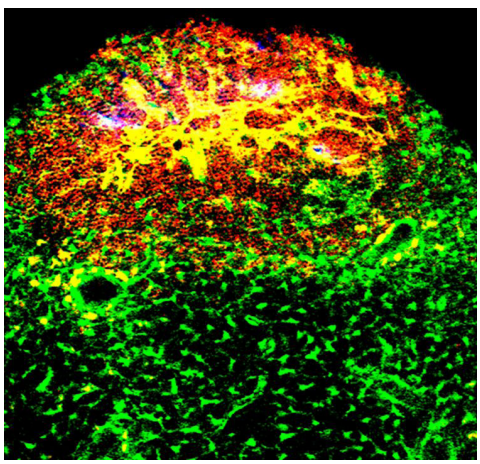
EMERGING IMMUNE FUNCTIONS OF NON-HEMATOPOIETIC STROMAL CELLS

Topic Editors:
Christopher G. Mueller, Centre National de la Recherche Scientifique, Strasbourg, France
Paul Kaye, University of York, United Kingdom
Mark C. Coles, University of York, United Kingdom
Burkhard Ludewig, Kantonal Hospital St. Gallen, Switzerland



Stromal cells were fate traced in the cervical lymph node using WNT1creRosa26eyfp mice. Neural crest derived mesenchymal cells give rise to all stromal cell lineages in lymph nodes from the head-neck region. Lymph nodes were stained with anti-GFP (Green) and anti-FDCM1 antibody to highlight the FDC cell network (red).

The development and function of the immune system is dependent on interactions between haematopoietic cells and non-hematopoietic stromal cells. The non-hematopoietic stromal cells create the microenvironment in which the immune system operates, providing an architectural landscape for hematopoietic cell-cell interactions and molecular cues governing haematopoietic cell positioning, growth and survival. Not surprisingly, therefore, aberrant stromal cell function has recently been shown to play a key role in the development of disease pathologies associated with immune dysfunction. For example, remodelling of lymphoid tissue stroma and the development of ectopic tertiary lymphoid tissues are characteristic of many infectious and inflammatory diseases and stromal cells have a recognised role in lymphoma and tumour development and resistance to therapy. An increased understanding of the molecular basis of stromal cell differentiation and function in these varied contexts will provide new tools to promote research on stromal cell biology and immune dysfunction, and potential new targets for therapeutic intervention in diseases with a major impact on public health. The importance of stromal cells and the molecular mechanisms of stromal cell function in the regulation of immune responses have only recently been appreciated and thus represent an exciting new area in immunology.

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Emerging immune functions of non-hematopoietic stromal cells

Christopher G. Mueller[1]* and Mark Christopher Coles[2]

[1] *Centre National de la Recherche Scientifique, Strasbourg, France*
[2] *University of York, York, UK*
**Correspondence: c.mueller@ibmc-cnrs.unistra.fr*

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Keywords: stromal cells, immune cells, lymphoid tissue, inflammation, development

The proper function of the immune system is dependent on interactions between hematopoietic cells and non-hematopoietic stromal cells, which comprise the ensemble of tissue-forming cells (fibroblasts, endothelial cells, epithelial cells, nerve cells, etc.) that form the microenvironments in which immune responses occur (1, 2). This collection of cells is also referred to as the immune stroma. It creates a microenvironment in which the immune system operates, providing an architectural landscape for hematopoietic cell–cell interactions and molecular cues governing hematopoietic cell positioning, growth, and survival (3). It is the organization of stromal cell networks that imposes order on adaptive and innate immune cells and thus drives efficient immune responses. This interdependence of stromal cells of different lineages with all types of hematopoietic cells is therefore an integral part of primary (bone marrow, thymus), secondary (lymph nodes, mucosal-associated lymphoid tissue), and tertiary lymphoid tissue (that arise in response to chronic inflammation). Even in healthy tissue the type of stroma and its cellular activity influences its hematopoietic composition and may determine whether or not tertiary lymphoid structures form in case of unresolved inflammation, characteristic of many infectious, autoimmune, hematological malignancies, and inflammatory diseases (4).

One of the earliest motives that have fueled the study of immune stroma has been the interest in immune system development and the quest to define the action of a set of molecules required for primary and secondary lymphoid organ formation (5, 6). Some effects were radical leading to a total absence or lymphoid organs, whereas some were mild, resulting in the reduction of a subset of hematopoietic cells such as B cells owing to the absence of follicular dendritic cells. These early works have also laid one of the principle distinctions between stroma and hematopoietic cells, namely, the relative radioresistance of stroma. This observation has been fundamental to many experimental analyses aimed at deciphering the molecular makeup of stroma leading for instance to the discovery of lymphotoxin-sensitive stroma. This approach is now being displaced by the advent of mouse models allowing stroma and hematopoietic specific gene deletion and reporter gene expression. In addition, the development of techniques to analyze stroma by flow cytometry together with high resolution 4Dimensional microscopic techniques have helped to loosen the knot that has hampered fast-track discovery science.

The study of immune stroma and its interaction with hematopoietic cells is a natural progress in our quest as immunologists to understand how the immune system protects tissue and the vital organs that it forms or – in opposition – causes morbidity and mortality by tissue-directed immunopathology and thus translation to the clinic. Yet, it adds an additional layer of complexity to immunology by supplanting our knowledge of the already complex network of hematopoietic cell communications with that of tissue-forming cells. Communication systems between fibroblasts may be used by hematopoietic cells, or messages sent by stromal cells may be read by a subset of hematopoietic cells. Indeed, the importance of stress-released neuro-substances has a well-recognized, but yet insufficiently understood impact on our immune system. Although these imprints may be subtle, they are one of the multiple ways to affect the immune system in the long term and coupled to our increased life-span these subtle impacts will end up visible. Another contribution to the gain of importance of stroma in immunology has been the acceptance that tissue-forming cells turn-over and are often dependent on a somatic stem cells to renew dying cells. For immunologists, who embrace the notion of cell activation, proliferation, and cell precursors as an integral part of their thinking, this realization has contributed to the acceptation that stroma may be an important player in immune plasticity. Thus, stromal plasticity is likely to rise to become a critical issue in the understanding of immune homeostasis.

A future challenge will be to focus our investigation on the meaningful interaction between stroma and hematopoietic cells that determine the outcome of immune responses and not to be side tracked by observing the bystander effect of stroma cross-talk. The second challenge will be to translate these findings to pathology, in particular, tertiary lymphoid structure formation arising during chronic inflammation. Indeed, a better understanding of the molecular basis of stromal cell differentiation and function will provide potential new targets for therapeutic intervention in these diseases. Taken together, the study of non-hematopoietic stromal cells is an emerging theme in biology because (i) immunologists have realized that the immune system is strongly influenced by the tissue-forming cells, (ii) novel genetic mouse models and experimental tools are emerging to better address this issue, and (iii) by definition, it is an interdisciplinary field making the link between the hematopoietic cells and the tissue.

This collection of mini-reviews, perspectives, and original articles on stroma aims to attract the general attention of immunologists. It therefore includes different types of stromal cells: fibroblastic (FDCs, TRCs, and MRCs), blood, and lymphatic endothelial cells. Moreover, it focuses on primary, secondary, and tertiary lymphoid structures. Admittedly, this collection is far from complete, but it is a first brick in the building of our understating of stroma in the complex immunological landscape.

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Mesenchymal cell differentiation during lymph node organogenesis

Andrea Brendolan*[1]* and *Jorge H. Caamaño*[2]

[1] *Division of Molecular Oncology, San Raffaele Scientific Institute, Milan, Italy*
[2] *School of Immunity and Infection, Institute for BioMedical Research-Medical Research Council Centre for Immune Regulation, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK*

Edited by:
Matthew Griffin, National University of Ireland, Ireland

Reviewed by:
Tom Cupedo, Erasmus University Medical Center, Netherlands
Christopher G Mueller, Centre National de la Recherche Scientifique, France

***Correspondence:**
Andrea Brendolan, Division of Molecular Oncology, San Raffaele Scientific Institute, Milan 20132, Italy.
e-mail: brendolan.andrea@hsr.it;
Jorge H. Caamaño, School of Immunity and Infection, Institute for BioMedical Research-Medical Research Council Centre for Immune Regulation, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK.
e-mail: j.caamano@bham.ac.uk

Secondary lymphoid tissues such as lymph nodes are essential for the interactions between antigen presenting cells and lymphocytes that result in adaptive immune responses that protect the host against invading pathogens. The specialized architecture of these organs facilitates the cognate interactions between antigen-loaded dendritic cells and lymphocytes expressing their specific receptor as well as B–T cell interactions that are at the core of long lasting adaptive immune responses. Lymph nodes develop during embryogenesis as a result of a series of cross-talk interactions between a hematopoietically derived cell lineage called lymphoid tissue inducer cells and stromal cells of mesenchymal origin to form the anlagen of these organs. This review will present an overview of the different signaling pathways and maturation steps that mesenchymal cells undergo during the process of lymph node formation such as cell specification, priming, and maturation to become lymphoid tissue stromal organizer cells.

Keywords: lymphoid tissues, stromal cells, lympho-mesenchymal interactions, lymphotoxin beta receptor, NF-κB

Lymph nodes (LN) develop during embryogenesis in mice and humans following a precise timing depending on anatomical location. Mesenteric LNs develop first in mouse embryos around embryonic day (E) 10.5, followed by the rest of these organs along the anterior-posterior body axis (Rennert et al., 1996, 1998; Mebius, 2003). Vertebrate organogenesis results from complex interactions of molecular and cellular networks in which progenitor cells become specified, proliferate and differentiate to ascertain organ formation and function (Brendolan et al., 2007; Costantini and Kopan, 2010). These sequential events are orchestrated by signaling molecules that activate cell-specific gene expression programs in uncommitted progenitors (Dressler, 2009). Thus, it is conceivable that LN development relies on similar mechanisms for the acquisition of cellular identity (specification) and that uncommitted mesenchymal cells assume a LN fate prior to proliferation and formation of the anlagen. Once specified, mesenchymal cells engage in cross-talk with lymphoid cells and this assures LN expansion coupled to mesenchymal cell differentiation (Roozendaal and Mebius, 2011). Two central cellular players required for the development of secondary lymphoid tissues during mouse embryogenesis have been identified (Honda et al., 2001; Mebius, 2003; Nishikawa et al., 2003). Lymphoid tissue inducer (LTi) cells, derived from lymphoid cell precursors and belonging to the family of innate lymphoid cells and mesenchymal progenitors cells whose origin have not been elucidated yet. LTi cells express CD45, CD4, interleukin-7 receptor α, integrin α4β7, receptor activator of NF-κB (RANK/TRANCE-R) and its ligand RANKL/TRANCE, lymphotoxin α1β2 (LT1β2), and the chemokine receptor CXCR5 and thus are attracted in response to the chemokine CXC-chemokine ligand 13 (CXCL13) secreted by mesenchymal cells.

Conversely, mesenchymal cells have been characterized as CD45$^-$, PDGF-receptor α$^+$, lymphotoxin β receptor$^+$ (LTβR), vascular cell adhesion molecule-1 (VCAM-1$^-$), and intercellular adhesion molecule-1 (ICAM-1$^-$). Analysis of different knockout mouse models has led to the discovery of several genes required for LN development and revealed a multistep process in which interactions between LTi cells and mesenchymal cells appear crucial to assure organ formation. However, the origin and identity of the signals that induce the specification of mesenchymal progenitor cells prior to the arrival of LTi cells at the site of LN formation remain largely unknown.

Lymphoid tissue inducer cell numbers appear to be the limiting factor controlling the development of LNs and other secondary lymphoid tissues as shown by the fact that over-expression of IL-7 *in vivo*, results in a significant increase of LTi cells and the number of LN (Meier et al., 2007).

MESENCHYMAL CELL SPECIFICATION

The mechanisms governing the spatial and temporal organization of the different LNs are poorly understood. It is currently unclear which signals assure LN organogenesis to take place at define locations along the body axis and ensure mesenchymal cell

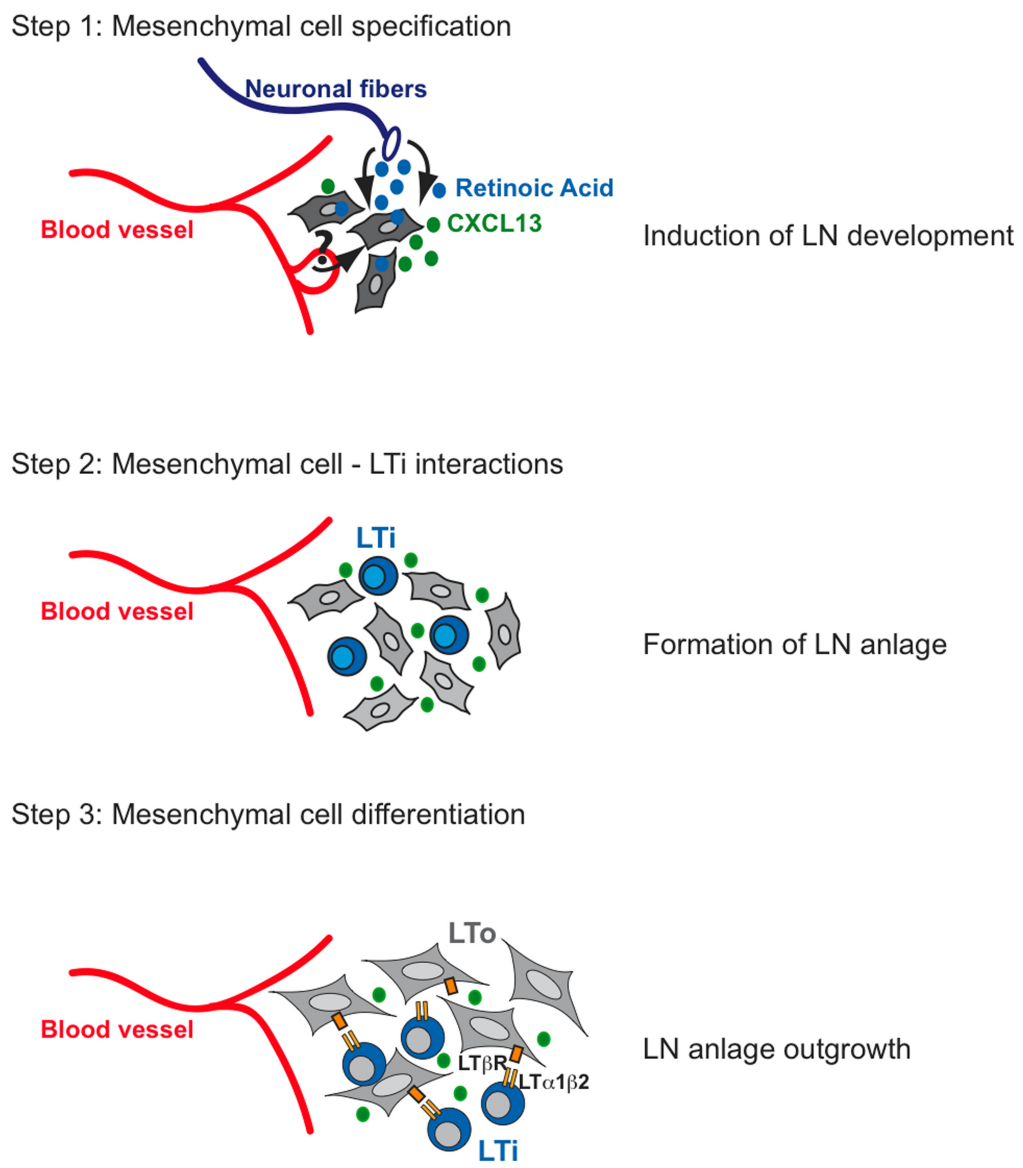

FIGURE 1 | Model of lymph node development. *Step 1*: Retinoic Acid produced by neurons stimulates mesenchymal cells to express the chemokine CXCL13. *Step 2*: CXCL13 expression by mesenchymal cells attracts lymphoid tissue inducer (LTi) cells to the site where lymph nodes will develop. LTi cells will cluster and they might signal in trans to each other through RANKL-RANK. *Step 3*: RANK signaling on LTi cells will induce high expression levels of LTα1β2. Binding of the latter to LTβR on mesenchymal cells will induce the expression of cell adhesion molecules VCAM-1, ICAM-1, and MAdCAM-1 as well as CXCL13, CCL21, and CCL19 to initiate a positive feedback loop that will attract large numbers of LTi cells to the LN anlage and thus result in the formation of the structure the organs.

specification prior to the clustering of LTi cells at sites where these organs will develop.

Given the different location of LNs along the antero-posterior axis, it is likely that different signals are required to commit progenitor cells toward a LN fate for each specific set of organs. Expression of CXCL13 by mesenchymal cells appears to be the first sign of mesenchymal cell specification (van de Pavert et al., 2009; **Figure 1**, step 1). This chemokine is required for the initial recruitment of LTi cells to form clusters with the former at the sites of LN formation (Ansel et al., 2000; Luther et al., 2003; Ohl et al., 2003). Recent work has shown that retinoic acid (RA) from neuronal cells induces CXCL13 expression in mesenchymal cells, indicating a possible role for RA in specifying, at least in part, the LN-mesenchyme (van de Pavert et al., 2009). These results

suggest a mechanism for specification of mesenchymal cells and for their initial condensation to form the LN anlagen that is LTi cell-independent. While RA was shown to induce CXCL13 expression in the mesenchymal LN anlage, it is presently unknown whether this morphogen has a similar action in the presumptive mesenchymal cells at the site of all LNs or different specification signals exist depending on the location of the organs. *Cxcl13*$^{-/-}$ and *Cxcr5*$^{-/-}$ embryos fail to form most LN anlagen due to the inability of recruiting LTi cells, yet mesenteric and cervical LNs are present in adult *Cxcl13*$^{-/-}$ mice, and *Cxcr5*$^{-/-}$ embryos develop rudimentary LN anlage (Ansel et al., 2000; Ohl et al., 2003). However, the finding that specification of mesenchymal cells can occur even in the absence of CXCL13 argues in favor of other signals required for this process.

Given the pleiotropic role of RA during vertebrate development including its capacity to regulate cell-fate and differentiation (Niederreither and Dolle, 2008), if RA derived from neuronal cells is required for specifying the mesenchymal cells of most LN, it is likely that, in addition to CXCL13, it activates a set of downstream targets including Hox genes (Wang et al., 2006; Duester, 2008). Hox genes encode a family of homeodomain transcription factors required for organ patterning and cell fate specification along the antero-posterior axis during embryogenesis thus they represent candidate factors involved in patterning the early LN anlage. It has been shown that the mature counterpart of the mesenchymal cells that form the LN anlage, lymphoid tissue organizer (LTo) cells from mesenteric LNs and Peyer's patches express different Hox genes (Okuda et al., 2007).

A century ago, Sabin (1902, 1909) identified a series of structures in pig embryos named lymph sacs from which it was hypothesized that LN develop. In support of this hypothesis is the recent finding that lymph sacs form during inguinal LN formation through endothelial-cell budding from the vasculature. It was proposed that invasion of the endothelial lumen by the surrounding mesenchyme will form the LN anlagen (Benezech et al., 2010). However, Vondenhoff et al. (2009b) have shown that in mouse embryos lacking lymphatic vasculature due to conditional ablation of *Prox1* in endothelial cells (*Tie2-Cre*; *Prox1*$^{f/f}$) the LN anlage still forms, although it is associated with hypoplasia and reduced number of LTi cells. While these findings indicate that lymphatic endothelial cells and lymph sacs may not be required for positioning LTi cells at the site of organ formation, they do not exclude a role for the lymphatic endothelium in specifying the surrounding mesenchyme. Thus, loss of lymphatic signals may affect the ability of mesenchymal cells to become fully specified and to attract a critical number of LTi cells at the site of LN anlage formation and this scenario may also explain the LN hypoplasia observed in *Prox1*$^{-/-}$ mice.

At present, however, despite a possible role of RA in the specification of mesenchymal progenitors, the source of the signals and the transcriptional program that ensures full commitment of progenitor cells into LN mesenchymal cells remains unknown.

LYMPH NODE ANLAGE FORMATION AND EXPANSION

Following mesenchymal cell specification and clustering of LTi cells, a series of cross-talk interactions between these cell populations appear to be crucial to assure organ development (**Figure 1**, step 2). Clustering of LTi cells expressing both RANK-L and its receptor RANK in the developing LN anlagen suggest that the RANK-L/RANK signaling pathway might be activated in trans by the close proximity of these cells (Kim et al., 2000; Vondenhoff et al., 2009a). RANK signaling up-regulates the expression of LTα1β2 thus enhancing the cross-talk interactions between LTi cells and the mesenchymal precursor cells (Yoshida et al., 2002; Vondenhoff et al., 2009a). A putative function for RANK signaling in LN stromal cells has been recently indicated (Hess et al., 2012; Sugiyama et al., 2012). Analysis of the CD45^{-} cell population in mesenteric and inguinal LN of E14 mouse embryos onward shows the presence of newly specified mesenchymal cells that are negative for VCAM-1 and ICAM-1 expression but positive for PDGFRα. By E15.5 these V^{-}I^{-} mesenchymal precursors differentiate into VCAM-1^{Int} ICAM-1^{Int} PDGFRα^{+} "primed" mesenchymal cells independently of the presence of LTi cells as accumulation of this intermediate population is present in *Rorc*$^{-/-}$ and *Ltβr*$^{-/-}$ embryonic LNs (Benezech et al., 2010). Importantly, the signal/s that prime the V^{-} I^{-} progenitors to become V^{int} I^{int} mesenchymal cells remains to be identified. By E17.5 primed V^{int} I^{int} mesenchymal cells differentiate into V^{high} I^{high} MAdCAM-1^{+} LTo cells (**Figure 1**, step 3). This maturation step requires LT signaling and LTi cells as LTo cells are absent in the LN anlagen of *Ltbr*$^{-/-}$ and *Rorc*$^{-/-}$ mice (lacking LTi cells). Accumulation of LTi cells in the LN anlagen coincides with the differentiation of V^{int} I^{int} mesenchymal cells into V^{high}I^{high} MAdCAM-1^{+} LTo cells (Cupedo et al., 2004b; Cupedo and Mebius, 2005; Benezech et al., 2010).

The tumor necrosis factor (TNF) family ligand LTα1β2 expressed on LTi cells engages its receptor, LTβR on V^{int} I^{int} mesenchymal cells, resulting in the activation of the NF-κB family of transcription factors through the classical/canonical (NF-κB1 p50/RelA) and the alternative/non-canonical pathways (NF-κB2 p52/RelB; Dejardin et al., 2002; Yilmaz et al., 2003). This process leads to increased expression of ICAM-1 and VCAM-1 and the homeostatic chemokines CXCL13, CCL19, and CCL21 creating a positive feedback loop for the continuous recruitment and retention of LTi cells and for the proliferation and homeostasis of LTo cells (Randall et al., 2008; Ruddle and Akirav, 2009; van de Pavert and Mebius, 2010). Interestingly, while the three stromal cell populations present in LN anlagen (V^{-}I^{-}, V^{int} I^{int}, V^{high}I^{high}) express LTβR, the NF-κB member RelB is only detected in V^{int} I^{int}, and V^{high}I^{high} cells correlating with their higher levels of expression of chemokines and cell adhesion molecules with respect to the V^{-}I^{-} cell population (Benezech et al., 2010). Lack of LTβR signaling in LTo cells, as observed in *Ltα*$^{-/-}$, *Ltβr*$^{-/-}$, and *Rorc*$^{-/-}$ mice, results in the absence of all LNs (De Togni et al., 1994; Futterer et al., 1998; Sun et al., 2000; Eberl and Littman, 2003; Eberl et al., 2004; White et al., 2007; Benezech et al., 2010). *Relb*$^{-/-}$ mice also fail to develop all LNs and *Nfkb2*$^{-/-}$ mice present with poorly developed inguinal and popliteal LNs due to impaired expression of chemokines, cell adhesion molecules and development of high endothelial venules (Weih and Caamaño, 2003; Carragher et al., 2004). Similarly, mice carrying a phosphorylation mutant kinase IKKα, that is essential for the

activation of the NF-κB alternative pathway, have a similar LN phenotype than the *Nfkb2*$^{-/-}$ mice (Drayton et al., 2004). LTβR signaling is required for the maturation and homeostasis of LTo cells and for expression of RANK-L, MAdCAM-1, and lymphoorganogenic chemokines (Yoshida et al., 2002; Eberl et al., 2004; Coles et al., 2006; White et al., 2007; Vondenhoff et al., 2009a; Benezech et al., 2010).

LN ORGAN EXPANSION AND LT ORGANIZER CELL DIFFERENTIATION

Lymphoid tissue organizer cells are thought to represent the precursors of mature stromal cells in adult LN. However, the contribution of the former to the stromal cell subsets in adult organs and the signals that induce their differentiation are still poorly defined.

Previous work showed that transplantation of neonatal LN cells under the skin of adult mice gave rise to distinct stromal cell networks, thus indicating that neonatal LTo cells from the transplanted cell suspensions were capable to differentiate into mature stromal cells (Cupedo et al., 2004a). Importantly depletion of LTi cells from the neonatal LN cell populations impaired their ability to develop an ectopic lymphoid structure in this system. Despite that these findings indicate that neonatal LN contains stromal progenitors, lineage-tracing experiments are required to unequivocally demonstrate the precursor-product relationship between embryonic LTo cells and adult LN stromal cells, including follicular dendritic cells as previously suggested (Katakai et al., 2008). In addition, it also needs to be determined whether the different stromal cell subsets originate from single multipotent mesenchymal progenitors or whether distinct progenitors exist for each stromal cell subpopulation.

MARGINAL RETICULAR CELLS

Lymphoid tissue organizer cells and marginal reticular cells (MRCs) are similar in the expression of several markers suggesting a precursor-product relationship between these cell types (Katakai et al., 2008; Roozendaal and Mebius, 2011), although formal proof that LTo cells can generate MRCs is still lacking. MRCs are located under the subcapsular sinus of LNs and in the spleen marginal zone where they are referred as marginal sinus lining cells. These stromal cells express MAdCAM-1, CXCL13, VCAM-1, ICAM-1, BP3, and RANK-L and their maturation does not appear to depend on signals from T- or B-cells as *Rag2*$^{-/-}$ mice have an intact LN MRC layer (Katakai et al., 2008). However, blocking LTβR signaling causes loss of CXCL13 and MAdCAM-1 expression on MRCs thus indicating that engagement of the LT pathway, possibly by LTi cells or their adult counterpart is required for maintaining the phenotypic characteristics of these cells. A recent report has shown that over-expression of RANK-L has an effect on MRCs and other stromal and endothelial cell types in adult LN resulting in enhance cell proliferation and organ expansion with increase numbers of B-cell follicles (Hess et al., 2012). Conversely, blocking of RANK-L in mouse embryos appears to disrupt B-cell follicle formation and induce HEV maturation in newborn mice (Sugiyama et al., 2012). In addition, differentiation of MRC appears to be strictly connected to signals associated to the development of secondary lymphoid organs since these cells are not found in ectopic lymphoid tissues and tertiary lymphoid organs (Katakai et al., 2008).

FOLLICULAR DENDRITIC CELLS

Follicular dendritic cells (FDCs) localize in the center of the B-cell follicle and appear a week after birth. Several studies have proposed a mesenchymal origin for FDCs although it is currently unclear whether FDCs arise from in situ embryonic mesenchymal precursors or from cells migrating to the organ postnatally (Allen and Cyster, 2008; e.g., bone marrow).

Recent studies show that FDCs originate from perivascular progenitor cells expressing *Mfge8* and *Pdgfrb* genes and that ablation of PDGFRβ^{+} cells induces the collapse of FDC networks. In addition, these findings also showed that PDGFRβ^{+} perivascular cells from non-lymphoid organs have the capacity to differentiate into FDCs *in vitro* and *in vivo*, thus suggesting that this cell population may be the source of FDC in tertiary lymphoid organ formation (Krautler et al., 2012). B-cell derived signals are required for FDC maturation as demonstrated by mice deficient for TNFα, $LT\alpha_1\beta_2$ and their receptors that fail to develop FDC networks and germinal centers (Allen and Cyster, 2008).

FIBROBLASTIC RETICULAR CELLS

Fibroblastic reticular cells (FRCs) are a heterogeneous population of stromal cells distributed in the T-zone of secondary lymphoid organs (Mueller and Germain, 2009). FRCs form the conduit system, a network of collagen-rich channels surrounded by fibroblasts that allows small molecules, such as chemokines and antigens to reach the T cell zones (Sixt et al., 2005; Bajenoff et al., 2006). Contrary to the spleen in which the formation of the FRC network depends on LTα1β2 from B-cells, LNs FRC networks develop normally in the absence of B-cells (Ngo et al., 2001), thus indicating that different signaling molecules and cell types may be required for proper FRC differentiation in different lymphoid organs. At present, it remains unclear whether FRCs originate from a common embryonic mesenchymal progenitor or if different lineages of mesenchymal cells generate the FRC network.

CONCLUDING REMARKS

Over the past several years, novel findings have highlighted the complexity of the cellular and molecular mechanisms governing lymphoid organ development and function. Central to these findings is the notion that interactions between lymphoid and mesenchymal cells are crucial for the development of secondary lymphoid organs. However, the cellular and molecular events underlying LN regionalization and those implicated in mesenchymal cell specification remain largely undefined.

It is also unknown at what point during lineage diversification mesenchymal cells become fully committed toward a specific fate and whether distinct stromal cell subsets arise from single multipotent progenitors or if different precursors exists for each stromal cell type. Despite these developmentally unsolved questions, recent work by several groups has shown that stromal cells are not merely passive inhabitants of lymphoid organs as previously thought, but instead are active players in modulating the activity of the immune system by providing structural support and signals

for survival, attraction, locomotion, and activation of immune cells (Mueller and Germain, 2009). The recent discovery that some stromal cell subsets contribute to tolerance induction further highlights their important function in the homeostasis of immune system (Fletcher et al., 2011). Thus, a full understanding of the ontogeny and function of the stromal microenvironment still requires that we uncover the genetic and transcriptional programs underlying mesenchymal cell differentiation and elucidate the molecular repertoire that characterize each stromal subsets during normal and pathological conditions.

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Marginal reticular cells: a stromal subset directly descended from the lymphoid tissue organizer

Tomoya Katakai*

Department of Molecular Genetics, Institute of Biomedical Science, Kansai Medical University, Moriguchi, Osaka, Japan

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Scott Mueller, The University of Melbourne, Australia
Tom Cupedo, Erasmus University Medical Center, Netherlands

***Correspondence:**
Tomoya Katakai, Department of Molecular Genetics, Institute of Biomedical Science, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan.
e-mail: katakait@takii.kmu.ac.jp

The architecture of secondary lymphoid organs (SLOs) is supported by several non-hematopoietic stromal cells. Currently it is established that two distinct stromal subsets, follicular dendritic cells and fibroblastic reticular cells, play crucial roles in the formation of tissue compartments within SLOs, i.e., the follicle and T zone, respectively. Although stromal cells in the anlagen are essential for SLO development, the relationship between these primordial cells and the subsets in adulthood remains poorly understood. In addition, the roles of stromal cells in the entry of antigens into the compartments through some tissue structures peculiar to SLOs remain unclear. A recently identified stromal subset, marginal reticular cells (MRCs), covers the margin of SLOs that are primarily located in the outer edge of follicles and construct a unique reticulum. MRCs are closely associated with specialized endothelial or epithelial structures for antigen transport. The similarities in marker expression profiles and successive localization during development suggest that MRCs directly descend from organizer stromal cells in the anlagen. Therefore, MRCs are thought to be a crucial stromal component for the organization and function of SLOs.

Keywords: CXCL13, fibroblastic reticular cell, follicular dendritic cell, lymph node, marginal reticular cell, organizer, secondary lymphoid organ, stromal cell

INTRODUCTION

Secondary lymphoid organs/tissues (SLOs) are essential for the efficient induction of adaptive immune responses. Several types of SLOs, including the lymph nodes (LNs), spleen, and mucosal-associated lymphoid tissues (MALTs) such as Peyer's patches (PPs), are strategically positioned throughout various places within the body. SLOs are an elaborate filter that samples antigens and is equipped with highly sensitive immune sensors. In order to collect and filtrate foreign antigens, SLOs contain specialized tissue structures that are associated with the endothelium or epithelium. Immune cells such as lymphocytes, dendritic cells (DCs), and macrophages accumulate to high densities and form compartments. A remarkable feature common to all SLOs is the segregated localization of B cells and T cells. The architecture of SLOs is supported by several types of non-hematopoietic stromal cells of mesenchymal origin, which construct networks and define compartments (Mueller and Germain, 2009; Roozendaal and Mebius, 2011). Stromal networks provide not only a functional foothold but also a space for immune cell activities, as well as a physical framework for the tissue. Moreover, growing evidence indicates that stromal cells also play critical roles in immune cell function and homeostasis (Link et al., 2007; Fletcher et al., 2010; Suzuki et al., 2010; Lukacs-Kornek et al., 2011). SLOs are programmed to develop from the anlagen that occur at certain places and during restricted periods in the fetus and infant. In addition, stromal cells are known to be important for SLO development and maintenance (Mebius, 2003). Here, I focus on a recently identified stromal subset, marginal reticular cells (MRCs), and summarize their characteristics. I also discuss the relationship between MRCs and tissue structure, other stromal subsets, and immunological functions.

Abbreviations: DC, dendritic cell; FAE, follicle-associated epithelium; FDC, follicular dendritic cell; FRC, fibroblastic reticular cell; HEV, high endothelial venue; IFC, interfollicular channel; IFR, interfollicular region; LN, lymph node; LT, lymphotoxin; LTi, lymphoid tissue inducer; LTo, lymphoid tissue organizer; MALT, mucosal associated lymphoid tissue; MRC, marginal reticular cell; MS, marginal sinus; MZ, marginal zone; PALS, periarteriolar lymphoid sheath; PP, Peyer's patch; SED, subepithelial dome; SLO, secondary lymphoid organ; SCS, subcapsular sinus; TLT, tertiary lymphoid tissue.

T AND B CELL COMPARTMENTS AND TWO CONVENTIONAL STROMAL CELL SUBSETS

As described above, B cells and T cells localize to distinct regions within SLOs. B cells accumulate to form follicles (B zone) in the outer cortex beneath the capsule in LNs or in the outer periarteriolar lymphoid sheath (PALS) in the spleen. A cluster of large follicles is the core tissue of PPs. During immune responses, activated B cells in the follicles form germinal centers, where they differentiate into high-affinity antibody producers (Cyster et al., 2000). In contrast, T cells localize to a separate area (T zone) adjacent to the follicles, i.e., the paracortex in LNs, inner PALS in the spleen, or interfollicular region (IFR) in PPs. DCs also accumulate in the T zone to present antigen and prime T cells (Steinman et al., 1997).

There are two different types of mesenchymal stromal subsets in the B and T zones (**Figure 1A**). Follicular dendritic cells (FDCs) form a dense network in the center of the follicles and have received considerable attention because of their importance in antibody production by B cells (Tew et al., 1997;

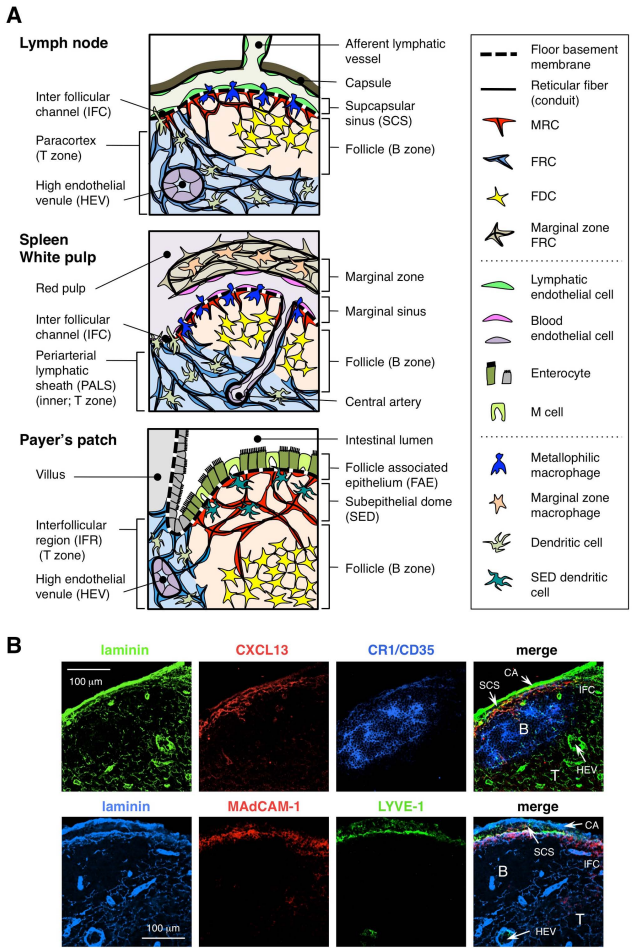

FIGURE 1 | Stromal cell subsets and tissue structures of SLOs. **(A)** Schematic representation of tissue structures in mouse SLOs, with an emphasis on stromal cells and the antigen-transporting apparatus. Stromal elements, including mesenchymal, endothelial, and epithelial cells as well as myeloid cells such as macrophages and dendritic cells (listed in the right panel), are drawn for emphasis. **(B)** Upper: The tissue framework constructed by three different types of stromal cells in the outer cortical region of LN. Fluorescent immunostaining of a mouse LN section. In the upper micrograph, there is a small resting follicle (B) without a germinal center, which is supported by FDCs expressing CR1/CD35 but undetectable levels of CXCL13. Instead, MRCs form a laminin$^+$CXCL13$^+$ reticular network in the outer edge of the follicle underneath the capsule (CA) and SCS. The network constructed by FRCs in the paracortex (T) is laminin$^+$CXCL13$^-$CR1/CD35$^-$. Lower: MAdCAM-1$^+$ MRC layer is closely associated with LYVE-1$^+$ lymphatic endothelial layer in the SCS. Note that MRC layer is extended to the IFC area.

Cyster et al., 2000; Victoratos et al., 2006; Suzuki et al., 2010). FDCs express CR1/CD35, CD23, and occasionally MAdCAM-1 (Szabo et al., 1997; Cyster et al., 2000). By contrast, an elaborate network of fibroblastic reticular cells (FRCs) comprises the scaffold of the T zone, which produces podoplanin/gp38 and various extracellular matrix (ECM) components (Gretz et al., 1997; Luther et al., 2000; Kaldjian et al., 2001; Katakai et al., 2004a,b). In general, immune cell migration and localization are regulated by a variety of chemokines (Mackay, 2001). Resting lymphocytes and mature DCs are highly responsive to "homeostatic chemokines" (Cyster, 1999; Müller et al., 2003). Consistent with this, there are clear correlations between chemokines produced by stromal cell subsets and the localization of immune cells expressing the corresponding receptors; CXCL13 produced by FDCs is an attractant of B cells expressing CXCR5, while CCL19 and CCL21 from T zone FRCs attract T cells and mature DCs through the common receptor CCR7 (Cyster, 1999; Cyster et al., 2000; Luther et al., 2000). Moreover, lymphocytes that migrate robustly in the tissue parenchyma to scan antigens are thought to use stromal networks as a foothold (Bajénoff et al., 2006). A variety of factors produced by stromal subsets are also required for the activation and survival of immune cells (Cyster et al., 2000; Huber et al., 2005; Link et al., 2007; Suzuki et al., 2010; Lukacs-Kornek et al., 2011; Malhotra et al., 2012). Therefore, the structure and function of the stromal network in each compartment is likely optimized for the activity of immune cell subsets.

The stromal network in the T zone is composed of FRCs and an ECM bundle known as a reticular fiber, which forms a "conduit" that facilitates the passage of low molecular weight substances (Gretz et al., 1997, 2000; Nolte et al., 2003). This conduit transports various factors and soluble antigens deep within the tissue, while particles and large molecules are predominantly excluded from the lymphocyte compartments. Indeed, lymph-borne chemokines from peripheral tissues are rapidly transported to the high endothelial venule through the conduit to control the mobilization of circulating cells into the LNs (Palframan et al., 2001). In addition, some resident DCs directly contact the conduit and capture lymph-borne antigens (Sixt et al., 2005).

A NEWLY IDENTIFIED STROMAL SUBSET, MARGINAL RETICULAR CELLS

The outer margin of the LN cortex, just beneath capsule, is surrounded by the subcapsular sinus (SCS; **Figure 1A**). The luminal surface of the SCS is covered with lymphatic endothelial cells and their cortical side is backed by the basement membrane, called the "floor" (Szakal et al., 1983). Particularly within the IFRs, reticular fibers spread out from the floor into the paracortex (Gretz et al., 1997). Importantly, a thin layer of reticular structure is also observed in the outermost region of the follicles. FRC-like stromal cells in the follicular reticulum express CXCL13 and MAdCAM-1 but not CCL21, which indicates that these cells are distinct from T zone FRCs, while the FDC marker CR1/CD35 was undetected or only weakly expressed in these cells (Katakai et al., 2008; **Figures 1B and 2A**). FDCs do not generate reticular fibers, and accordingly, the reticular marker ER-TR7 is virtually absent in the center of the follicles (Katakai et al., 2004a,b). In addition, reticular cells in the subcapsular region specifically express RANKL/TRANCE, which is a TNF family cytokine that is essential for LN development (Dougall et al., 1999; Kong et al., 1999). Therefore, these stromal cells were thought to be a new stromal subset and were designated marginal reticular cells (MRCs; Katakai et al., 2008). Of note, a substantial amount of CXCL13 is constitutively expressed in MRCs in resting small follicles even if FDCs express undetectable levels of CXCL13 (**Figure 1B**). It is well established that the outer edge of PALS in the spleen, especially that which lies over the follicles, is bordered by a MAdCAM-1$^+$

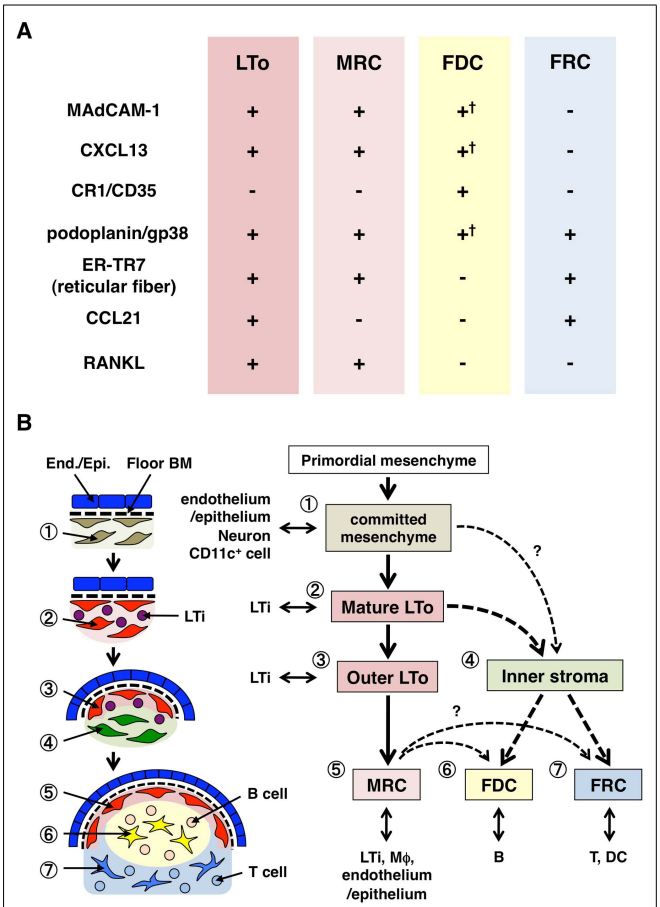

FIGURE 2 | Relationship between MRCs and other stromal cell subsets in SLOs. (A) Pattern of marker expression in stromal cell subsets. Expression levels of MAdCAM-1, CXCL13, and podoplanin/gp38 in FDCs are often weak or undetectable by immunohistochemistry (†). **(B)** Schematic of a generalized model of SLO development with the locations of stromal cell subsets (left) and a putative genealogy of stromal cell subsets (right). Numbers indicating cell elements in the left drawing represent stromal cell subsets shown in the right scheme. BM, basement membrane; End./Epi., endothelial or epithelial layer.

stromal layer. The cells that constitute the lining of basement membrane beneath the marginal sinus (MS) also express CXCL13 and RANKL, indicating that these cells are a type of MRCs (Katakai et al., 2008). In MALTs, reticular cells similar to MRCs form a network in the subepithelial dome (SED) region just under the basement membrane of the follicle-associated epithelium (FAE; Katakai et al., 2008; Knoop et al., 2009). Taken together, MRCs are thought to be a stromal subset common to SLOs but distinct from FDCs and FRCs.

MRCs AND THE ANTIGEN ENTRY ROUTE IN SLOs

Secondary lymphoid organs are characterized by peculiar structures that filtrate and transport antigens into the lymphocyte compartments (**Figure 1A**). LNs are connected with lymphatic vessels to survey lymph-borne antigens. Because the afferent lymphatics are open to the SCS, the sinus lymphatic endothelium and floor basement membrane are, as it were, the front surface of the LN filter. The spleen filters blood, in which branches of the central artery open to the MS and marginal zone (MZ), where immune cells survey the blood contents. In these specialized sinus structures, the border barriers are the lymphatic endothelium in the SCS of the LN and the blood endothelium in the MS of the spleen, both of which are supported by the MRC reticulum. Interestingly, $CD169^+$ metallophilic macrophages are selectively distributed near the sinus lining over the follicles, some of which settle across the endothelial barrier and convey particulate antigens from the sinus lumen into the lymphocyte compartment (Szakal et al., 1983; Taylor et al., 2005; Carrasco and Batista, 2007; Phan et al., 2009). DCs that carry antigens from the tissues via the lymphatic vessels arrive at the SCS and subsequently move into the paracortex across the floor of the interfollicular channel (IFC; Steinman et al., 1997; Katakai et al., 2004b; Braun et al., 2011). In MALTs, the FAE functions as the barrier in which a specialized epithelial cell called M cells transfers bacteria or particles from the gut lumen to underlying DCs or macrophages (Kraehenbuhl and Neutra, 2000). Therefore, MRCs support the frontline antigen-transporting apparatus in each SLO. The basic design of SLOs is that the follicles are primarily arranged toward the site of antigen entry and are accompanied by T zones. Thus, from a stromal viewpoint, the whole tissue architecture appears to be organized toward the MRC layer.

Although the immunological functions of MRCs remain elusive, it was previously shown that the conduit network constructed by MRCs in the outer follicle transports small soluble antigens to follicular B cells and FDCs (Bajénoff and Germain, 2009; Roozendaal et al., 2009). Most $CD169^+$ macrophages are positioned at the cortical side of the SCS floor and protrude an extension into the sinus lumen to capture particles (Phan et al., 2009). Thus, MRCs might be involved in the localization, morphology, and function of these macrophages. M cell development in PPs requires RANKL-RANK signaling, which is likely controlled by a RANKL-expressing MRC network just beneath the FAE (Knoop et al., 2009). Inhibiting the LT pathway, which abolishes MRC signatures in the splenic white pulp, disturbs the MS structure (Koike et al., 1996; Balogh et al., 2007; Katakai et al., 2008; Zindl et al., 2009). Moreover, elevated ICAM-1, VCAM-1, and CXCL13 expression implies that MRCs are involved in the dynamic interstitial migration of follicular B cells as a functional scaffold. $CD169^+$ macrophages have been shown to directly deliver particulate antigens to antigen-specific B cells in this area (Carrasco and Batista, 2007; Phan et al., 2009), suggesting that MRC network-mediated control of B cell migration may impact this process. Since MRCs are also present in the IFC region, they possibly regulate the transmigration of DCs from the SCS toward the T zone.

MRCs AND ORGANIZER STROMAL CELLS IN SLO DEVELOPMENT

Secondary lymphoid organs develop from the anlagen, which are aggregates of mesenchymal and hematopoietic cells associated with vessels or epithelium, at a defined site and period in the fetus or infant (Mebius, 2003). A critical event in the development of the SLO anlagen is the accumulation of $CD45^+$ $CD4^+$ $CD3^-$ hematopoietic cells, which are also known as lymphoid tissue

inducer (LTi) cells that interact with mesenchymal stromal cells called lymphoid tissue organizer (LTo) cells (Mebius et al., 1997). A TNF family cytokine, lymphotoxin (LT)-α1β2 that is expressed by LTi cells transmits signals to LTo cells via the LT-β receptor. LTo cells subsequently induce the expression of adhesion molecules, including ICAM-1, VCAM-1, and MAdCAM-1, and chemokines CXCL13, CCL19, and CCL21 (Honda et al., 2001; Cupedo et al., 2004a; Bénézech et al., 2010). In particular, CXCL13 is especially important in attracting LTi cells to the anlagen via its receptor CXCR5 (Finke et al., 2002; Luther et al., 2003; Ohl et al., 2003). It is assumed that a positive feedback loop, i.e. newly immigrating LTi cells that produce LT further activate LTo stromal cells, promoting the organization of the anlagen. As lymphocytes began to accumulate after birth, the tissue expands and the compartments supported by different stromal cell subsets are induced (Cupedo et al., 2004b; Bajénoff and Germain, 2009).

Marginal reticular cells and LTo cells express a very similar pattern of various markers (**Figure 2A**), suggesting that there is some relationship between these two stromal cells. LTo cells seem to be more concentrated in the marginal region of the LN anlagen adjacent to the lymphatic sinus of the presumptive SCS (Finke et al., 2002; Cupedo et al., 2004a; Eberl et al., 2004; Katakai et al., 2008). This LTo cell layer appears to expand outwardly with the growth of the anlagen, which ultimately appears to become the MRC layer (Katakai et al., 2008). Likewise, as lymphocytes accumulate around the central artery in the postnatal spleen, LTo cells expressing MAdCAM-1 and RANKL expand with the layer and become MRCs in the white pulp. These findings strongly suggest that MRCs are a direct descendant of the LTo stroma, which preserve the characteristics of LTo cells at specialized sites within SLOs. Even RAG-deficient mice exhibit a subcapsular MRC layer in atrophic LNs and shrunken periarterial MRC sheathes in the spleen, indicating that MRC development occurs independently of B and T cells and is programmed before their colonization (Katakai et al., 2008). Since SLOs efficiently sample antigens to trigger immune responses, the antigen-collecting structures that are constructed during development need to be maintained thereafter. Accordingly, it is reasonable that these tissue structures are maintained by organizer-like stromal cells throughout adulthood.

RELATIONSHIP BETWEEN MRCs AND OTHER STROMAL SUBSETS

Figure 2B shows the possible genealogy of stromal subsets in SLOs. In the embryo, the primordial mesenchyme that is in close proximity to the vasculature or epithelium is committed to form the anlage core and attracts LTi cells. Nerve cells or other less characterized cells are also involved in the initial process (Veiga-Fernandes et al., 2007; van de Pavert et al., 2009). The accumulation of LTi cells facilitates the maturation of mesenchymal cells into LTo cells, which ultimately facilitates the construction of the basic architecture. Typically, LTo cells tend to concentrate at the periphery of the anlage and expand outward with tissue growth, while stromal cells exhibiting weak or no LTo signatures conversely increase in the inner portion of the anlage. Postnatal colonization of B and T cells leads to compartments with the induction of conventional stromal subsets.

If this scheme is correct, it follows that all the stromal subsets in adult SLOs are originally derived from LTo cells. Thus, do MRCs that preserve the characteristics of LTo cells have the ability to differentiate into FDCs or FRCs? MRCs share many signatures with other subsets (**Figure 2A**), suggesting that this is possible. One speculation is that MRCs function as stromal stem cells that continuously supply all of the stromal subsets throughout adulthood. Although this idea is intriguing, it will require cautious consideration and further validation in the future. Mesenchymal cells are generally highly flexible in nature depending on the surrounding environment, and thus specific features can be easily changed. Extended culturing of stromal cells isolated from LNs results in lost expression for many genes, particularly homeostatic chemokines (Katakai et al., 2004a; Tomei et al., 2009). This suggests that the *in vivo* phenotypes of stromal cell subsets are optimally maintained by the tissue circumstances, which are reversible and not due to terminal differentiation. In addition, inflammatory stimuli induce the robust proliferation of stromal cells and the dramatic remodeling of SLOs (Katakai et al., 2004a; Chyou et al., 2011). Therefore, although MRCs could be converted to other subsets, conventional stromal subsets might also self-renew and be interchangeable in adult SLOs.

REMODELING OF SLOs AND TERTIARY LYMPHOID STRUCTURES

During immune responses, stromal structures within SLOs are dramatically remodeled (Gretz et al., 1997; Katakai et al., 2004a). Some infections cause a severe disruption of tissue structures within the LNs and splenic white pulp within a few days, which is restored as the pathogens are eradicated (Mueller et al., 2007a,b; Scandella et al., 2008; St John and Abraham, 2009). This restoration process likely recapitulates a self-organizing process via a feedback reaction similar to SLO development. Importantly, LTi-like cells are also present in adult tissues and regulate infection-associated remodeling of SLOs (Kim et al., 2003; Scandella et al., 2008). Although the role of MRCs in such processes is unknown, they possibly have an organization role in collaboration with LTi-like cells and determine the outer frame of SLOs during reconstruction.

In chronic inflammation associated with various organ pathologies, large numbers of infiltrating lymphocytes often lead to organizations that are similar to SLOs, known as tertiary lymphoid tissues (TLTs; Drayton et al., 2006). B and T cells are segregated and corresponding networks of stromal cell subsets are induced. It would be interesting to determine whether MRCs are present in these ectopic lymphoid structures; however, MRC-like cells and related tissue structures are not observed in TLTs that developed during mouse autoimmune gastritis (Katakai et al., 2006, 2008). It should be emphasized that TLTs are fundamentally not programmed lymphoid organizations and naturally do not associate with the antigen-transporting structures. Therefore, MRCs are likely absent in TLTs. Even though stromal cells that are phenotypically similar to MRCs might be induced in some lesions, they would not be identified as MRCs unless they closely associate with the specific endothelial or epithelial structure and form a layered reticulum at the outer follicles.

CONCLUSION

Marginal reticular cells are a unique stromal cell subset common to SLOs. MRCs are clearly different from conventional subsets that are induced or maturated through interactions with lymphocytes after birth, and are directly derived from LTo stromal cells in the anlagen and independent of lymphocytes. The network of MRCs is closely associated with the antigen-transporting apparatus of SLOs and is thought to directly or indirectly control antigen delivery to lymphocyte compartments as well as the localization and migration of immune cells. MRCs likely play pivotal roles in maintaining SLO structures as the outer framework and may be converted to other stromal subsets at steady state or during tissue remodeling. It will be important to collectively consider all the stromal subsets, tissue structures, and immunological microenvironments in order to comprehensively understand the SLO system.

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Stroma cell priming in enteric lymphoid organ morphogenesis

Manuela Ferreira, Rita G. Domingues and Henrique Veiga-Fernandes*

Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Lisboa, Portugal

Edited by:
Mark Christopher Coles, University of York, UK

Reviewed by:
Mark Christopher Coles, University of York, UK
Sanjiv A. Luther, University of Lausanne, Switzerland
Tom Cupedo, Erasmus University Medical Center, Netherlands

***Correspondence:**
Henrique Veiga-Fernandes, Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Avenida Professor Egas Moniz, Edifício Egas Moniz, 1649-028 Lisboa, Portugal.
e-mail: jhfernandes@fm.ul.pt

The lymphoid system is equipped with a network of specialized platforms located at strategic sites, which grant strict immune-surveillance and efficient immune responses. The development of these peripheral secondary lymphoid organs (SLO) occurs mainly *in utero*, while tertiary lymphoid structures can form in adulthood generally in response to persistent infection and inflammation. Regardless of the lymphoid tissue and intrinsic cellular and molecular differences, it is now well established that the recruitment of fully functional lymphoid tissue inducer (LTi) cells to presumptive lymphoid organ sites, and their consequent close and reciprocal interaction with resident stroma cells, are central to SLO formation. In contrast, the nature of events that initially prime resident sessile stroma cells to recruit and retain LTi cells remains poorly understood. Recently, new findings revealed early phases of SLO development putting emphasis on mesenchymal and lymphoid tissue initiator cells. Herein we discuss the main tenets of enteric lymphoid organs genesis and focus in the most recent findings that open new perspectives to the understanding of the early phases of lymphoid morphogenesis.

Keywords: enteric lymphoid organ morphogenesis, stroma cells, LTin cells

INTRODUCTION

The lymphoid system possesses highly specialized peripheral organs formed at strategic anatomical sites that constitute three-dimensional platforms ensuring efficient immune-surveillance, rapid immune responses and maintenance of protective immunity. Secondary lymphoid organs (SLO), such as lymph nodes (LN) and Peyer's patches (PP), develop during the embryonic life, but can also assemble after birth as it occurs with enteric cryptopatches and isolated lymphoid follicles (Randall et al., 2008; Eberl and Sawa, 2010; van de Pavert and Mebius, 2010; Neyt et al., 2012).

Remarkably, while LN develop at strictly invariable locations along lymphatic vessels, PP develop in variable number and position in the anti-mesenteric side of the mid-intestine (5–12 in mice; Nishikawa et al., 2003). Similarly, cryptopatches appear confined to intestinal lamina propria but they also distribute randomly within the gut wall (Kanamori et al., 1996). Despite these intrinsic differences, SLO development relies on an antigen-independent process where presumptive regions are colonized by lymphoid tissue inducer (LTi) cells that cross-talk with resident mesenchymal cells through lymphotoxin (LT) $\alpha1\beta2$ and LTβ receptor (LTβR) interactions, thus creating a positive feed-back loop that culminates on the anlagen formation.

Although the mechanisms of SLO development have been extensively characterized throughout the years (Randall et al., 2008; van de Pavert and Mebius, 2010; Cupedo, 2011), most studies have been powerless to scrutinize early events preceding LTi cell colonization and clustering. Thus, putative early triggering events preceding LTi cell ingress into lymphoid organ anlagen remain poorly understood (Nishikawa et al., 2003).

GENESIS OF LYMPHOID ORGAN PRIMORDIA: THE LTi PARADIGM

Fetal hematopoietic cells colonize pre-defined sites between embryonic day 9.5 (E9.5) and 16.5 (E16.5) according to the type and location of the prospective lymphoid organ (Rennert et al., 1996; Adachi et al., 1998; Mebius et al., 2001; Yoshida et al., 2001; Veiga-Fernandes et al., 2007; Possot et al., 2011; Tachibana et al., 2011; Cherrier et al., 2012). Hematopoietic cells include $CD3^{-}CD4^{-/+}cKit^{+}IL7R\alpha^{+}\alpha4\beta7^{+}Ror\gamma t^{+/-}$ LTi cells (Kelly and Scollay, 1992; Adachi et al., 1997, 1998; Mebius et al., 1997; Yoshida et al., 1999; Sawa et al., 2010; Possot et al., 2011; Cherrier et al., 2012) and a distinct population of $CD3^{-}CD4^{-}cKit^{+}IL7R\alpha^{-}CD11c^{+}$ lymphoid tissue initiator (LTin) cells (Hashi et al., 2001; Fukuyama and Kiyono, 2007; Veiga-Fernandes et al., 2007; Patel et al., 2012). PP development depends on LTi and LTin cells, while LN genesis relies on LTi cells although the role of LTin in LN formation remains elusive. Upon arrival to prospective sites, LTin and LTi cells are believed to establish an interplay with their mesenchymal cell counterparts, lymphoid tissue organizers (LTo) cells, in order to trigger lymphoid organ formation.

The presence of fully functional LTi and LTin cells is necessary for the development of enteric SLO. Absence of LTi cells, as described in mice deficient for Ikaros, Inhibitor of DNA-binding 2 (Id2), retinoic acid-related orphan receptor γt (Rorγt), and RUNT-related transcription factor 1 (Runx1)/core-binding factor, beta 2 subunit (Cbfb2), result in PP developmental failure (Wang et al., 1996; Yokota et al., 1999; Sun et al., 2000; Tachibana et al., 2011). Similarly, depletion of LTin cells or deficiency of *Ret* expression on these cells results in impaired PP formation (Fukuyama and Kiyono, 2007; Veiga-Fernandes et al., 2007).

Lymphoid tissue inducer cells express the chemokine receptors CXCR5 and CCR7 that specifically bind to the homeostatic chemokines CXCL13 and CCL21/19, respectively. These chemokines create gradients that coordinate LTi cell migration and colonization of presumptive lymphoid organ sites (Forster et al., 1996, 1999; Honda et al., 2001; Luther et al., 2003; Mebius, 2003). In addition, the expression of the adhesion molecules ICAM-1, VCAM-1, and MAdCAM-1 by stroma organizer cells ensures retention of hematopoietic cells through the ligation of the integrin receptors α4β1 and α4β7 expressed by LTi and LTin cells surface (Mebius et al., 1996; Hashi et al., 2001; Finke et al., 2002; Veiga-Fernandes et al., 2007). Thus, it is commonly accepted that chemokines and adhesion molecules contribute to a productive and persistent communication between hematopoietic and mesenchymal cells (van de Pavert and Mebius, 2010).

The engagement of LTα1β2 expressed by LTi cells with stromal cell LTβR leads to activation of the classical and alternative NF-κB signaling pathways, which are critical to stroma cell maturation and lymphoid organ development (Weih et al., 1995; Yamada et al., 2000; Alcamo et al., 2001, 2002; Paxian et al., 2002; Yilmaz et al., 2003; Carragher et al., 2004; Lovas et al., 2008). In agreement, mice deficient for LTα, LTβ, LTβR, or molecular players of the NF-κB signaling pathways fail to develop LN and PP (Rennert et al., 1996, 1997, 1998).

The activation of LTβR results in the maturation of stroma cells, inducing the expression of adhesion molecules MAdCAM-1, VCAM-1, and ICAM-1 (Cuff et al., 1999; Dejardin et al., 2002; Yoshida et al., 2002; Ame-Thomas et al., 2007; Vondenhoff et al., 2009a), as well as the homeostatic chemokines CCL19, CCL21, and CXCL13 (Ansel et al., 2000; Luther et al., 2003). In addition, IL-7 and TRANCE induce the expression of LTα1β2 and generate a positive feed-back loop that sustains a continuous supply of signals between stroma and LTi cells granting maturation of the former (Ansel et al., 2000; Honda et al., 2001; Yoshida et al., 2002; Luther et al., 2003; Mebius, 2003).

MATURATION OF MESENCHYMAL CELLS: THE STROMACENTRIC VIEW

The general mechanism of SLO development, whereby LTi cells colonize lymphoid organ primordia, is similar among PP and LN anlagen (Yoshida et al., 2002; Randall et al., 2008; van de Pavert and Mebius, 2010; Cupedo, 2011). However, despite the obvious parallels there are also remarkable differences between the morphogenesis of these organs. Examples of such differential processes are provided by IL7/IL7R and TRANCE/TRANCE-R signaling. Thus, while IL7R signal is critical to PP development, as revealed by *Il7r*$^{-/-}$ mice, brachial, axillary, and mesenteric LN develop normally in these animals (Adachi et al., 1998; Yoshida et al., 1999; Luther et al., 2003). Furthermore, while in *Trance*$^{-/-}$ and *Traf6*$^{-/-}$ mice LN development is severely compromised, PP form normally in these mice (Dougall et al., 1999; Naito et al., 1999). Finally, the tyrosine kinase receptor RET also plays a differential role in LN and PP genesis. This is revealed by the absence of PP in *Ret* null embryos, which have seemingly normal LN anlagen development (Veiga-Fernandes et al., 2007).

Interestingly, mesenchymal organizer cells from LN and PP also exhibit distinctive genetic features (Yoshida et al., 2002; Cupedo et al., 2004; Okuda et al., 2007). This genetic heterogeneity, suggests that LTo cells may also provide different cues to hematopoietic cells. Nevertheless, it remains unclear whether the acquisition of such divergent genetic profiles are cell autonomous or derived from paracrine cellular interaction with different hematopoietic cell subsets.

The distribution of mesenchymal cells within lymphoid organs differs between PP and LN. In the intestine, stromal cells are distributed throughout the gut tissue that becomes colonized by highly motile hematopoietic cells between day E12.5 and E15.5. At this stage rare VCAM-1^{+} cells are detected in the gut wall (Adachi et al., 1997). However, by E16.5, VCAM-1^{+}/ICAM-1^{+} clusters of stroma cells are clearly visible forming PP primordia (Adachi et al., 1997; Yoshida et al., 1999; Hashi et al., 2001; Veiga-Fernandes et al., 2007). Conversely, LN invariably develop within lymph sacs, where ICAM-1^{+}VCAM-1^{+} mesenchymal stromal cells initially surround endothelial cells and by E16.5 start to invade the endothelium core to form a proper compartment of the anlagen (Okuda et al., 2007). Surprisingly, although lymphatic endothelial cells are essential to the correct formation of LN and lymphatic vasculature, they are dispensable for the initial aggregation of LTi and LTo cells (Cupedo et al., 2004; Vondenhoff et al., 2009b; Benezech et al., 2010).

Interestingly, mounting evidence indicates that LTo cells are very heterogeneous. In PP genesis, VCAM-1^{+}/ICAM-1^{+} organizer cells express LTβR, CCL19, and CXCL13 (Adachi et al., 1997; Yoshida et al., 1999; Hashi et al., 2001; Honda et al., 2001; Veiga-Fernandes et al., 2007), and further analysis revealed that this cell population comprises VCAM-1^{in}ICAM-1^{in} and VCAM-1^{hi}ICAM-1^{hi} subpopulations (Okuda et al., 2007). Similarly, these populations were also identified in LN (Cupedo et al., 2004; Okuda et al., 2007; Benezech et al., 2010). The comparison of genetic expression between PP and LN VCAM-1^{hi}ICAM-1^{hi} cells shows that mesenteric LN LTo cells have surface expression of TRANCE, whereas their PP counterparts lack the expression of this ligand (Cupedo et al., 2004; Okuda et al., 2007). Furthermore, microarray analysis revealed that their genetic signatures are distinct. Mesenteric LN stroma cells express significantly higher levels of cytokines and chemokines such as IL6, IL7, CCL7, CXCL1, and CCL11 (Okuda et al., 2007). Conversely, the homeostatic chemokines CCL21, CCL19, and CXCL13 are more abundant in enteric stroma cells. Interestingly, genes implicated in morphogenesis, such as Meox2, Lhx8, and Prrx1, were significantly higher in mesenteric LN when compared to PP counterparts, yet their functional relevance in lymphoid organogenesis is unclear (Okuda et al., 2007).

In addition to previously described VCAM-1^{in}ICAM-1^{in} and VCAM-1^{hi}ICAM-1^{hi} stroma cells, another population of VCAM-1^{neg}ICAM-1^{neg}, expressing PDGFRα but gp38/podoplanin and VEGFR3 negative was identified in LN (Benezech et al., 2010). Although, VCAM-1^{in}ICAM-1^{in} and VCAM-1^{hi}ICAM-1^{hi} cells have been described in PP, the existence of a VCAM-1^{neg}ICAM-1^{neg} counterpart remains to be investigated (Cupedo et al., 2004; Okuda et al., 2007). VCAM-1^{neg}ICAM-1^{neg} stroma cells express *Ccl21* and *Tnfr1* while VCAM^{hi}ICAM^{hi} express the highest levels of *Ccl21*, *Ccl19*, *Cxcl13*, *Trance*, and *Il7*, as compared with VCAM-1^{in}ICAM-1^{in}, confirming their greater potential to attract

LTi (Cupedo et al., 2004; Okuda et al., 2007; Benezech et al., 2010). Interestingly, the treatment of LN with αLTβR Ab agonist significantly increased the frequency of VCAM-1^{hi}ICAM-1^{hi} cells (Benezech et al., 2010). In addition, *Ltbr*$^{-/-}$ and Rorc(γt)$^{-/-}$ mice absolutely lacked these VCAM-1^{hi}ICAM-1^{hi} cells in inguinal and mesenteric LN (Benezech et al., 2010), thus confirming the implication of LTi cells and the engagement of LTβR on the maturation of residential stroma cells. Surprisingly, the emergence of VCAM-1^{in}ICAM-1^{in} is seemingly normal in the absence of LTβR and LTi cells (White et al., 2007; Benezech et al., 2010). These data indicate that while the maturation of VCAM-1^{in}ICAM-1^{in} into VCAM-1^{hi}ICAM-1^{hi} absolutely depends on LTβR and LTi cells, the transition from VCAM-1^{lo}ICAM-1^{lo} to VCAM-1^{in}ICAM-1^{in} is LTβR and LTi cell independent (Benezech et al., 2010). These conclusions are supported by the fact that the recruitment of LTi cells to lymphoid organ is observed in the absence of LTβR signaling (Yoshida et al., 2002; Coles et al., 2006; Vondenhoff et al., 2009a; Benezech et al., 2010), and by the LTβR-independent expression of homeostatic chemokines CCL21 and CXCL13, and the cytokine IL7 (Ansel et al., 2000; Luther et al., 2003; Cupedo et al., 2004; Moyron-Quiroz et al., 2004; Benezech et al., 2010). Finally, while agonist anti-LTβR treatment rescues LN development in *LTα*$^{-/-}$ mice, the same treatment in LTi deficient *Rorc(γt)*$^{-/-}$ mice, fail to promote SLO development (Rennert et al., 1998; Eberl et al., 2004). Altogether, these observations suggest that early LTβR-independent events precede LTi cell arrival, initiating a specific genetic expression profile in mesenchymal cells.

In agreement with this hypothesis, it was shown that CXCL13 can be induced by the vitamin A metabolite retinoic acid independently of LTα1β2/LTβR signaling (van de Pavert et al., 2009). Interestingly, analysis of mice deficient for RALDH-2, a crucial retinoic acid-synthesizing enzyme, revealed that at E14.5 the majority of LN were absent and CXCL13 expression was undetectable (van de Pavert et al., 2009). Additionally, neurons-expressing RALDH-1/2 were observed near the LN anlagen, suggesting potential neuronal source of retinoic acid (van de Pavert et al., 2009). Whether this signaling axis has a functional relevance for PP development remains unclear. However, *Gdnf* and *Gfra1* null embryos fail to develop a myenteric nervous system but still have normal PP development arguing against such hypothesis (Moore et al., 1996; Cacalano et al., 1998; Veiga-Fernandes et al., 2007). Nevertheless, the presence of parasympathetic and/or sympathetic neurons still present in the guts of these mutants, may provide such retinoic acid cues for PP formation. Interestingly, given the role of retinoic acid role in intestinal immune responses, a direct effect of retinoic acid in LTin and/or LTi cell subsets cannot be discarded at this stage (Hall et al., 2011a,b).

THE EARLY PRIMING EVENTS OF ENTERIC SLO: THE LTin CELL REIGN

In the intestine CD3$^-$CD4$^+$IL7Rα$^+$ LTi cells and VCAM-1^+/ICAM-1^+ stromal organizer cells cluster together with CD3$^-$CD4$^-$IL7Rα$^-$cKit$^+$CD11c$^+$ cells (Fukuyama and Kiyono, 2007; Veiga-Fernandes et al., 2007). Mice partly depleted of CD3$^-$CD4$^-$IL7Rα$^-$cKit$^+$CD11c$^+$ cells have impaired PP development and mice deficient for the receptor tyrosine kinase RET (*Ret*$^{-/-}$), expressed by this population do not develop PP (Veiga-Fernandes et al., 2007). Thus, CD3$^-$CD4$^-$IL-7Rα$^-$cKit$^+$ CD11c$^+$ cells were suggested to be involved in early phases of enteric lymphoid tissue formation and were named LTin cells (Fukuyama and Kiyono, 2007; Veiga-Fernandes et al., 2007). Supporting this concept, the RET ligand ARTN induces the formation of ectopic lymphoid structures, and LTin cells are the first hematopoietic cellular entity to cluster together with VCAM-1 expressing stroma cells (Veiga-Fernandes et al., 2007; Patel et al., 2012). Although, LTi cells are scarcely detected at very early phases of enteric organ formation, an extensive accumulation of LTi cells occurs subsequently to LTin cell aggregation (Patel et al., 2012). Interestingly, LTin cells respond unconventionally *in trans* to all RET ligands, reducing their motility upon contact with mesenchymal cells, in an adhesion-dependent manner (Patel et al., 2012). Furthermore, while *Ccl19*, *Ccl21*, and *Cxcl13* chemokine expression is not required in this early triggering phase, VCAM-1 blockage results in a profound reduction of cell clustering efficiency, indicating that subsequent up-regulation of VCAM-1 in stroma organizer cells is essential to recruit and retain the first coming LTi cells (Patel et al., 2012). Thus, in opposition to the LTi action mechanism, where chemokines and LT/LTβR are key (Hashi et al., 2001; Finke et al., 2002; Luther et al., 2003; Ohl et al., 2003), LTin cells act at very early phases determining early maturation of enteric mesenchymal cells in a RET-dependent, chemokine-independent manner (Patel et al., 2012). Strikingly, in agreement with previous reports in the LN, the initial induction of VCAM-1 expression in enteric stroma cells might not rely on the engagement of LTβR, since RET ligand stimulation does not up-regulate LTβ on LTin cells and blockage of LTβR signaling does not impair VCAM-1 induction on stromal cells (Patel et al., 2012). Thus, we would like to propose that PP development is a multi-step, multi-cellular process relying on an initial RET-dependent and adhesion-dependent interaction between LTin and mesenchymal cells, which result in stroma cell priming, ultimately leading to efficient LTi cell recruitment (**Figure 1**). Although, CD11c$^+$ cells have been detected in anlagen LN, *Ret*$^{-/-}$ mice develop peripheral LN (Veiga-Fernandes et al., 2007). Thus it remains unknown whether LTin cells are also implicated in early stroma cell priming of LN. LTin cells have been phenotypically characterized. These cells present some features of dendritic cells, expressing CD11c, CD11b, and MHC class II, but lack DEC205 and express NK1.1 and Gr-1 (Veiga-Fernandes et al., 2007). Thus, it would be very interesting to understand the precursor-product relationship between LTin cells and other cell lineages. Finally, it would be exceedingly exciting to determine whether LTin and RET responses may also initiate enteric cryptopatches or lymphoid tissue induced in chronic inflammation.

CONCLUDING REMARKS

Over the last two decades, remarkable findings have consolidated our knowledge on lymphoid organogenesis. Despite differences between diverse lymphoid organs, we can now appreciate that recruitment of fully functional LTi cells is central in LN and PP organogenesis and that, upon their arrival, an intimate and productive cross-talk is established with

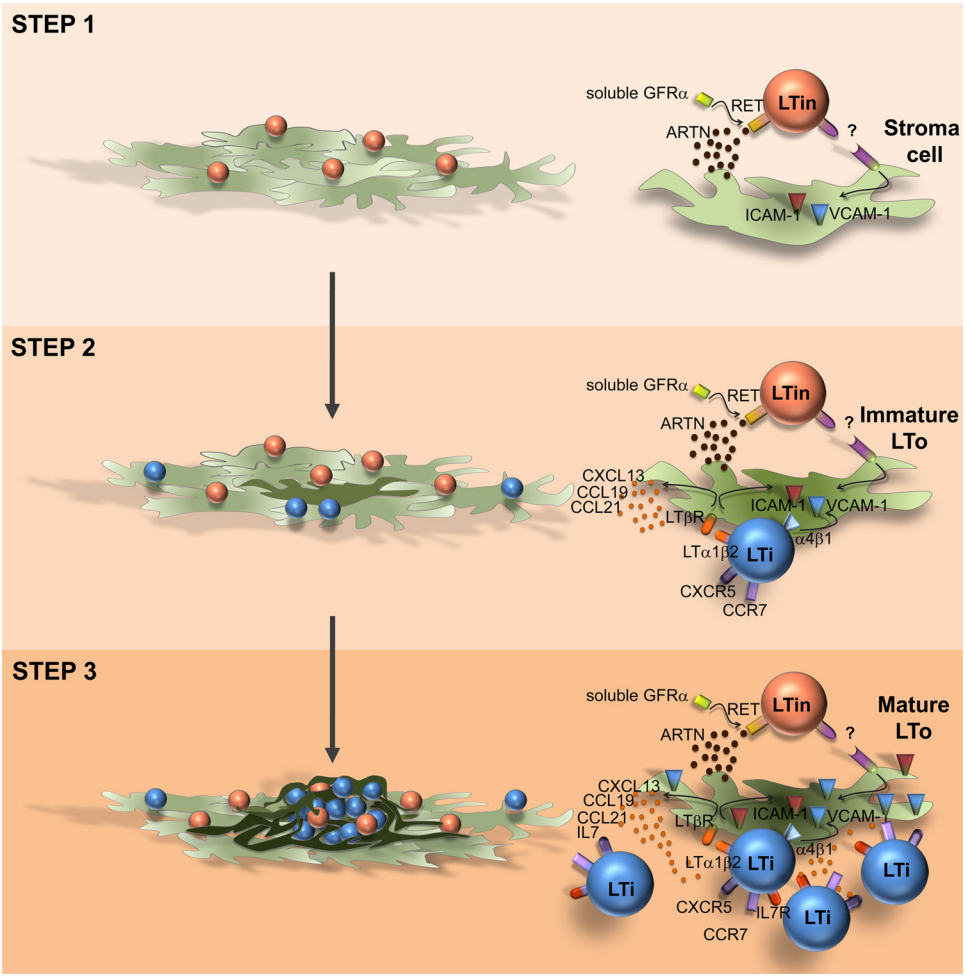

FIGURE 1 | Model of Peyer's patch development. Peyer's patch development relies on a multi-step, multi-cellular process. Step 1: RET-dependent, adhesion-dependent interaction between LTin and mesenchymal cells, results in stroma cell priming and VCAM1 induction (immature LTo cell). Step 2: retention of resident LTi cells through a VCAM1 mediated process; LTi/LTo interaction through LTαβ/LTβR inducing a chemokine and adhesion molecule competent LTo cell (mature LTo cell). Step 3: Positive feed-back loop generating fully mature LTo cells and additional LTi cell recruitment and retention into the primordium.

stroma cells. However, new insights have recently shed light on early initiating events that imprint stroma cells to create an attractive milieu for LTi cell recruitment. These findings emphasize a subtle phase, yet crucial to enteric stroma cell maturation, this is the step where LTin cells reign. We foresee the identification of early key players to stroma cell priming in adulthood and inflammatory settings as important challenges in the future.

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Lymphotoxin-sensitive microenvironments in homeostasis and inflammation

Bryant Boulianne, Elisa A. Porfilio, Natalia Pikor and Jennifer L. Gommerman*

Department of Immunology, University of Toronto, Toronto, ON, Canada

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Burkhard Ludewig, Cantonal Hospital St. Gallen, Switzerland
Andrey Kruglov, German Rheumatism Research Center, Germany

***Correspondence:**
Jennifer L. Gommerman, Department of Immunology, University of Toronto, Toronto, ON, Canada M5S 1A8.
e-mail: jen.gommerman@utoronto.ca

Stromal cell microenvironments within lymphoid tissues are designed to support immune cell homeostasis and to regulate ongoing immune responses to pathogens. Such stromal cell networks have been best characterized within lymphoid tissues including the spleen and peripheral lymph nodes, and systems for classifying stromal cell phenotypes and functions are emerging. In response to inflammation, stromal cell networks within lymphoid tissues change in order to accommodate and regulate lymphocyte activation. Local inflammation in non-lymphoid tissues can also induce *de novo* formation of lymphoid aggregates, which we term here "follicle-like structures." Of note, the stromal cell networks that underpin such follicles are not as well characterized and may be different depending on the anatomical site. However, one common element that is integral to the maintenance of stromal cell environments, either in lymphoid tissue or in extra-lymphoid sites, is the constitutive regulation of stromal cell phenotype and/or function by the lymphotoxin (LT) pathway. Here we discuss how the LT pathway influences stromal cell environments both in homeostasis and in the context of inflammation in lymphoid and non-lymphoid tissues.

Keywords: lymphotoxin, follicular dendritic cell, fibroblastic reticular cell, lymph node, chemokine, follicle-like structures

INTRODUCTION

Within the secondary lymphoid tissues, stromal cell networks are an integral scaffold for complex immune cell interactions necessary to mount an effective immune response to pathogens. The maintenance of the phenotype and function of some stromal cell types is critically dependent on constitutive signaling of the lymphotoxin-beta receptor (LTβR). LTβR is a member of the tumor necrosis factor (TNF) superfamily of receptors and is triggered by two ligands: membrane-bound $LT\alpha_1\beta_2$ heterotrimers and LIGHT, resulting in the activation of both the canonical and alternative NFκB pathways (Bista et al., 2010). During embryogenesis, the LTβR-dependent activation of NFκB within lymphoid tissue organizer (LTo) cells is achieved by interaction with LTαβ-expressing lymphoid tissue inducer (LTi) cells, thus facilitating lymph node (LN) and Peyer's patched (PP) development (Mebius, 2003; Ruddle and Akirav, 2009).

In the adult animal, stromal cell phenotype and function must be constitutively maintained for the lifetime of the host in order to maintain the integrity of lymphoid tissue, and much of this maintenance is accomplished by continual LTβR signaling (Gommerman and Browning, 2003). The cell types which provide LTαβ are generally lymphocytes, in particular B cells (Tumanov et al., 2002, 2004), but can also be LTi-like innate lymphoid cells, especially in the context of the gut (Eberl, 2005). The moment such a homeostatic program is interrupted, as achieved by a single injection of the LT pathway antagonist LTβR-Ig, stromal cell networks collapse and the lymphoid tissues become disorganized (Mackay and Browning, 1998). When the drug is cleared, however, aspects of the lymphoid tissue stromal cell environment can be re-established (Gommerman et al., 2002).

These findings have important implications for how we view stromal cells. First, it suggests that stromal cells are highly dynamic and rely on continual input from LTαβ-expressing cells. Second, since LTαβ is up-regulated on activated lymphocytes (Summers-DeLuca et al., 2007), lymphocytes that have been triggered by foreign or self-antigen (Ag) may have the potential to provide stromal cell differentiation cues. Finally, the ability to manipulate stromal cell biology via the LT pathway allows one to study the potential function of LT-sensitive stromal cell types during tissue homeostasis and during inflammation. Here, we outline the role of LTβR signaling in the homeostatic maintenance of non-lymphoid cell types within LN and in the small intestine, and explore how LTβR signaling influences changes in stromal cell phenotype/function during inflammation within lymphoid tissues and in ectopic sites of follicle development.

LTβR-DEPENDENT REGULATION OF STROMAL CELLS IN PERIPHERAL LYMPHOID TISSUES

Lymph nodes are composed of a variety of stromal cell types whose phenotype and function are being increasingly elucidated (Malhotra et al., 2012). In general, marginal reticular cells are located in the sub-capsular sinus (SCS), under which follicular dendritic cells (FDCs) populate the follicle. Fibroblastic reticular cells (FRCs) are located in the T cell-rich paracortex area and LN medullary fibroblasts are found in the medullary cords. Vascular and lymphatic endothelial cells are an additional source of non-lymphoid

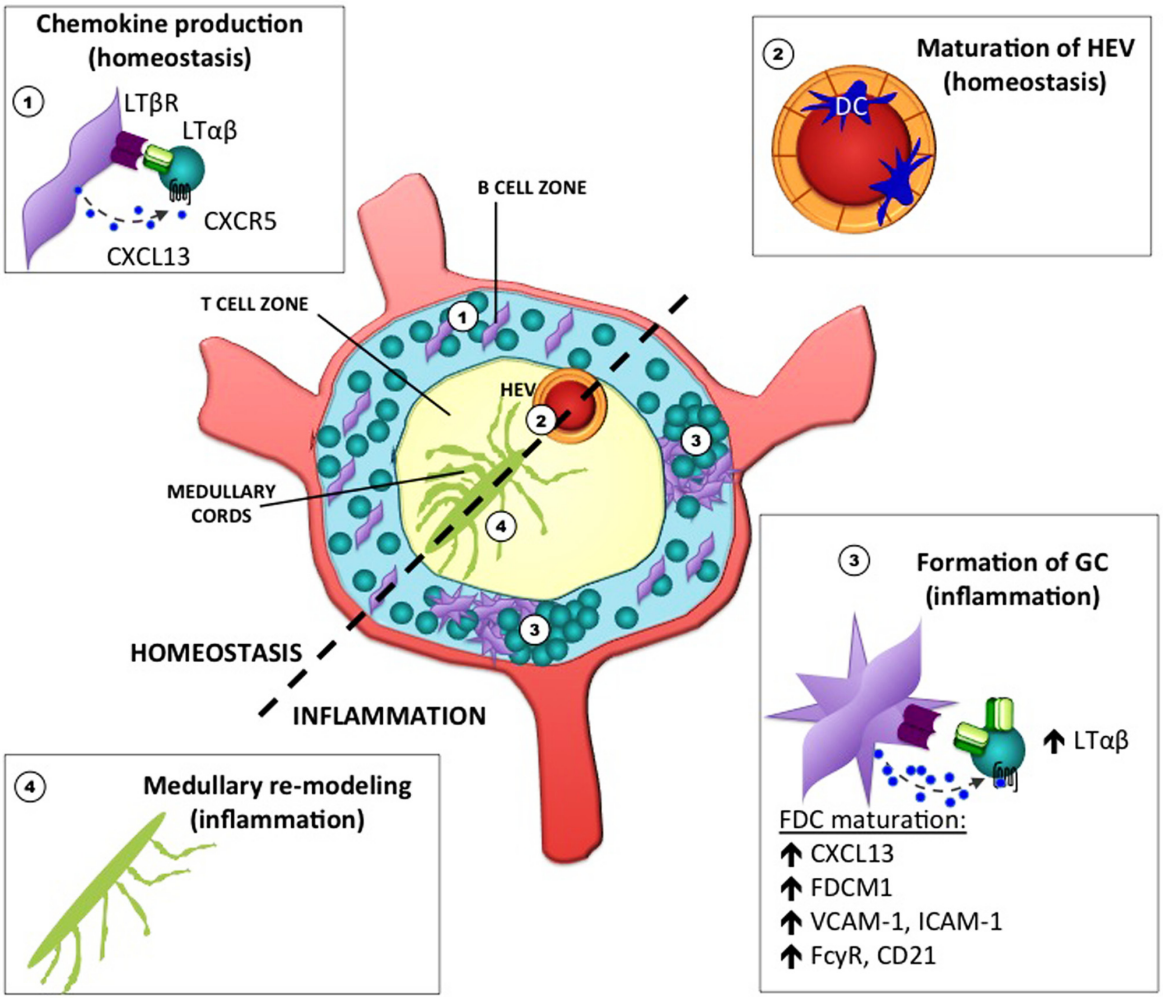

FIGURE 1 | Stromal cell elements in the lymph node under lymphotoxin control during homeostasis and inflammation. The LT pathway is critical for the proper maintenance and function of various stromal cell elements in the LN. During homeostasis, chemokine production by FDC in the primary follicle is required for B cell positioning (1). LTβR signaling in endothelial cells of HEV is also required for the expression of sulfotransferases that promote the proper glycosylation of PNAd (2). During inflammation, the LN becomes enlarged, stromal cells acquire new functions, and increased vascularization occurs (not depicted). In addition, clusters of B and T cells aggregate within germinal centers during T-dependent immune responses, and highly differentiated FDC within the GC environment require LTβR signaling (3). To facilitate the output of plasma cells that emerge from these GC reactions, remodeling of the medullary region has been shown to occur (4).

cell types. In the context of the non-inflamed LN, we focus on FDCs, FRCs, and the endothelial cells that form high endothelial venules (HEV) since the role of the LT pathway in these cell types has been well described. Depicted in **Figure 1** are examples of LN stroma that are under LT control in both the steady state and during inflammation.

FOLLICULAR DENDRITIC CELLS

B cell follicles in lymphoid tissues are largely defined by FDC (Allen and Cyster, 2008). FDCs are an important source of the B cell chemo-attractant CXCL13 which helps to establish the polarity between B and T cell zones in lymphoid tissues. FDCs also aid in germinal center responses by secreting the B cell survival factor BAFF and by trapping immune complexes for display to activated B cells (Suzuki et al., 2010). Though the exact identity of the FDC precursor is still unclear, it is thought that FDCs derive from mesenchymal cells *in situ* (Munoz-Fernandez et al., 2006; Allen and Cyster, 2008). It is well established that mature primary FDCs are maintained within B cell follicles by virtue of the interaction between LTαβ on B cells and LTβR on a resident radioresistant stromal cell precursor (Fu et al., 1998; Gonzalez et al., 1998; Endres et al., 1999). LTβR signals stimulate FDCs to secrete CXCL13, which attracts more B cells and induces them to upregulate LTαβ, thereby initiating a positive feedback loop (Ansel et al., 2000). Constitutive signaling is required for FDC maintenance and disruption of LTαβ-LTβR signaling *in vivo* results in the rapid disappearance of FDCs along with a disorganization of the B cell and T cell zones (Mackay et al., 1997).

FIBROBLASTIC RETICULAR CELLS

Fibroblastic reticular cells are found predominantly in the T cell areas of LN (Balogh et al., 2008; Turley et al., 2010). FRCs secrete fibronectin, laminin, and ER-TR7 antigen, which bind ECM collagen fibers to produce a reticular network (Katakai et al., 2004). This reticular network serves as a scaffold for cell migration and retention (Bajenoff et al., 2006), provides a source of IL-7 (Link et al., 2007), creates conduits that facilitate movement of chemokines and small soluble Ag (Roozendaal et al., 2009), and influences T cell tolerance in the steady state (Fletcher et al., 2011). Like FDCs, FRCs are thought to derive *in situ* from a mesenchymal precursor, and multipotent mesenchymal stem cells isolated from human tonsils and bone marrow stimulated with recombinant TNFα and LTαβ develop an FRC phenotype *in vitro* (Ame-Thomas et al., 2007). Murine FRCs cultured alone *in vitro* do not secrete ER-TR7 but upon co-culture with $CD4^+$ T cells FRCs produce large amounts of reticula that are coated with ER-TR7 in an LT- and TNFα-dependent manner (Katakai et al., 2004). Similarly, LTβR-Ig treatment diminished FRC networks in pancreatic infiltrates of diabetic CXCL13-RIP mice *in vivo* (Link et al., 2011). However, it is unclear if the development and/or maintenance of an intact ER-TR7-producing FRC network within LN requires constitutive LTβR signaling, although the loss of T cells concomitant with a decrease in LTαβ is correlated with FRC collapse in human immunodeficiency virus (HIV) infection (Zeng et al., 2012).

HIGH ENDOTHELIAL VENULES

High endothelial venules are the portals of entry for naive lymphocytes into LN. This is because the endothelium of HEV displays adhesion molecules, notably peripheral node addressin (PNAd). Mice that receive LTβR-Ig treatment have hypo-cellular LN due to the requirement of LTβR signaling in regulating the expression of sulfotransferase enzymes that mediate post-translational modification of PNAd. Without these modifications, PNAd is aberrantly expressed in HEV and naive L-$selectin^+$ lymphocytes transmigrate into LN tissues inefficiently (Browning et al., 2005). A similar paradigm is observed for ectopic lymphoid aggregates in the pancreas (Drayton et al., 2003). Recently, it was shown that dendritic cells (DC) are an important source of LTαβ in providing the maturation signal for HEV. This suggests there could be intimate cross-talk between DC and HEV (Moussion and Girard, 2011). Whether DC can communicate with other LTβR-expressing stromal cell elements within lymphoid tissues remains to be determined.

LTβR-DEPENDENT REGULATION OF STROMAL CELLS IN THE SMALL INTESTINE

The LT pathway plays a critical role in regulation of IgA production in the gut (Kang et al., 2002), and this has been linked to the activity of LTβR signaling in gut-resident stromal cells in different types of gut-associated lymphoid tissues (Tsuji et al., 2008). Such lymphoid tissues include PP, which are located along the small intestine. PP contains large B cell follicles along with smaller T cell regions in "inter-follicular" zones. Not unlike the case in LN, FDC and T/B segregation within the PP are likewise dependent on LTβR signaling in PP stromal cells, primarily by virtue of expression of LTαβ on B cells (Tumanov et al., 2004). PP-resident FDCs are somewhat different than LN FDCs in that they produce mediators that particularly encourage IgA class switch recombination (Suzuki et al., 2010). Overarching the PP follicles is the sub-epithelial dome that hosts a rich community of DC. Interestingly, expression of the chemokine CCL20 in the follicle-associated epithelium which overlies the DC-rich sub-epithelial dome is also LT sensitive (Rumbo et al., 2004). The CCL20/CCR6 axis may be important for the recruitment of B cells to the PP, and since B cells can express LTαβ, this could potentially drive the subsequent organization of the PP architecture (Williams, 2006). Microfold (M) cells, which are also partially dependent on the LT pathway (Debard et al., 2001), are interspersed within the follicle-associated epithelium. Along with dome-resident DC, M cells play an important role in shuttling Ag from the gut lumen into the PP for sampling and generation of immune responses. In general, the stroma in PP is less well characterized than in the LN.

Also within the small intestine are lymphoid tissue structures that develop strictly after birth called cryptopatches. In the presence of commensal bacteria, these cryptopatches mature to become isolated lymphoid follicles (ILF; Taylor and Williams, 2005). LTαβ- and LTβR-deficient animals lack both ILF and cryptopatches. It is thought that IL-7 release by the underlying stroma in the small intestinal lamina propria induces the expression of LTαβ on LTi-like innate lymphoid cells. This in turn results in the triggering of LTβR to form the cryptopatch which matures into an ILF (Eberl, 2005). Like PP, ILF development also requires the CCL20/CCR6 axis (Bouskra et al., 2008). Such ILF can be an alternative location for the generation of mucosal IgA^+ cells (Tsuji et al., 2008).

LTβR-DEPENDENT CHANGES IN LYMPHOID STROMAL CELLS DURING INFECTION AND INFLAMMATION

Several changes occur in the draining inflamed LN following exposure to Ag in adjuvant: systems for Ag transport are mobilized, stromal cells acquire new functions, the LN becomes enlarged, neo-vascularization occurs to accommodate increased cellular input, and specialized niches that support T/B interactions are formed. In this section we describe these changes, how such changes are influenced by different types of stromal cells, and the role of the LT pathway in orchestrating dynamic changes in the inflamed LN.

ANTIGEN TRANSPORT

Lymph-borne Ag enters LN into the SCS. There, Ag complexes are bound by $CD169^+F4/80^-$ SCS macrophages (SCS Mφ) that extend their processes into the SCS lumen to pick up Ag complexes (Carrasco and Batista, 2007; Junt et al., 2007). Non-cognate B cells subsequently pick up Ag complexes from SCS Mφ, carry them deeper into follicles, and deposit the Ag on FDCs in germinal centers (Phan et al., 2007). Interruption of this transport chain results in early dissipation of germinal centers and impaired affinity maturation. SCS Mφ express LTβR and their presence in the SCS region requires signals from LTαβ on B cells (Phan et al., 2009). As such, the expression of LTαβ on B cells is an important form of innate defense due to its ability to signal LTβR on cells

within the SCS: the first point of Ag entry (Moseman et al., 2012). Stromal cells within the SCS have been described (Katakai et al., 2008), and it will be of interest to learn how these stromal cells interact with the Ag transport chain.

LYMPHOID TISSUE REMODELING DURING INFLAMMATION AND INFECTION

Dramatic changes occur in lymphoid tissues in response to viral infections. For example, during lymphocytic choriomeningitis virus (LCMV) infection, lymphoid tissue architecture becomes disorganized but is eventually restored in a manner that depends on LTαβ expression on LTi-like innate lymphoid cells (Scandella et al., 2008). In addition to this dramatic remodeling, lymphoid stroma can be an important source of type I interferons during viral infection, and LTβR signaling in splenic stroma can drive such a Type I interferon response independent of MyD88 or TRIF-derived signals (Schneider et al., 2008).

In the LN, inflammation also greatly increases the size of the LN and this LN hypertrophy is accompanied by endothelial cell proliferation that can be promoted by the production of VEGF. FRC is a source of VEGF and this is dependent on LTαβ/LTβR signaling (Chyou et al., 2008) as well as input by the alternative LTβR ligand LIGHT (Zhu et al., 2011). Furthermore, LTαβ expression on B cells can also drive HEV network extension/remodeling in response to LCMV infection independent of VEGF (Kumar et al., 2010). Thus, through various mechanisms, the LT pathway is important for accommodating the increased flow of lymphocytes into a draining reactive LN. The medullary stroma, which supports lymphocyte egress from the LN, also becomes remodeled during an immune response. This may be important for providing a niche for the incredible burst in plasma cell output that is generated following a germinal center response. In this process, collagen-poor and collagen-rich areas are created, with plasma cells settling in the collagen-rich regions, presumably to take advantage of stromal cell factors that may enhance their survival (Zhu et al., 2011).

GERMINAL CENTER FORMATION

As mentioned, mature primary FDCs are located throughout B cell follicles and rely on constitutive, low-level LTβR signaling (Fu et al., 1998; Gonzalez et al., 1998; Endres et al., 1999). During an immune response, activated Ag-specific B cells that receive co-stimulation from T cells up-regulate LTαβ even further and provide stronger LTβR signals to FDCs (Vu et al., 2008). This elevated LTβR signaling prompts FDCs to mature into secondary FDCs within germinal centers. Secondary FDCs up-regulate complement receptors CD21 and CD35 as well as FcγRIIB to enhance capture of Ag complexes (Allen and Cyster, 2008). While the exact role for Ag complexes on FDCs is still debated, it is likely that they help sustain the germinal center response and enhance affinity maturation. Secondary FDCs also begin to express FDC-M1 antigen (Mfg-e8), which may play a role in the clearance of apoptotic germinal center B cells (Kranich et al., 2008).

INFLUENCE OF LTβR SIGNALING ON ECTOPIC LYMPHOID TISSUE

Inflammation in peripheral tissues can create an environment that is permissive to the formation of follicle-like structures (FLS). These structures have been observed in a wide variety of settings and display differing levels of organization, and in some cases have been shown to support local immune responses (Aloisi and Pujol-Borrell, 2006). In this section, we review two examples of FLS and speculate on how the LT pathway may support such structures.

INDUCIBLE BRONCHIAL LYMPHOID TISSUE

Inducible bronchus-associated lymphoid tissues (iBALT; Randall, 2010) are FLS that form in the lungs in response to respiratory inflammation due to infection (Moyron-Quiroz et al., 2004; Lugade et al., 2011), chronic inflammation (Hogg et al., 2004), or autoimmunity (Rangel-Moreno et al., 2006). The content of such structures varies from highly organized niches beneath a dome epithelium with defined T cell and B cell areas and FDC capable of supporting germinal centers, to small clusters of lymphocytes containing mostly B cells and some FDC (Moyron-Quiroz et al., 2004). Local production of CXCL13, CCL19, and CCL21 drives the recruitment of lymphocytes to iBALT follicles (Foo and Phipps, 2010). Fully formed iBALT require approximately 10 days to become organized niches in adult mice post-infection (Moyron-Quiroz et al., 2004; Halle et al., 2009) but are maintained for months (Moyron-Quiroz et al., 2006).

Unlike LN and PP which require LTαβ–LTβR signaling for their formation, studies using *LTα*$^{-/-}$ mice have shown that LTβR signaling is not required for iBALT formation or induction of CXCL13, CCL19, and CCL21 during acute inflammation (Moyron-Quiroz et al., 2004). Instead, Randall and colleagues determined that CD4^{+}IL-17^{+} cells are necessary to initiate iBALT formation (Moyron-Quiroz et al., 2004). However, once established, CD4^{+}IL-17^{+} cells are insufficient for optimal organization and maintenance of iBALT which instead is dependent on LTβR signaling.

FLS IN THE CENTRAL NERVOUS SYSTEM

Follicle-like structures have been documented at sites of chronic inflammation in several autoimmune diseases including: rheumatoid arthritis, Sjörgen's syndrome, systemic lupus erythematosus, and Multiple Sclerosis (MS; Aloisi and Pujol-Borrell, 2006). There is a range in the level of lymphoid-like organization of these structures: from perivascular infiltrates, to diffuse aggregates with HEV-like vessels, to organized follicles with T and B cell segregation and underlying FDC networks (Browning, 2008). The disease relevance of FLS is associated with local tissue injury and cell death. In MS, FLS preferentially accumulate in the meninges in patients at the later progressive stage of the disease (Serafini et al., 2004), and meningeal FLS are associated with increased demyelination and neuronal loss (Magliozzi et al., 2007, 2010).

A role for the LT pathway in attenuating clinical disease has previously been described in the rodent model of MS, experimental autoimmune encephalomyelitis (EAE; Gommerman et al., 2003). Pharmacological disruption of LT signaling reduces the size and number of meningeal FLS compared with control treatment (Columba-Cabezas et al., 2006). Impaired FLS formation following LT inhibition is concomitant with reduced mRNA levels of CXCL10 and CXCL13 in the brain, suggesting that LT regulates chemokine induction at peripheral sights of inflammation. However, not unlike iBALT, emerging studies in EAE also support

the notion that distinct pathways may culminate in orchestrating FLS. For example, adoptively transferred myelin-specific Th17 cells induce EAE concomitant with FLS formation (Peters et al., 2011). How signals from the LT pathway and from Th17 cells co-integrate to induce and/or maintain FLS structures in the CNS is unknown.

CONCLUSION

It is clear that LTβR-generated signaling underpins the maintenance and in some cases the function of stromal cell types within lymphoid tissues. Not discussed here are examples of how LTβR signaling is also important in myeloid/DC biology (Deluca and Gommerman, 2012), and DC have been implicated in regulating stromal cells and the formation of FLS (GeurtsvanKessel et al., 2009; Halle et al., 2009; Moussion and Girard, 2011). Thus, it will be of interest to learn more about the connections between DC and stromal cells with respect to the LT pathway. Moreover, many questions remain unanswered regarding how the LT pathway integrates with other forms of input, such as Th17 cells, to orchestrate distinct stages of FLS formation (i.e., initiation versus maintenance), and which LTαβ and LTβR-expressing cell types support FLS. Indeed, while exciting advances have been made toward understanding the nature of stromal cell types in peripheral LN, this question has barely been addressed in the mucosal lymphoid tissues and in the context of FLS. Unraveling the many facets of LTβR signaling in regulating and fine-tuning the immune response is a tall order, but of value for considering the therapeutic potential of LT inhibitors in treatment of chronic diseases.

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Emerging functions of RANKL in lymphoid tissues

Christopher G. Mueller*[1] *and* ***Estelle Hess*[1,2]**

[1] *CNRS, Laboratory of Therapeutic Immunology and Chemistry, Institut de Biologie Moléculaire et Cellulaire, University of Strasbourg, Strasbourg, France*
[2] *Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix Namur, Namur, Belgium*

Edited by:
Burkhard Ludewig, Kantonal Hospital St. Gallen, Switzerland

Reviewed by:
Annette Oxenius, Federal Institute of Technology Zurich, Switzerland
Jorge Caamano, University of Birmingham, UK
Ifor Williams, Emory University, USA

****Correspondence:***
Christopher G. Mueller, CNRS, Laboratory of Therapeutic Immunology and Chemistry, UPR9021, Institut de Biologie Moléculaire et Cellulaire, 15 Rue René Descartes, University of Strasbourg, 67084 Strasbourg, France.
e-mail: c.mueller@cnrs-ibmc.unistra.fr

The tumor necrosis factor superfamily (TNFSF) members play pivotal roles in embryonic development of lymphoid tissue and their homeostasis. RANKL (Receptor activator of NF-κB ligand, also called TRANCE, TNFSF11) is recognized as an important player in bone homeostasis and lymphoid tissue formation. In its absence bone mass control is deregulated and lymph nodes fail to develop. While its function in bone is well described, there is still little functional insight into the action of RANKL in lymphoid tissue development and homeostasis. Here we provide an overview of the known functions of RANKL, its signaling receptor RANK and its decoy receptor OPG from the perspective of lymphoid tissue development and immune activation in the mouse. Expressed by the hematopoietic lymphoid tissue inducing (LTi) cells and the mesenchymal lymphoid tissue organizer (LTo) cells, RANKL was shown to stimulate Lymphotoxin (LT) expression and to be implicated in LTi cell accumulation. Our recent finding that RANKL also triggers proliferation of adult lymph node stroma suggests that RANKL may furthermore directly activate LTo cells. Beyond bone, the RANKL-RANK-OPG triad plays important roles in immunobiology that are waiting to be unraveled.

Keywords: TRANCE, TNFSF11, OPG, lymphoid organs, lymph node, stroma, LTi, LTo

INTRODUCTION

Tumor necrosis factor (TNF) and Lymphotoxin (LT) were identified as the first members of a large family, now called the TNF-superfamily (SF). Not surprisingly, the receptors for these proteins also constitute a SF with sequence homology, named TNF Receptor (TNFR) SF. A hallmark of these ligand-receptor pairs lies in a threefold symmetry, where by the oligomeric binding arrangement amplifies their avidity and introduces flexibility. Further complexity arises through different partner affinities and generation of soluble ligand and receptor forms (Bodmer et al., 2002). RANKL (TNFSF11) is the ligand of two receptors, RANK (TNFRSF11a) and OPG (TNFRSF11b). OPG (osteoprotegerin) was the first of this protein triad to be discovered (Simonet et al., 1997) in a search for an inhibitor of osteoclastogenesis (Tsuda et al., 1997). OPG-ligand was then isolated and cloned using OPG as bait (Lacey et al., 1998; Yasuda et al., 1998). OPG-ligand turned out to be identical to TRANCE (TNF-related activation induced cytokine), cloned during a search for apoptosis-regulatory genes in T cells (Wong et al., 1997b), and RANKL (Receptor activator of NF-κB) identified as the ligand for RANK that had attracted attention for its homology to CD40 (Anderson et al., 1997). The affinity of RANKL for OPG is 1000-fold higher than for RANK (Nakagawa et al., 1998), which is dependent on the ability of OPG to homodimerize (Schneeweis et al., 2005). OPG is also a ligand for TNF-related apoptosis-inducing ligand (TRAIL; Emery et al., 1998), however, its affinity for TRAIL is 10,000 times less compared to RANKL (Body et al., 2006) questioning the *in vivo* relevance of OPG-TRAIL interaction. There is now an emerging consensus to refer to the receptor as RANK and, as a consequence and for simplicity, its ligand is called RANKL. The acronym OPG has remained in use.

The discovery of RANK, RANKL, and OPG in bone and the immune system raises the question of its evolutionary origins. The genes arose simultaneously during ontogeny of bony fish as evidenced by gene sequence identification and presence of resorption and remodeling activity of vertebrate mineralized tissue (Witten and Huysseune, 2009). They therefore postdate the formation of the primordial immune system comprising a primitive thymus and lymphoid structures associated with exposed sites. However, they preceded the development of lymph nodes (LNs) and germinal centers arising in amphibians and the emergence of LT β receptor, a key molecule in lymphoid development (Glenney and Wiens, 2007; see below). It is therefore likely that the RANK-RANKL-OPG protein triad was co-opted by the advanced immune system for higher order structure together with an efficient regulation of immune cell output from the bone marrow before genesis of LT β receptor-regulated lymphoid tissues.

RANKL is a type-II transmembrane protein but can also exist in a soluble form by ectodomain shedding and alternative splicing (Ikeda et al., 2001; Hikita et al., 2006; Baud'huin et al., 2007). OPG comprises two C-terminal regions homologous to death domains of TNFR1 or TRAIL receptor, which were found to be functional when OPG was expressed with a transmembrane sequence (Yamaguchi et al., 1998). Natural OPG is unlikely to transmit signals because it misses the transmembrane sequence and is secreted (Simonet et al., 1997). RANK comprises a transmembrane region and a large cytoplasmic domain. Upon interaction with the RANKL trimer RANK undergoes homotrimerization and activates recruitment of TNFR-associated factors (TRAFs; Galibert et al., 1998; Wada et al., 2006). RANK binds five of the six known TRAF-proteins but TRAF6 seems particularly important

for RANK signaling, because TRAF6$^{-/-}$ mice present similar phenotypes as *Rank*$^{-/-}$ mice (Naito et al., 1999). RANK signaling cascades were mostly deciphered in the myeloid lineage and include the canonical and the non-canonical NF-κB pathways (Raju et al., 2011). In mammary glands RANK-activation intersects with proliferative cues through cyclin D1, Id2, and Id4 (Schramek et al., 2011). RANK was recently found to play a role in mammary and in hair follicle epithelial stem cell activation (Schramek et al., 2010; Duheron et al., 2011) and to induce intestinal microfold cells (M cells) differentiation via the Ets transcription factor Spi-B (Kanaya et al., 2012).

RANKL IN BONE AND HEMATOPOIESIS

A number of reviews have been published on the role of these proteins in regulating bone mass (Suda et al., 1999; Walsh and Choi, 2003; Baud'huin et al., 2007; Leibbrandt and Penninger, 2010). *Rankl*$^{-/-}$ and *Rank*$^{-/-}$ mice present osteopetrosis and lack of teeth (Dougall et al., 1999; Kong et al., 1999), whereas *Opg*$^{-/-}$ animals exhibit osteoporosis (Bucay et al., 1998; Mizuno et al., 1998; Yun et al., 2001). RANK activates the differentiation of bone matrix degrading osteoclasts (OCL) from myeloid precursor cells (Yasuda et al., 1998; Hsu et al., 1999; **Figure 1**). RANKL and OPG are synthesized by the bone mesenchymal lineage and are under inflammatory and hormonal control (Udagawa et al., 1999; Takeda et al., 2003; Nakashima et al., 2011; Xiong et al., 2011). Another source of RANKL is activated T cells that can cause abnormal bone resorption by triggering osteoclastogenesis (Takayanagi et al., 2000; Sato et al., 2006).

T cell lymphopoiesis itself underlies RANK regulation as *Rank*$^{-/-}$ mice present a block in the progression to CD4$^-$CD8$^-$CD44$^-$CD25$^-$ thymocytes (Kong et al., 1999). Recently it was shown that also Vγ5$^+$ T cells are under RANK regulatory action (Roberts et al., 2012). In fact RANK signaling is a key event in the early stages of medullary thymic epithelial cell (mTEC) formation, and its cooperation with LT and CD40 signals is required to establish a fully developed medullary microenvironment (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka et al., 2008; Mouri et al., 2011). mTEC play a crucial role in self-tolerance by eliminating self-reactive αβT cells and by regulating the early production of γδT cells. Thymic CD3$^-$CD4$^+$ lymphoid tissue inducing (LTi) cells and Vγ5$^+$ thymocytes as well as later arising CD4$^+$CD8$^-$ single positive thymocytes and γδT cells are equipped with RANKL (Rossi et al., 2007; Hikosaka et al., 2008; Roberts et al., 2012). RANK and OPG are expressed by mTECs (Hikosaka et al., 2008). Animals defective in RANK signaling also display abnormal B cell hematopoiesis and hypogammaglobulinemia (Dougall et al., 1999; Kong et al., 1999). Although B cells express RANK, in particular in response to activation (Yun et al., 1998; Perlot and Penninger, 2012), a B cell-specific RANK knock-out mouse does not reproduce this phenotype (Perlot and Penninger, 2012), suggesting that the defect lies in bone marrow or splenic stroma.

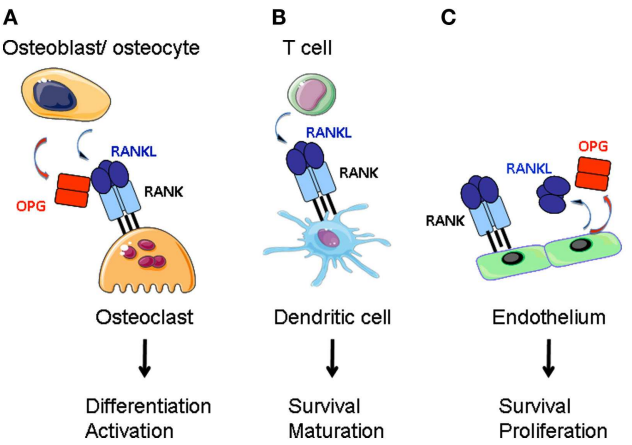

FIGURE 1 | RANKL function in the bone, the adaptive immune system and the vascular system. (A) RANKL produced by bone marrow stroma (osteoblasts and osteocytes) directs the differentiation of preosteoclasts of the myeloid lineage into mature osteoclasts. Stroma-derived OPG negatively regulates RANKL-RANK interaction. **(B)** Activated T cells express RANKL that stimulates dendritic cell survival and maturation. **(C)** Endothelium is a source for RANKL and OPG. RANK-activation of endothelial cells supports cell survival and promotes angiogenesis.

RANKL IN EARLY STAGES OF SECONDARY LYMPHOID ORGAN DEVELOPMENT

RANK and RANKL-deficient animals display a complete absence of LNs, defects in Peyer's patches (PPs) and cryptopatches (CPs) and abnormalities of the spleen (Dougall et al., 1999; Kong et al., 1999; Kim et al., 2000; Knoop et al., 2011; Perlot and Penninger, 2012). Therefore the RANK-RANKL-OPG axis shares with the LT and TNFα pathways the control of molecular and cellular processes determinant in secondary lymphoid organ (SLO) development (Tumanov et al., 2003; Fritz and Gommerman, 2010). SLO formation is initiated around embryonic day (E) 15 with the recruitment of the hematopoietic LTi cells to a rudimentary organ anlage composed of mesenchymal and endothelial stroma (White et al., 2007; Vondenhoff et al., 2009; Benezech et al., 2011). The recruitment process is dependent on the chemokine CXCL13 produced by precursors of lymphoid tissue organizer (LTo) cells stimulated by neuronal production of retinoic acid (van de Pavert et al., 2009). This step is followed by a cross-talk between LTi cells that express RANK, RANKL, and LT, and LTo precursors that carry the LT receptor LTβR. LTβR engagement induces LTo cells to express RANKL and chemokines to attract larger numbers of LTi cells that upon clustering with LTo cells initiate LN organization (Cupedo and Mebius, 2005; Koning and Mebius, 2011; **Figure 2**).

In view of the finding that LTi cell recruitment is LT independent (Eberl et al., 2004; White et al., 2007; Vondenhoff et al., 2009) the question arises whether LTi cell accumulation is regulated by RANK. Both *Rankl*$^{-/-}$ and *Lt*α$^{-/-}$ mice have lower number of LTi cells in mesenteric LNs of newborn mice (Kim et al., 2000). TRAF6$^{-/-}$ mice display fewer LTi cells in mesenteric LNs at E 17.5 but not at E15.5 (Yoshida et al., 2002). Administration of RANK-Fc antagonist led to a partial reduction in LTi cells, with a more prominent effect in mesenteric LNs (Eberl et al., 2004). Therefore, although current data do not unambiguously support RANKL as a direct regulator of LTi cell numbers, they sustain the concept that RANKL is instrumental for LTi cell accumulation. Of note,

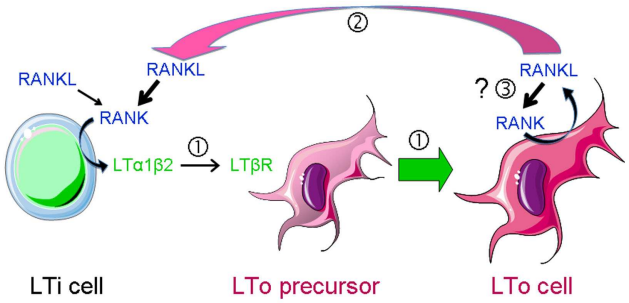

FIGURE 2 | RANKL-dependent amplification loops in SLO development. SLO development during embryogenesis is initiated by the recruitment of lymphoid tissue inducer (LTi) cells to a rudimentary organ anlage composed of mesenchymal lymphoid tissue organizer (LTo) cell progenitors. This step is followed by a cross-talk between LTi cells that express RANKL, RANK, and LTαβ and LTo cells that carry LTβR ①. Engagement of the latter induces the maturation of LTo cells ① to express RANKL and chemokines that attract larger numbers of LTi cells that upon clustering with LTo cells initiate LN organization. LTo cells also express RANKL. The current model of LN development indicates that a positive feedback loop takes place between LTo and LTi cells via RANKL-RANK and LTαβ-LTβR amplifying the early stages of LN development ②. If LTo cells expressed also RANK, a second (autocrine) loop may occur, leading to a direct activation of LTo cells ③.

RANK signaling mediators include Id2 (Kim et al., 2011), a factor indispensable for LTi cell formation (Yokota et al., 1999). It is hence plausible that Id2 is implicated in RANK regulation of LTi cell numbers and function.

In the current model of LN development a positive feedback loop takes place between LTi and LTo cells (**Figure 2**). RANK signaling in LTi cells increases the expression of LT that upon binding to LTβR of LTo cells induces RANKL production, thus amplifying the early stages of LN development (Yoshida et al., 2002; Koning and Mebius, 2011; Roozendaal and Mebius, 2011). In support for such a feedback loop is the observation that RANKL expression is up to 10-fold higher in LTo cells than in LTi cells (Sugiyama et al., 2012). However, it is unlikely that the function of RANKL in SLO development is limited to the induction of LT by LTi cells and the creation of the amplification loop. Firstly, LT is upregulated by a number of other factors, such as IL-7, TNFα, and CXCL13 (Ansel et al., 2002). Indeed PPs are LT-dependent but develop in *Rankl*$^{-/-}$ mice. Second, mucosal LNs develop in LTβ$^{-/-}$ mice but not in *Rankl*$^{-/-}$ mice (Alimzhanov et al., 1997; Koni et al., 1997). Finally, administration of an LTβR-agonistic antibody to *Rankl*$^{-/-}$ embryos cannot rescue LN genesis (Kim et al., 2000).

LTβR signaling in mTECs induces RANK expression (Mouri et al., 2011). We have recently shown that RANK is expressed in adult LN stroma and induces hyperproliferation of reticular fibroblastic and vascular cells (Hess et al., 2012). Expression of both RANK and RANKL by LTo cells may therefore trigger a second (autocrine) loop leading to a direct activation of these cells (**Figure 2**). In addition, vascular endothelial cells express RANK, RANKL, and OPG that together regulate angiogenesis (Kim et al., 2003; Benslimane-Ahmim et al., 2011; Sugiyama et al., 2012; **Figure 1**).

RANKL AND SLO GROWTH

A number of observations support a role of RANKL in SLO growth. The LNs that developed after neutralization of RANK signaling in embryos were smaller (Eberl et al., 2004; Sugiyama et al., 2012). PPs, CPs, and isolated lymphoid follicles (ILFs) were reduced in size in *Rankl*$^{-/-}$ mice (Knoop et al., 2011), and postnatal RANKL overproduction led to massive LN hyperplasia (Hess et al., 2012). Recruitment of immune cells and stromal cell division stand out among possible regulatory mechanisms of SLO size. Lymphocyte recruitment into SLO anlage coincides with maturation of LTo cells to produce high levels of chemokines and cell adhesion molecules (Honda et al., 2001; Finke et al., 2002; Luther et al., 2003; Cupedo et al., 2004b; White et al., 2007; Benezech et al., 2011). In mice with postnatal LN hyperplasia RANKL upregulates CXCL13, CCL19, MAdCAM-1, and VCAM-1 gene transcription in adult fibroblastic reticular cells (FRCs) and vascular cells (Hess et al., 2012). Therefore, RANKL could directly boost immune cell accumulation by increased chemokine and adhesion factor output. While recruitment of stromal precursors from surrounding tissue or bone cannot be excluded, CD45-negative cells label for the cell division marker Ki-67 as early as E16 (Eberl et al., 2004; White et al., 2007). Although this proliferation appears to be dependent on LT (White et al., 2007), it is unclear whether the proliferating cells are endothelial cells, precursor or mature LTo cells. We have found that RANKL stimulates FRC and endothelial cell proliferation (Hess et al., 2012). Further support for a functionally important role of RANKL in cell proliferation stems from findings that thymic mTECs and skin keratinocyte cell growth is accelerated in response to RANK stimulation (Hikosaka et al., 2008; Duheron et al., 2011).

TOWARD A ROLE OF RANKL IN B CELL RECRUITMENT AND FOLLICLE ORGANIZATION

B cell recruitment and organization into follicles occur in a CXCL13-dependent manner at later stages of SLO formation (Ansel et al., 2000; Cupedo et al., 2004a). B cell follicular dendritic cells (FDCs) and the recently identified marginal reticular cells (MRCs), both mesenchymal cell types, are the main producers of this chemokine (Ansel et al., 2000; Katakai et al., 2008). First supportive evidence for a role of RANKL in B cell recruitment and organization was provided after the rescue of LNs by exogenous IL-7 in TRAF6$^{-/-}$ mice: it was noted that in these LNs B cells and FDCs were absent (Yoshida et al., 2002). However, because TRAF6 is also a signaling component for TNFR, a critical receptor for FDC formation (Rennert et al., 1998; Endres et al., 1999), a role of RANK signaling in B cell recruitment cannot be directly invoked. More direct evidence was provided by administration of a RANKL-neutralizing antibody to embryos. This resulted in reduced LN B cells numbers, misplaced FDCs, and reduced VCAM-1 staining (Sugiyama et al., 2012). In addition, Knoop et al. (2011) noted an absence of B cells in small intestine CPs of *Rankl*$^{-/-}$ mice and observed that most stromal cells in the B cell compartment lacked VCAM-1 and CXCL13 expression. Finally, postnatal RANKL overexpression resulted in an increase in small but clearly defined B cell follicles, which all comprised FDCs (Hess et al., 2012). Three possible scenarios can be advanced to explain these phenomena: (i) RANKL increases the bone marrow B cell output, (ii)

CXCL13 production by FDCs and/or MRCs is under RANKL positive control, (iii) RANK-signaling promotes MRC and/or FDC differentiation. Although the first scenario appears plausible in view of the known action of RANKL in the bone, so far, there is no experimental support for this idea. The rise in LN B cell numbers in response to RANKL overproduction is not accompanied by an expansion in splenic transitional B cell subsets (Hess et al., 2012). As for the second model, there is evidence that RANKL upregulates CXCL13 gene transcription in FRCs, however the level of induction was low (Hess et al., 2012). Lastly, reduction of VCAM-1 expression by FDCs is indicative of a requirement of RANK-signaling for terminal differentiation of FDCs. In keeping with this idea, reduced CXCL13 expression by FDCs could be the consequence of FDC dysfunction. It is intriguing that MRCs, which have been proposed to function as FDC precursors, express RANKL (Katakai et al., 2008). Cells that bear resemblance to LN MRCs have also been found in the spleen, PP, and ILF on the grounds of RANKL expression and independence of LTβR signaling (Taylor et al., 2007; Katakai et al., 2008). The polarized expression of RANKL beneath the follicle-associated epithelium may be necessary to focus its activity of inducing differentiation of intestinal M cells, cells specialized in the transport of antigen to the underlying lymphoid tissue (Knoop et al., 2009). It is plausible that RANKL jointly regulates FDC differentiation and (native) antigen access.

RANKL AND THE ADAPTIVE IMMUNE RESPONSE

Activated CD4 and CD8 T cells express surface and soluble RANKL (Josien et al., 1999; Wang et al., 2002; **Figure 1**). Dendritic cells are of the same lineage as OCL and express RANK (Anderson et al., 1997). RANKL confers to DCs better survival with more notable effects on *in vitro* generated DCs and in combination with other TNFSF members (Wong et al., 1997a; Dougall et al., 1999; Josien et al., 2000; Williamson et al., 2002). Stimulation of DCs results in production of pro-inflammatory cytokines IL-6, IL-1β, and T cell differentiation factors IL-12, IL-15 (Josien et al., 1999). However, other reports have noted anti-inflammatory activity for RANKL. In a model of oral tolerance, RANKL stimulation of DCs has been associated with tolerance induction (Williamson et al., 2002). An anti-inflammatory effect was also noted for RANKL-stimulated Langerhans cells and macrophages (Maruyama et al., 2006; Yoshiki et al., 2009). This discrepancy may be due to low RANK expression level in immature DCs; its expression being upregulated in response to Toll-like receptor (TLR) ligands or inflammatory cytokines (Hochweller and Anderton, 2005). Another explanation could be a redundancy with other TNFRSF members such as its close homolog CD40 (Bachmann et al., 1999). Alternatively, activated DCs express OPG, thus inhibiting RANKL (Schoppet et al., 2007). Except for a reduction in Langerhans cell numbers (Barbaroux et al., 2008), there is little experimental support that the RANK-RANKL-OPG triad controls DC development *in vivo* (Dougall et al., 1999).

Th17 T cells represent an important osteoclastogenic T cell type by robust RANKL production and activation of RANKL release by mesenchymal cells (Sato et al., 2006). This T cell type is of particular importance in progressive periodontitis, a dental disease characterized by destruction of alveolar bone with high prevalence of bacteria such as *Porphyromonas gingivalis* (Kajiya et al., 2010). In this disease, periodontal ligament fibroblasts are an important source of RANKL when stimulated by microbial products including TLR ligands. TLRs are also expressed by osteoclast precursors and OCL and their stimulation promotes osteoclastogenesis and maturation of OCL. Interestingly, gingival Langerin-expressing DCs have recently been shown to control inflammation in *P. gingivalis*-induced periodontitis and therefore reduce alveolar bone loss (Arizon et al., 2012). It is yet unclear whether this occurs via a direct RANKL-induced DC anti-inflammatory activity.

CONCLUSION

The RANK-RANKL-OPG axis plays a recognized role in bone homeostasis through the regulation of osteoclastogenesis. It is also implicated in SLO development and regulation of the immune response. There are many incentives to answer remaining questions. In addition to a restless curiosity of the researcher, tertiary lymphoid tissues that arise in inflamed tissue rely on similar if not identical cellular dialogs as those found in SLO development. Defining RANKL function in lymphoid tissue development will open new therapeutic avenues to treat inflammatory diseases and provide new strategies for vaccine development.

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TNF receptor family signaling in the development and functions of medullary thymic epithelial cells

Taishin Akiyama*, Miho Shinzawa and Nobuko Akiyama

Division of Cellular and Molecular Biology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Graham Anderson, University of Birmingham, UK
Xinjian Chen, University of Utah, USA

****Correspondence:***
Taishin Akiyama, Division of Cellular and Molecular Biology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan.
e-mail: taishin@ims.u-tokyo.ac.jp

Thymic epithelial cells (TECs) provide the microenvironment required for the development of T cells in the thymus. A unique property of medullary thymic epithelial cells (mTECs) is their expression of a wide range of tissue-restricted self-antigens, critically regulated by the nuclear protein AIRE, which contributes to the selection of the self-tolerant T cell repertoire, thereby suppressing the onset of autoimmune diseases. The TNF receptor family (TNFRF) protein receptor activator of NF-κB (RANK), CD40 and lymphotoxin β receptor (LtβR) regulate the development and functions of mTECs. The engagement of these receptors with their specific ligands results in the activation of the NF-κB family of transcription factors. Two NF-κB activation pathways, the classical and non-classical pathways, promote the development of mature mTECs induced by these receptors. Consistently, TNF receptor-associated factor (TRAF6), the signal transducer of the classical pathway, and NF-κB inducing kinase (NIK), the signal transducer of the non-classical pathway, are essential for the development of mature mTECs. This review summarizes the current understanding of how the signaling by the TNF receptor family controls the development and functions of mTEC.

Keywords: medullary thymic epithelial cells, TNF receptor family, NF-κB, signal transduction, self-tolerance, autoimmune disease

INTRODUCTION

The development of self-tolerant T cells and regulatory T cells in the thymus requires a microenvironment of both non-hematopoietic stroma cells and cells of hematopoietic origin (Gill et al., 2003; Takahama, 2006; Anderson and Takahama, 2012). Thymic epithelial cells (TECs), essential components of this unique microenvironment, are subdivided into two major subtypes according to their localization: medullary thymic epithelial cells (mTECs) and cortical thymic epithelial cells (cTECs). IL-7 and Delta-like 4 expressed by TECs promote their proliferation and commitment to the T-cell lineage, thereby leading to the differentiation of $CD4^+CD8^+$ double-positive (DP) T cells expressing a diverse repertoire of T-cell antigen receptors (TCRs) (Hozumi et al., 2008; Koch et al., 2008; Hong et al., 2012). Subsequently, this T-cell repertoire is scrutinized by the TECs displaying the complex of self-peptides and MHC molecules (self-pMHCs) (Kyewski and Klein, 2006; Anderson et al., 2007; Klein et al., 2009). The presentation of self-pMHCs by cTECs is required for the survival and differentiation of DP cells to CD4- or CD8-single-positive (CD4SP or CD8SP) T cells. This process, positive selection, is achieved when a TCR binds to a self-pMHC with moderate avidity, whereas T cells expressing a TCR recognizing a self-pMHC with high avidity undergo apoptosis, i.e., negative selection. Several studies suggest that mTECs are involved in negative selection, thereby preventing the onset of autoimmune diseases (Mathis and Benoist, 2004; Kyewski and Klein, 2006; Anderson et al., 2007; Klein et al., 2009). Moreover, mTECs might contribute to the development of thymic regulatory T cells (Tregs) expressing the transcription factor Foxp3 (Hsieh et al., 2012), which suppress autoimmune responses and excessive immune reactions (Wing and Sakaguchi, 2010; Josefowicz et al., 2012). Given the connections with autoimmune diseases, researchers have been striving to uncover the molecular mechanisms underlying the functions and development of mTECs. In this review, we primarily focus on the role of signaling by the TNF receptor family (TNFRF) members RANK, CD40, and LtβR in the development and functions of mTECs in the establishment of self-tolerance.

FUNCTIONS OF mTECs

It is well established that mTECs ectopically express a wide variety of self-antigens that are normally expressed in a tissue-specific fashion (TSAs, for example, insulin, c-reactive protein, and caseins) (Kyewski and Klein, 2006). Because mature mTECs express high levels of MHC class II, co-stimulatory molecule CD80 and antigen-processing enzymes (Gray et al., 2006; Guerder et al., 2012), it is likely that mTECs directly perform negative selection by presenting TSA-peptides, as was confirmed in a recent study (Hinterberger et al., 2010) (**Figure 1**). Additionally, the TSAs expressed in mTECs are transferred to and presented by cDCs for negative selection (Gallegos and Bevan, 2004; Koble and Kyewski, 2009) (**Figure 1**). Consequently, the T cells that are potentially reactive to the TSAs in the periphery would undergo apoptosis in the thymus. It is important future study to clarify the relative contributions of mTECs and cDCs to negative selection.

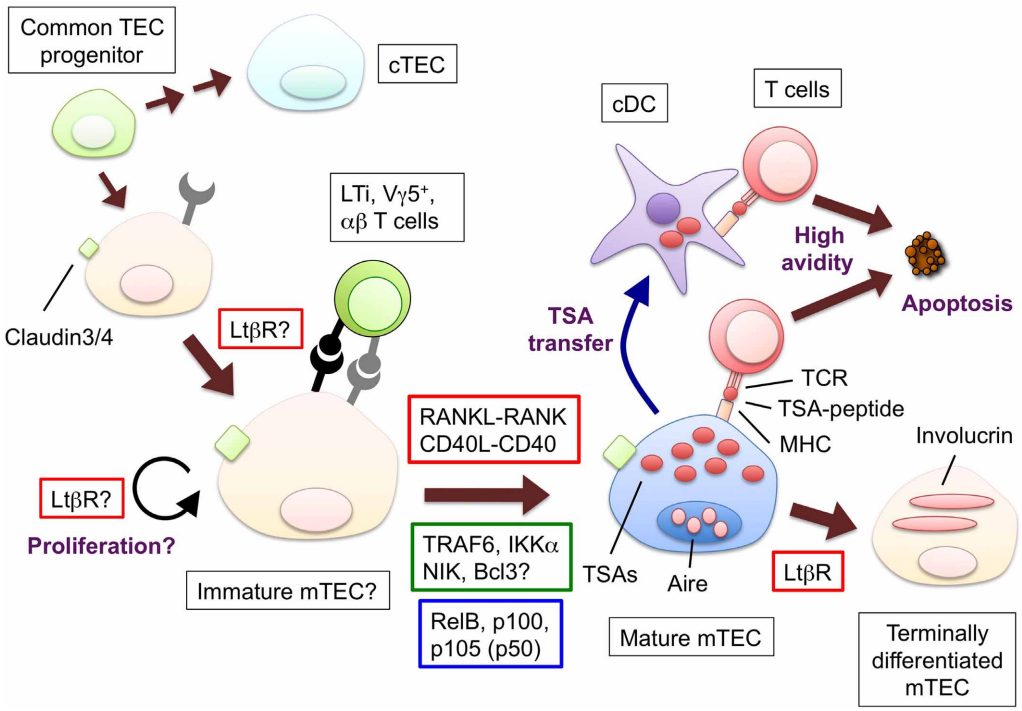

FIGURE 1 | The hypothetical model for the development and functions of mTECs and molecules to promote the mTEC development. Bipotent common progenitors give rise to the mTEC progenitors expressing claudin-3 and -4. Lymphoid tissue inducer (LTi), $V\gamma5^+$ DETC progenitor ($V\gamma5^+$) or positively selected αβ T cells produce RANKL; CD40L is supplied only by αβ T cells. The interaction between RANKL and RANK promotes the differentiation and/or proliferation of the mTECs. The similar contribution of the interaction of CD40L and CD40 begins at the neonatal stage. RANK and CD40 signaling results in the translocation of the NF-κB family member RelB through the activation of signal transducers TRAF6 and NIK. The mature mTECs express a wide variety of tissue-specific antigens (TSAs) and AIRE. The TSAs are in part transferred to conventional DCs; as a result, both the mTECs and DCs would display the TSA-peptides to the developing T cells. The T cells expressing TCR bound to the complex of the TSA-peptide and MHC molecules with high avidity undergo apoptosis. Mature mTECs might further differentiate into involucrin-expressing mTECs in the LtβR and AIRE-dependent process. The LtβR signaling might regulate the up-regulation of RANK in the TECs and/or enhance the proliferation of the RANK-expressing TECs. Ligands and receptors involved in each process are surrounded by red line rectangles, signal transducers are by green line rectangles, transcription factors are by blue line rectangles.

Autoimmune regulator (AIRE), for which loss of function mutations cause an inherited human autoimmune disease (Consortium, 1997; Nagamine et al., 1997; Michels and Gottlieb, 2010), is expressed in mTECs and promotes TSA expression, thereby suppressing the onset of autoimmune diseases (Anderson et al., 2002; Peterson et al., 2008; Mathis and Benoist, 2009). Although the involvement of AIRE is evident, the molecular mechanisms underlying a broad range of TSA expression in mTECs remain unclear. The unique epigenetic regulation in mature mTECs appears to regulate the diversity of TSA expression (Derbinski et al., 2005; Tykocinski et al., 2010). Interestingly, individual mTECs appear to have a different and transient TSA expression pattern, implying the probabilistic nature of the expression of TSAs (Derbinski et al., 2008; Villaseñor et al., 2008).

The requirement of TSA expression in preventing the onset of autoimmune diseases was suggested by the results of several studies (Kyewski and Klein, 2006). It was reported that the polymorphisms in the promoter region of the muscle acetylcholine receptor gene (*CHRNA1*), which is associated with the early onset of human autoimmune myasthenia gravis, leads to the reduction of *CHRNA1* expression in human mTECs (Giraud et al., 2007). Two studies provide more direct evidence for the correlation between the expression of TSAs in mTECs and autoimmunity in the corresponding tissues. The lack of interphotoreceptor retinoid-binding protein in thymic stroma cells is sufficient for inducing spontaneous autoimmunity in the eyes (DeVoss et al., 2006). Moreover, deletions of insulins in AIRE-expressing mTECs spontaneously provoked autoimmune diabetes in mice (Fan et al., 2009).

The high avidity interaction between TCRs and self-pMHCs is the determinant of the development of Treg (Hsieh et al., 2012; Josefowicz et al., 2012). Previous studies revealed that the cTEC-specific expression of MHC class II (MHC II) is sufficient for Treg generation in the thymus (Bensinger et al., 2001). Moreover, a recent study revealed that the diversity of TCRs in Tregs was not altered by the lack of AIRE (Daniely et al., 2010). These data suggest that TSA presentation by mTECs is dispensable for thymic Treg development. However, almost all $Foxp3^+$ cells in the thymus are CD4SP T cells localized in the medulla (Lee and Hsieh, 2009), and $Foxp3^+$ expression initiates at the stage of the CD4SP progenitor (Burchill et al., 2008; Lio and Hsieh, 2008). Thus, the contribution of mTECs to Treg differentiation and/or proliferation cannot be ruled out (Hsieh et al., 2012). Indeed,

several mTEC-deficient mice showed a partial reduction in the Treg frequency in the thymus (Kajiura et al., 2004; Shimo et al., 2011).

The chemokines CCL19 and CCL21 secreted from mTECs attract positively selected T cells expressing the chemokine receptor CCR7 into the medulla (Ueno et al., 2002, 2004). A recent study revealed that mTECs also express the chemokine XCL to attract cDCs in the medulla (Lei et al., 2011). Interestingly, the expression of CCL19, CCL21, and XCL appear to be regulated by AIRE (Laan et al., 2009; Lei et al., 2011). These findings might be consistent with the idea that AIRE regulates the differentiation of mTECs as well as the TSA expression (Gillard et al., 2007; Dooley et al., 2008; Yano et al., 2008).

DEVELOPMENT OF mTECs

The endodermal epithelial cells in the ventral part of third pharyngeal pouch differentiate into TECs (Blackburn and Manley, 2004; Holländer et al., 2006). Then, both the mTECs and cTECs are differentiated from bipotent TEC progenitors (Bleul et al., 2006; Rossi et al., 2006). The existence of mTEC-committed progenitors was initially proposed from the finding that the mTECs form clusters expressing a single MHC II in the thymuses of chimeric mice with two different types of MHC II molecules (Rodewald et al., 2001). Furthermore, it was shown that the TEC fraction expressing claudin-3 and -4, components of tight junction, differentiates into AIRE^{+} mTECs but not into cTECs (Hamazaki et al., 2007). Another study suggested that CD80^{-} UEA-1-lectin^{+} TECs emerging in embryonic thymus contain the mTEC progenitors (Gäbler et al., 2007). The rapid turnover of mTECs (Gray et al., 2006, 2007) and post-mitotic nature of the AIRE-expressing cells (Gray et al., 2007) in the adult thymus imply the presence of progenitors that continuously provide mature mTECs. Indeed, the CD80^{-} UEA-1^{+} TECs prepared from postnatal thymuses are converted into CD80^{+} UEA-1^{+} mTECs in re-aggregated fetal thymus organ cultures (Gäbler et al., 2007). Currently, the ratio of mTEC progenitors in the claudin-3/4^{+} CD80^{-} UEA-1^{+} TEC fraction is unclear, and it remains elusive whether the mTEC progenitors in the adult thymus are identical to those in the embryonic thymus.

In the adult thymus, the mTECs are separated into mainly two subsets (Gray et al., 2006). The first subset expresses relatively higher levels of MHC II and CD80, usually referred to as mTEC^{high}; this subset contains AIRE-expressing cells and is regarded as the mature type (Kyewski and Klein, 2006). The other subset, mTEC^{low}, shows relatively lower expression levels of MHC II and CD80. The mTEC^{high} subset would be expected to play a role in negative selection because of the expression of AIRE, the wide range of TSAs as well as their high antigen-presenting ability, whereas the contribution of the mTEC^{low} subset on thymic tolerance remains to be addressed. In addition to these two subsets, the mTECs expressing involucrin, a keratinocyte terminal differentiation marker, are regarded as terminally differentiated mTECs (Yano et al., 2008; White et al., 2010). Moreover, a recent fate mapping of AIRE expression suggested another developmental stage of mTECs in which the expression of AIRE ceases (Nishikawa et al., 2010).

TNF RECEPTOR FAMILY SIGNALING

The binding of TNF family ligands to the TNFRF proteins leads to various cellular responses, such as proliferation, differentiation, inflammatory responses, and apoptosis (Aggarwal, 2003; Hehlgans and Pfeffer, 2005; Aggarwal et al., 2012). Several members of the TNFRF activate NF-κB family transcription factors, inducing the expression of the genes required for these cellular responses (Aggarwal, 2003; Hehlgans and Pfeffer, 2005; Vallabhapurapu and Karin, 2009). Several lines of evidence have indicated that the signaling of the TNFRF members RANK, CD40, and LtβR play critical roles in the development and function of mTECs.

CD40L-CD40

The engagement of CD40 with CD40L initiates and progresses acquired immune responses through the activation and survival of B cells, macrophages, and DCs (Elgueta et al., 2009; Ma and Clark, 2009). The expression of CD40 in human TECs was initially reported almost two decades ago (Galy and Spits, 1992), and, because the CD40 signal exerts profound effects on antigen presenting cells, several studies on the function of CD40 in the thymus have focused on the roles in proliferation and the selection of T cells (Foy et al., 1995; Ruggiero et al., 1996; Williams et al., 2002) and Treg development (Kumanogoh et al., 2001; Spence and Green, 2008; Martín-Gayo et al., 2010).

Several studies have provided evidence that CD40 signaling controls the development of mTECs. The forced expression of CD40L by the lck promoter caused a reduction of the cTECs and an increase of mTECs (Dunn et al., 1997), and both CD40L-deficient (CD40L-KO) mice and CD40-KO mice showed a reduction of mTECs (Gray et al., 2006; Akiyama et al., 2008; White et al., 2008). In addition, the *in vitro* development of mTECs is elicited by the addition of recombinant CD40L protein to fetal thymic stroma organ culture (Akiyama et al., 2008), which is prepared from fetal thymus by the elimination of cells of hematopoietic origin. Notably, whereas CD40 is expressed in the TECs of fetal thymus (Dunn et al., 1997; Akiyama et al., 2008; Shakib et al., 2009), the expression of CD40L was not detected in the fetal thymus but only began in the neonatal mouse (Dunn et al., 1997; Akiyama et al., 2005). This expression pattern suggested that the contribution of the CD40L-CD40 interaction to the development of mTECs should be limited following birth. The expression of CD40L in the thymus was predominantly detected in the medulla (Dunn et al., 1997). Consistently, some studies indicated that the expression of CD40L is up-regulated in positively selected CD4SP T cells (Hikosaka et al., 2008; Irla et al., 2008).

RANKL-RANK

The RANKL and RANK interaction regulates a diverse set of physiological events, such as bone homeostasis (Yasuda et al., 1998; Dougall et al., 1999; Kong et al., 1999), lymph node development (Kong et al., 1999; Kim et al., 2000), mammary gland development (Fata et al., 2000), hair follicle anagen (Duheron et al., 2011), and DC survival (Anderson et al., 1997; Wong et al., 1997). RANKL-deficient (RANKL-KO) and RANK-deficient (RANK-KO) mice exhibited a reduction in mature mTECs (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka

et al., 2008), which was more remarkable for the mTEC^{high} subset. Moreover, recombinant RANKL protein efficiently induces the development of mTECs expressing AIRE and TSAs in fetal thymic stroma culture (Rossi et al., 2007; Akiyama et al., 2008). Although a lack of the RANKL-RANK interaction causes a significant reduction of mature mTECs, a small population of mature mTECs still exists in these mutant mice (Akiyama et al., 2008; Hikosaka et al., 2008), a finding that suggests the presence of another signal for inducing mature mTECs in addition to RANK signaling. Indeed, mature mTECs were completely absent in RANKL- and CD40-double deficient (RANKL/CD40 DKO) mice (Akiyama et al., 2008). Thus, RANK and CD40 signaling have an overlapping function in the development of mature mTECs. Osteoprotegerin, OPG, a natural inhibitor of RANKL, is expressed in mTECs (Hikosaka et al., 2008), and, consistently, OPG-deficient mice showed an increase in mTECs in the thymus (Hikosaka et al., 2008).

RANKL is produced by certain types of thymic cells. An initial study revealed that lymphoid tissue inducer cells (LTi) express RANKL in the fetal thymus (Rossi et al., 2007). Furthermore, a recent study uncovered the role of the progenitors of Vγ5 chain-positive dendritic epidermal T cells (DETC), a subset of γδ T cells, in supplying RANKL in the embryonic thymus (Roberts et al., 2012). Interestingly, the RANKL signal in turn induces the expression of Skint-1 in mTECs, promoting the selection and generation of the monoclonal DETC compartment (Barbee et al., 2011), which suggests a role for the mTECs in DETC selection. In addition, RANKL is provided by positively selected αβ T cells in adult thymus (Hikosaka et al., 2008).

As described above, because the CD40L expression is practically absent in the fetal thymus, RANKL is essential for the mature mTEC development in embryo (Akiyama et al., 2008). Currently, the relative contributions of LTi and Vγ5$^+$ thymocytes to the RANKL expression remain to be determined. Moreover, other lymphoid cells might be involved in the expression of RANKL in the fetal thymus because small numbers of AIRE$^+$ mTECs were present in the mice lacking both LTi and Vγ5$^+$ thymocytes. In the postnatal thymus, large numbers of αβ T cells supply both RANKL and CD40L. Therefore, contributions of LTi and Vγ5$^+$ thymocytes might be limited due to their low frequency.

RANKL KO and RANK KO mice did not show any appreciable autoimmune phenotypes, which may be because a small amount of AIRE and low number of mature mTECs are sufficient for suppressing autoimmunity. Another possibility is that the interaction of RANKL and RANK in other immune cells is required for the progression of autoimmunity in peripheral organs. Consistent with the latter possibility, a transplantation of RANK-KO fetal thymic stroma (Rossi et al., 2007) or an adoptive transfer of splenocytes from RANKL-KO or RANKL/CD40 DKO mice (Akiyama et al., 2008) provoked autoimmunity in the recipient nude mice.

LYMPHOTOXIN β RECEPTOR

LtβR is essential for the organogenesis of secondary lymphoid organs (Mebius, 2003; Drayton et al., 2006; Randall et al., 2008). There are at least two types of ligands that bind to LtβR: a heterotrimer consisting of Ltα and Ltβ and the Light homotrimer. Although LtβR-deficient mice show a defect in the development of mTECs (Boehm et al., 2003; Venanzi et al., 2007), the developmental processes regulated by LtβR signaling remain unclear (Zhu et al., 2010). The injection of an agonistic LtβR antibody into mice increases the expression of AIRE within a few hours (Chin et al., 2003), and the treatment of an mTEC line with the same antibody induces the expression of AIRE in the presence of a DNA methylation inhibitor (Chin et al., 2003). However, the expression levels of AIRE in the mTECs isolated from LtβR-KO mice are comparable to wild-type mice (Venanzi et al., 2007), and the treatment of fetal thymic stroma with an agonistic LtβR antibody failed to induce the expression of AIRE (Akiyama et al., 2008). Thus, a direct linkage between LtβR signaling and AIRE expression seems to be unwarranted. It is probable that the LtβR signal controls the development of mTECs by a process i.e., different from that mediated by the RANK and CD40 signals, which are more directly linked to the development of mature AIRE-expressing mTECs. Indeed, LtβR is involved in the expression of AIRE-independent TSAs and chemokines in the thymus (Chin et al., 2006; Zhu et al., 2007; Seach et al., 2008), and a recent study revealed the requirement of LtβR in the development of involucrin-expressing mTECs (White et al., 2010). In addition, another recent study revealed that LtβR signaling induces the expression of RANK, in turn facilitating the development of the mature mTECs (Mouri et al., 2011). These mutually non-exclusive studies suggest that LtβR signaling has multiple roles in the development and functions of mTECs.

LtβR-KO mice exhibit inflammatory cellular infiltration in their peripheral organs (Chin et al., 2006). The fetal thymic stroma transplantation confirmed the requirement of LtβR signaling in the thymic stroma cells for the suppression of autoimmunity (Chin et al., 2006). In addition, the LtβR signal regulates the expression of the chemokines that attract lymphocytes in the medulla (Zhu et al., 2007; Seach et al., 2008). Because the failure in the movement of the positively selected T cells to the medulla results in autoimmunity (Kurobe et al., 2006), this function may be linked to the autoimmune phenotypes observed in LtβR-KO mice.

INTRACELLULAR SIGNAL TRANSDUCERS AND NF-κB

RANK, CD40, and LtβR signaling activates two distinct NF-κB activation pathways: the classical and non-classical NF-κB pathways (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009) (**Figure 2**). In the classical pathway, the engagement of the receptor recruits the TRAF proteins to induce the activation of the downstream serine threonine kinases (e.g., TGF-β activating kinase 1), which in turn activate the IKK complex comprising IKKα, IKKβ, and NEMO. Subsequently, the IKK complex phosphorylates IkBα, sequestering NF-κB in the cytoplasm. The phosphorylation of IkBα triggers its degradation by the ubiquitin-dependent 26S-proteasome complex, which in turn results in the translocation of the NF-κB family RelA complex to the nucleus for transcriptional activation. Conversely, in the non-classical pathway, the engagement of the receptors induces the release of NIK from the protein complex comprising cIAP1/2, TRAF2, and TRAF3, a complex that degrades NIK in an unstimulated state. The released NIK phosphorylates IKKα, which in

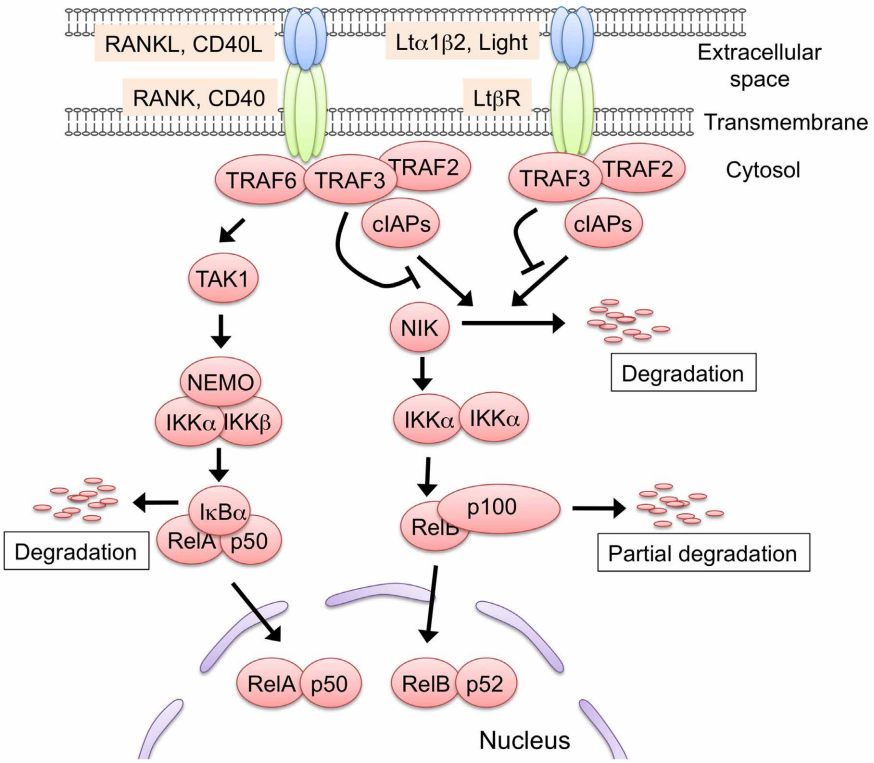

FIGURE 2 | The two NF-κB activation pathways induced by RANK, CD40, and Ltβ R signaling. Either RANK, CD40, or LtβR signaling is capable of activating the non-classical NF-κB pathway. In the steady state, the protein complex consisting of TRAF2, TRAF3, and cIAP1/2 binds to and poly-ubiquitinates NF-κB inducing kinase (NIK). The poly-ubiquitinated NIK is immediately degraded by the 26S-proteasome machinery. The engagement of the receptors with their ligands recruits the TRAF2, TRAF3, and cIAP1/2 complex, leading to the release of NIK from the complex. The stabilized NIK activates the IKK α complex by phosphorylation, which in turn phosphorylates p100, thus sequestering RelB in the cytoplasm. Subsequently, p100 is poly-ubiquitinated and converted to p52 by partial degradation, an event that leads to the nuclear translocation of the active RelB/p52 complex. The RANKL-RANK and CD40L-CD40 interaction recruits TRAF6 to their cytoplasmic tails. TRAF6 activates the downstream serine/threonine kinase, typically TGF-β activating kinase 1 (TAK1), and activated TAK1 causes the activation of the IKK complex, which consists of IKK α, IKK β, and NEMO. Thereafter, the activated IKK complex phosphorylates IkB α, thereby inducing the degradation of IkB α to lead to the nuclear translocation of the RelA complex. The TRAF6-mediated NF-κB activation might induce the expression of RelB. Although LtβR also can activate the classical pathway, there is no supporting evidence to date that the LtβR-mediated classical pathway regulates mTEC development. Such protein modifications as phosphorylation and ubiquitination are omitted in this figure for simplicity.

turn phosphorylates the p100 that sequesters the NF-κB family member RelB in the cytoplasm. The phosphorylation of p100 triggers its partial degradation to p52, leading to the nuclear translocation of the RelB/p52 complex to induce gene expression.

RelB-deficient (RelB-KO) mice (Burkly et al., 1995; Weih et al., 1995; Zuklys et al., 2000) and *aly/aly* mice (Miyawaki et al., 1994; Kajiura et al., 2004; Shinzawa et al., 2011), which have a dysfunctional point mutation in the gene encoding NIK, showed severe defects in the development of mTECs expressing AIRE and TSAs. Consistently, these mutant mice exhibit autoimmune phenotypes. Although IKK α-deficient (IKK α-KO) mice die immediately following birth, a defect in mTEC development was still identified (Kinoshita et al., 2006; Lomada et al., 2007). Furthermore, p100 (also named NF-κB2)-deficient (p100-KO) mice showed a partial reduction of the mTEC developmental and autoimmune phenotypes (Zhang et al., 2006; Zhu et al., 2006). Therefore, these data strongly suggested that the non-classical NF-κB pathway is essential for the development of mTECs and mTEC-mediated self-tolerance. Interestingly, whereas the defect in mTEC development is mild in the p100-KO mice, p100 and p105 (also named NF-κB1)-double-deficient mice (Franzoso et al., 1997) and p100 and Bcl-3-double-deficient mice (Zhang et al., 2007) exhibited more severe defects in the development of mTEC compared to each single mutant, suggesting redundant roles among these NF-κB members and the nuclear IκB. Because the non-classical NF-κB pathway can be activated by either RANK, CD40, or LtβR signaling, the developmental stages at which these molecules control mTEC development need to be clarified.

TRAF6, an E3-ubiquitin ligase, activates the classical NF-κB pathway (Inoue et al., 2007), which indirectly facilitates the non-classical NF-κB activation pathway by inducing RelB (Bren et al., 2001; Akiyama et al., 2005) and p100 (Dejardin et al., 2002). Several studies indicated that TRAF6 mediates RANK signaling (Darnay et al., 2007) and CD40 signaling (Bishop et al., 2007) but not LtβR signaling (Qin et al., 2007). Consistently, TRAF6-deficient mice showed a severe defect in mTEC development that was comparable to RANKL and CD40 DKO mice

(Akiyama et al., 2005, 2008). Thus, it is likely that, as a downstream molecule of RANK and CD40 signals, TRAF6 is required for the development of mature mTECs expressing AIRE, TSAs, and RelB (Akiyama et al., 2005, 2008).

CONCLUDING REMARKS

It is now widely accepted that, despite of the very low frequency in the population of total thymic cells, mTECs play critical roles in preventing autoimmunity in the body. Several studies have provided evidence that RANK, CD40, and LtβR signaling are critical for the development of mTECs. It is most likely that the engagement of these receptors activates the two NF-κB activation pathways. The ensuing transcriptional activation of the NF-κBs induces the genes that promote the development and function of mTECs. However, literature on the downstream target genes induced by these signals in mTECs is currently scarce (Ohshima et al., 2011), and the identification of these targets is important for future research because these target genes would define the unique properties of mTECs. Moreover, the developmental stages of the mTECs receiving this signaling are not fully characterized, and the mechanisms and signals that determine the commitment to the mTEC lineage also remain to be addressed. Future studies, on the biology of mTECs would be informative for the understanding of the mechanism involved in the establishment of self-tolerance and the development of the thymic medullary environment.

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Podoplanin: emerging functions in development, the immune system, and cancer

Jillian L. Astarita[1,2], Sophie E. Acton[1,3] and Shannon J. Turley[1,4]*

[1] *Department of Cancer Immunology and AIDS, Dana Farber Cancer Institute, Boston, MA, USA*
[2] *Division of Medical Sciences, Harvard Medical School, Boston, MA, USA*
[3] *Department of Cell and Developmental Biology, University College London, London, UK*
[4] *Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA*

Edited by:
Burkhard Ludewig, Cantonal Hospital St. Gallen, Switzerland

Reviewed by:
Veronika Lukacs-Kornek, Saarland University Hospital, Germany
Lucas Onder, Cantonal Hospital St. Gallen, Switzerland
Luisa Cervantes-Barragan, Washington University School of Medicine, USA

***Correspondence:**
Shannon J. Turley, Department of Cancer Immunology and AIDS, Dana Farber Cancer Institute, 450 Brookline Avenue, Dana 1440A, Boston, MA 02115, USA.
e-mail: shannon_turley@dfci.harvard.edu

Podoplanin (PDPN) is a well-conserved, mucin-type transmembrane protein expressed in multiple tissues during ontogeny and in adult animals, including the brain, heart, kidney, lungs, osteoblasts, and lymphoid organs. Studies of PDPN-deficient mice have demonstrated that this molecule plays a critical role in development of the heart, lungs, and lymphatic system. PDPN is widely used as a marker for lymphatic endothelial cells and fibroblastic reticular cells of lymphoid organs and for lymphatics in the skin and tumor microenvironment. Much of the mechanistic insight into PDPN biology has been gleaned from studies of tumor cells; tumor cells often upregulate PDPN as they undergo epithelial-mesenchymal transition and this upregulation is correlated with increased motility and metastasis. The physiological role of PDPN that has been most studied is its ability to aggregate and activate CLEC-2-expressing platelets, as PDPN is the only known endogenous ligand for CLEC-2. However, more recent studies have revealed that PDPN also plays crucial roles in the biology of immune cells, including T cells and dendritic cells. This review will provide a comprehensive overview of the diverse roles of PDPN in development, immunology, and cancer.

Keywords: podoplanin, CLEC-2, lymph node stromal cells, lymphatic endothelial cells, platelets, cancer-associated fibroblasts

INTRODUCTION

Podoplanin (PDPN) is a 36- to 43-kDa mucin-type transmembrane protein. It has homologues in humans, mice, rats, dogs, and hamsters and is relatively well conserved between species. PDPN has a wide variety of functions including regulation of organ development, cell motility, and tumorigenesis and metastasis (Wicki and Christofori, 2007; Suzuki-Inoue et al., 2011). PDPN has been identified and studied in many different contexts; thus, it has been given several names. PDPN was first described on lymphatic endothelial cells (LECs) as the E11 antigen (Wetterwald et al., 1996) and on fibroblastic reticular cells (FRCs) of lymphoid organs and thymic epithelial cells as gp38 (Farr et al., 1992a,b; **Table 1**). PDPN is also homologous to T1a/rTI$_{40}$, one of the first molecular markers of alveolar type I epithelial cells (Rishi et al., 1995; Williams et al., 1996; **Table 1**), PA2.26, which is upregulated in skin keratinocytes upon injury (Scholl et al., 1999), OTS-8, a molecule induced in osteoblasts upon phorbol ester treatment (Nose et al., 1990), and Aggrus, a platelet-aggregating factor (Kato et al., 2003). Finally, this molecule was given the name podoplanin due to its expression on kidney podocytes and possible involvement in the flattening of podocyte foot processes (Breiteneder-Geleff et al., 1997).

While PDPN expression patterns in many of these cells have been well characterized, there is still little known about the physiological functions of this protein. PDPN has been reported to bind to the C-type lectin receptor CLEC-2, which is highly expressed by platelets and immune cells. However, this interaction has only been extensively studied with regard to platelets. Engagement of PDPN by CLEC-2 on platelets leads to platelet aggregation and activation, and this process is critical for the maintenance of normal lymphatic vessels (Bertozzi et al., 2010; Suzuki-Inoue et al., 2010). PDPN has also been used as a marker for FRCs in the lymph node (LN) and spleen, but there is limited data on whether PDPN expression is required for the function of these cells or influences their interactions with leukocytes.

The majority of data examining the function and signaling pathways of PDPN are from studies of PDPN overexpression in tumor cells. While these studies certainly provide critical insight into cellular and molecular aspects of PDPN biology, it is important to understand whether PDPN functions similarly in non-pathological settings and in cell types where it is naturally expressed. Here, we will review what is currently known about the structure, molecular interactions, and *in vivo* roles of PDPN. We will focus on the function of PDPN on stromal cells, including epithelial cells, endothelial cells, and fibroblasts but will also describe recent studies of PDPN expression by immune cells.

PDPN IN DEVELOPMENT

Podoplanin is first expressed in the developing mouse embryo on day E9 in the foregut, proepicardial organ, and central nervous system (CNS; Williams et al., 1996; Mahtab et al., 2009; **Table 1**). Throughout development, it is also expressed in the fetal rat kidney, choroid plexus, intestine, and esophagus (Williams et al., 1996; **Table 1**). Over time, PDPN expression is increasingly restricted such that in an adult animal, PDPN is predominantly

Table 1 | Podoplanin expression and function in organs and immune cells.

Organ	Time of expression	PDPN function	Reference
Central nervous system	Beginning day E9, becomes restricted to choroid plexus in adult mouse	No specific function reported during development; high PDPN expression in brain tumors	Williams et al. (1996), Kaji et al. (2012), Peterziel et al. (2012)
Heart	Expressed in entire organ on day E9; continued expression in adult heart	Required for normal heart development, specifically for EMT in epicardium-derived cells	Martín-Villar et al. (2005), Mahtab et al. (2008, 2009), Douglas et al. (2009)
Lungs	Appears in foregut on day E9 before lung buds; subsequently restricted to alveolar type I epithelial cells	Required for lung development; specifically the effective maturation of alveolar type I epithelial cells	Ramirez et al. (2003)
Intestine	Expressed on day E9 in foregut; continued expression in lamina propia	No specific function determined	Farr et al. (1992a), Williams et al. (1996)
Lymphoid organs	Present in spleen 4 days postnatally; in adult, expression by FRCs, LECs, and FDCs in lymph node and spleen, and thymic medullary epithelial cells	Required for proper formation and organization of lymph nodes and spleen; necessary for efficient DC migration to and within lymph nodes; highly expressed by stroma and some T cells in ectopic lymphoid tissue	Farr et al. (1992a), Bekiaris et al. (2007), Raica et al. (2010), Peters et al. (2011), Acton et al. (2012), Yu et al. (2007)
Immune cell	**Expression pattern**	**Function**	**Reference**
T cell	Expressed only on T_H17 cells, not other subsets	Plays a role in T_H17-driven development of ectopic germinal centers in EAE	Peters et al. (2011)
Macrophages	Expressed by inflammatory macrophages, such as thioglycollate-elicited peritoneal macrophages	Possibly plays a role in response to fungal infections; can activate platelet aggregation	Hou et al. (2010), Kerrigan et al. (2012)

expressed in alveolar type I cells, mature osteoblasts, LECs, and FRCs in the T cell zone of lymphoid organs (Wetterwald et al., 1996; Williams et al., 1996; Schacht et al., 2003; **Table 1**). PDPN is critical for normal development of some of these organs and has been well studied in PDPN-deficient animals. *Pdpn*$^{-/-}$ mice develop normally until around day E10, which coincides with the appearance of PDPN protein. From days E10–16, approximately 40% of *Pdpn*$^{-/-}$ embryos die; the ones that survive to birth die within a few days (Mahtab et al., 2008). However, interestingly, when the mice are crossed onto a C57Bl/6 background, many more embryos survive to birth, and although 50% die in the first week, approximately 20% of the mice do survive to adulthood (Uhrin et al., 2010). The reason why the genetic background affects the severity of the defects suffered by the *Pdpn*$^{-/-}$ mice is intriguing and warrants further study. Furthermore, it would be of great use to the field to have a conditional knockout of PDPN to avoid these survival defects.

The defect in blood-lymphatic vascular separation is the phenotype most extensively studied in PDPN-deficient mice. On day E11.5, PDPN first appears in the developing circulatory system on Prox-1^{+} lymphatic cells (Schacht et al., 2003). It was first reported by Schacht et al. (2003) that *Pdpn*$^{-/-}$ mice have abnormal lymphatic vessels that cannot properly regulate lymph flow and that this defect did not appear in blood vessels. These findings were further supported by Fu et al. (2008), who reported that endothelial cell expression of PDPN was responsible for a blood-lymphatic misconnection. Furthermore, continued expression of PDPN into adulthood was required to maintain proper vascular architecture, as an inducible deletion of T-synthase, a major glycosyltransferase required for O-glycan synthesis and normal levels of PDPN expression, showed similar blood-lymph mixing (Fu et al., 2008).

This non-separation phenotype is also observed in mice where hematopoietic cells lack Syk, SLP-76, PLCγ2, and CLEC-2 (Abtahian et al., 2003; Sebzda et al., 2006; Suzuki-Inoue et al., 2010). While platelets and neutrophils both express CLEC-2, it was initially believed that platelets could not be involved in this phenotype because mice lacking nearly all platelets had normal lymphatic vasculature (Shivdasani et al., 1995). However, elegant recent studies have proven that CLEC-2 expression and downstream signaling through SLP-76 are required specifically in platelets (Bertozzi et al., 2010; Osada et al., 2012). The interaction of platelet CLEC-2 and PDPN on LECs induces platelet aggregation and prevents blood from flowing into new lymphatic vessels budding from the cardinal vein. Furthermore, injecting a PDPN-blocking antibody or otherwise inhibiting platelet aggregation is sufficient to disrupt lymphatic development (Uhrin et al., 2010). Overall, the model that has emerged indicates that during the budding of the

lymph sac from the cardinal vein, PDPN becomes upregulated on Prox-1$^+$Lyve-1$^+$ LECs and binds with CLEC-2 on platelets. This interaction activates downstream signaling in platelets, which results in platelet aggregation. This aggregation then allows for a complete separation of the budding lymphatic vessels from the developing blood vessels.

In addition to its role in lymphatic vessel development, PDPN may play a role in the development or maintenance of lymphoid organ architecture. In the spleens of mice lacking lymphocytes, no PDPN expression is observed, although FRCs are still present as indicated by VCAM-1 and ER-TR7 staining (Ngo et al., 2001; Bekiaris et al., 2007). It appears that this lack of expression is due to a lack of lymphotoxin, but it remains unclear exactly which cell type provides that signal during development of the spleen. A more striking phenotype has been observed by Peters et al. (2011) in that *Pdpn*$^{-/-}$ mice lack nearly all LNs, and the ones that develop are extremely disorganized. The spleens of these mice were present, but were also disorganized. It is interesting to speculate whether this phenotype indicates an important function for PDPN on FRCs and T cells; however, it is also possible that the lack of LNs is due to impaired lymph flow caused by the malformed lymphatic vessels. Thus, further work is needed to dissect this phenotype.

The first defects described in *Pdpn*$^{-/-}$ mice were in the lung (**Table 1**), as these mice die shortly after birth due to an inability to inflate the lungs (Ramirez et al., 2003). This defect stems from an impairment in the development of alveolar type I cells. These cells cover the majority of the lung surface and play a key role in the proper development of the alveoli, which are the major gas exchange centers of the lung (Williams, 2003). In normal lung development, alveolar type I cells exhibit a high proliferation rate during early and mid-gestation periods, but this high growth rate slows a few days before birth (Ramirez et al., 2003). However, when alveolar type I cells lack PDPN, they continue proliferating in later stages of embryonic development, which is partially explained by a decrease in the negative cell cycle regulator, p21, at birth (Millien et al., 2006).

Podoplanin is also necessary for proper development of the heart (**Table 1**). PDPN is first expressed in the proepicardial organ on day E9.5 and by day E12.5, it is expressed in most of the heart. Without PDPN expression, the hearts of developing mice exhibit hypoplasia in the pulmonary vein, left atrium dorsal wall, and the atrial septum (Douglas et al., 2009). In this setting, the lack of PDPN leads to a dysregulation of epithelial-mesenchymal transition (EMT), a process that involves the transition of sessile epithelial cells into more motile mesenchymal cells through the downregulation of epithelial markers, such as adhesion molecules like E-cadherin (Thiery, 2002). In PDPN-deficient mice, the epicardium-derived cells responsible for cardiac development show increased levels of E-cadherin and decreased levels of RhoA compared with their WT counterparts, which is indicative of impaired EMT (Mahtab et al., 2008, 2009). While PDPN has been shown to play a role in regulating EMT (Martín-Villar et al., 2006), these studies are the first evidence that PDPN may play a role in physiological instances of EMT in non-transformed cells.

Overall, PDPN is crucial for the development of multiple organs, including the lymphatic system, lungs, and heart. Interestingly, PDPN serves diverse functions in these organs. In some instances it is required for CLEC-2-dependent platelet aggregation, but in others it seems to have an intrinsic effect on proliferation or differentiation in a specific cell type. This raises the question of whether PDPN function could to some degree be tissue specific. The range of physiological effects downstream of PDPN expression may be due to different protein interactions and binding partners in diverse cell types.

MOLECULAR INTERACTIONS AND SIGNALING OF PDPN

Podoplanin contains a single transmembrane domain, a short, nine amino acid cytoplasmic tail, and a heavily glycosylated extracellular domain (Martín-Villar et al., 2005). While there are no obvious conserved protein domains in PDPN, several studies have identified specific residues on PDPN that mediate interactions with other proteins (**Figure 1**). The first hints at the cellular function of PDPN came from Scholl et al. (1999), who discovered that PDPN was upregulated in keratinocytes from induced epidermal carcinogenesis and was localized to membrane protrusions such as filopodia and lamellipodia. PDPN co-localized with ezrin, radixin, and moesin (ERM) family proteins, and was later found to directly bind ezrin and moesin. This interaction requires a conserved motif of three basic residues in the cytoplasmic tail (see **Figure 1**) and overexpression of PDPN resulted in increased phosphorylation of ERM proteins (Martín-Villar et al., 2006; Wicki et al., 2006). The ERM proteins function as connectors between integral membrane proteins and the actin cytoskeleton. Phosphorylation causes a conformational change that exposes binding sites for actin and other proteins (Fehon et al., 2010). Thus, this interaction likely underlies many of the effects that PDPN has on cytoskeleton. A closer examination of the effects of PDPN upregulation revealed that overexpression of PDPN in epithelial cell lines caused them to become more mesenchymal in appearance, with decreased stress fibers and increased filopodia (Martín-Villar et al., 2006; Wicki et al., 2006). These changes, in addition to a downregulation of E-cadherin and other epithelial markers, are indicative of cells undergoing EMT, which is indeed what Martín-Villar et al. (2006) observed. However, Wicki et al. (2006) demonstrated that while PDPN overexpression resulted in increased motility, it did not result in an E-cadherin switch or EMT. Discrepancies were also found when the involvement of Rho family small G proteins was examined. Martín-Villar et al. (2006) reported that PDPN overexpression resulted in an increase in RhoA and no change in Rac-1 or Cdc42, while Wicki et al. (2006) found a downregulation in RhoA, Rac-1, and Cdc42. In addition, Navarro et al. (2010) found that knocking down PDPN in LECs resulted in decreased levels of activated RhoA and increased levels in Cdc42. While it is clear that the expression of PDPN has an effect on the activity levels of these proteins, more work must be done to fully elucidate the mechanism. As described above, it is possible that PDPN exerts different effects and utilizes distinct signaling cascades in various cell types, which could partially explain the observed discrepancies.

Recently, it was discovered that PDPN resides in lipid rafts in the plasma membrane. Barth et al. (2010) found that PDPN resides in detergent-insoluble fractions of alveolar type I epithelial cells, but its function within these rafts remains unknown. It was subsequently reported that human PDPN expressed in Madin–Darby Canine Kidney (MDCK) type II cells is localized to lipid rafts

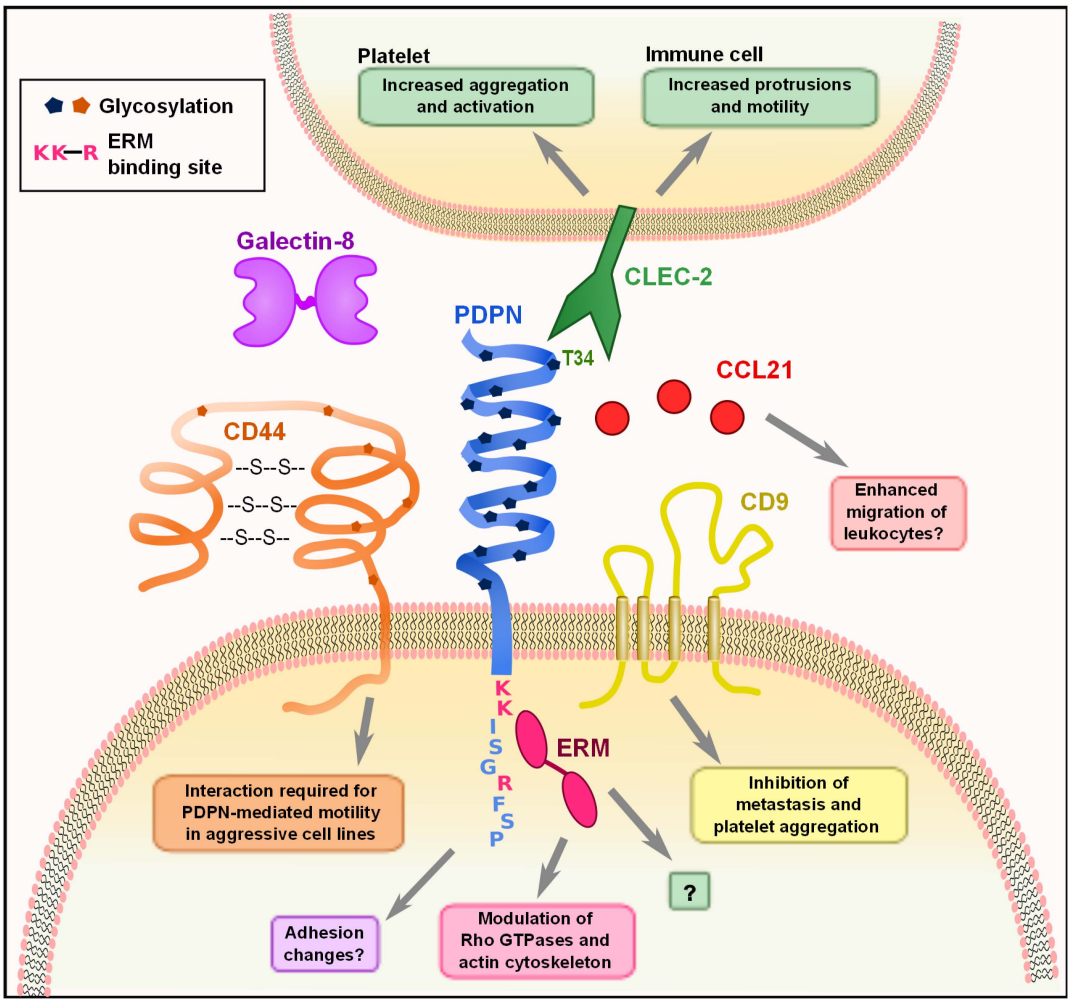

FIGURE 1 | Molecular interactions of PDPN. PDPN interacts with a variety of intracellular and transmembrane proteins to mediate effects on cell migration and adhesion. The binding of PDPN to CD44 or ERMs results in increased cell migration and rearrangement of the actin cytoskeleton to generate actin-rich protrusions of the membrane. The three amino acids colored in pink (K, K, R) are the basic residues requires for ERM protein binding. Interactions between PDPN and CD9 affect metastasis and platelet aggregation. The engagement of PDPN by CLEC-2 causes increased motility in DCs and aggregation and activation of platelets. PDPN binds with high affinity to the chemokine CCL21 and while the consequences of this effect have not been examined, it may play a role in facilitating leukocyte migration. Finally, PDPN binding to galectin-8 may modulate adhesion of LECs.

(Fernández-Muñoz et al., 2011). In these cells, the transmembrane and cytoplasmic domains of PDPN were necessary for association with lipid rafts. Furthermore, manipulation of this localization by substituting the transmembrane domain with that of other proteins inhibited PDPN-mediated increases in EMT, migration, and phosphorylation of ERMs (Fernández-Muñoz et al., 2011). Interestingly, cytoskeletal interactions are not required for PDPN to get into lipid rafts (Barth et al., 2010); however, the cytosolic domain is necessary (Fernández-Muñoz et al., 2011) and one way this might be explained is via interactions with ERMs, given that ezrin is also raft-associated.

Given that the cytoplasmic tail of PDPN is extremely short, it is difficult to imagine that there is much direct signaling downstream of PDPN other than through the ERM proteins, simply due to spatial restrictions. Interestingly, however, PDPN also interacts with two integral membrane proteins that could help to further explain how it affects cell motility and metastasis. CD44, which is widely expressed, affects many cellular functions such as migration and adhesion, and the expression of some isoforms is linked to more invasive cancers. Martín-Villar et al. (2010) noted that CD44 and PDPN were coordinately upregulated in aggressive cancer cell lines and subsequently found that they directly bind to one another. This interaction is dependent on correct glycosylation of the extracellular domain of PDPN, and CD44 expression is required for PDPN-induced cell migration (Martín-Villar et al., 2010). Additionally, Nakazawa et al. (2008) found that PDPN directly interacts with the tetraspanin CD9 through transmembrane domains 1 and 2 of CD9. CD9 acts as a tumor suppressor in many cancers (Zöller, 2009), and co-expression of CD9 and PDPN resulted in a CD9-mediated decrease of PDPN-induced metastasis. CD9 also inhibited PDPN-mediated platelet aggregation without directly interfering with CLEC-2 binding of PDPN

(Nakazawa et al., 2008). This finding indicates that CD9 potentially disrupts CLEC-2 multimerization, which is required for downstream signaling. These interactions provide some insight into how PDPN can exert striking effects on actin cytoskeleton rearrangement, cell motility, and metastasis. Still however, much remains to be elucidated such as the downstream signaling changes that occur upon PDPN binding to CD9 or CD44, how PDPN overexpression results in an increase of ERM phosphorylation, and how that in turn modulates the activity of the Rho family small G proteins.

The only known receptor for PDPN is CLEC-2, a C-type lectin that is expressed by platelets, neutrophils, and dendritic cells (DCs) (Colonna et al., 2000; Sobanov et al., 2001; Kerrigan et al., 2009; Acton et al., 2012). Glycosylation of T34 on PDPN is required for CLEC-2 binding of PDPN. This amino acid resides in the platelet-aggregation stimulating (PLAG) domain, which is highly conserved between PDPN homologues (Kaneko et al., 2006). The effect of CLEC-2 engagement by PDPN has been extensively studied in platelets; however, the effect of this interaction in PDPN-expressing cells has not been addressed. This is an area that warrants further exploration, given that *in vivo*, many PDPN$^+$ cells will be exposed to CLEC-2 signals, whether they are tumor cells interacting with CLEC-2$^+$ platelets or FRCs interacting with CLEC-2$^+$ DCs.

Lymphatic endothelial cells and FRCs, the two major subsets of lymphoid stromal cells, express high levels of PDPN (Malhotra et al., 2012; **Table 1**), but only a few studies have examined the molecular function of PDPN in these cells. PDPN interacts with galectin-8 on LECs, and this interaction is also dependent on PDPN glycosylation (Cueni and Detmar, 2009). Galectin-8 can have varying effects on adhesion depending on whether it is secreted or membrane-bound (Zick et al., 2004); it seems that PDPN binding to galectin-8 may affect LEC adhesion, but additional studies are needed to fully elucidate the consequences of this interaction. PDPN also binds CCL21 with high affinity, and this interaction is also dependent on glycosylation of PDPN (Kerjaschki et al., 2004). This interaction has interesting implications for lymphocyte trafficking, as both LECs and FRCs express CCL21 to direct lymphocyte and DC trafficking to the T zone of LNs (Luther et al., 2000; Bajénoff et al., 2006; Turley et al., 2010).

It has yet to be examined whether the above binding partners of PDPN are cell-type specific or how interaction with one protein affects the binding of PDPN to another interacting molecule. With the exception of the ERMs and CD44, it remains unclear whether PDPN can bind to several of these proteins at one time or whether such interactions might be mutually exclusive. A more global understanding of these various interactions is critical to our overall understanding of PDPN's molecular functions and downstream signaling.

TRANSCRIPTIONAL CONTROL OF PDPN EXPRESSION

Information about the transcriptional control of PDPN first came from the early studies of the role of PDPN in the development of the lymphatic system. The fact that PDPN was specifically expressed on differentiating LECs but not nearby BECs led to the discovery that Prox-1, the major regulator of LEC differentiation, controlled the induction of PDPN (Hong et al., 2002). In fact, forced expression of Prox-1 was sufficient to induce a LEC-like phenotype in differentiated BECs, including the upregulation of PDPN (Hong et al., 2002). Furthermore, it was later found that IL-3, which is involved controlling the differentiation of a variety of hematopoietic cells and is produced by LECs but not BECs, was capable of upregulating Prox-1 and PDPN (Gröger et al., 2004). However, Prox-1 is not expressed in FRCs or in many of the other cells types expressing PDPN. Therefore alternative pathways must be involved in PDPN expression in tissues other than lymphatics. This may be another reason why the physiological functions of PDPN are so varied between different systems.

In skin cancers, osteosarcomas, and gliomas, PDPN is regulated by the AP-1 transcription factor (Durchdewald et al., 2008; Kunita et al., 2011; Peterziel et al., 2012). AP-1 is a heterodimeric complex comprised of Fos and Jun proteins. Both Fos and Jun are critical for progression of many carcinomas, including models of skin carcinogenesis (Eferl and Wagner, 2003). Durchdewald et al. (2008) compared genetic profiles of skin tumors from mice that had either WT Fos expression or Fos specifically deleted in keratinocytes and found that PDPN was one of the most highly upregulated genes in the Fos-sufficient samples. Furthermore, they demonstrated that Fos directly binds to the PDPN promoter. This interaction was further characterized in gliomas, and it was found that PTEN expression, a negative regulator of the PI3K-AKT-AP-1 pathway, was inversely correlated with PDPN expression (**Figure 2**; Peterziel et al., 2012). Furthermore, the PDPN promoter is heavily methylated, which keeps it repressed (Peterziel et al., 2012). Thus, it appears that a major pathway of PDPN upregulation in malignant conditions depends on the activity of Fos and Jun (AP-1) transcription factors.

Finally, there have been two reports of pro-inflammatory cytokines resulting in PDPN upregulation in disease. In rheumatoid arthritis (RA), fibroblast-like synoviocytes are the main mediators of inflammation and tissue destruction and undergo a process resembling EMT during RA progression (Huber et al., 2006). Ekwall et al. (2011) recently reported that while PDPN is absent from the synovium of healthy subjects and patients with osteoarthritis, it is highly upregulated in RA patients. Furthermore, expression of PDPN in cultured synoviocytes is increased upon treatment with IL-1β, TNF-α, or TGF-β1 (Ekwall et al., 2011). Similarly, PDPN upregulation was observed in keratinocytes treated *in vitro* with TGF-β, IL-6, IL-22, or IFN-γ (Honma et al., 2012). The TGF-β-mediated PDPN upregulation required Smad2/3 and 4 signaling, while STAT1 and STAT3 were necessary for IFN-γ signaling and STAT1 was required for IL-6 and IL-22 signaling (Honma et al., 2012).

Overall, it appears that a multitude of stimuli can drive PDPN expression, including normal differentiation factors such as Prox-1 and potentially malignant factors such as pro-tumorigenic signaling pathways and pro-inflammatory cytokines. It is possible that the different pathways controlling PDPN upregulation could result in the activation of distinct downstream signaling pathways and therefore different cellular outcomes. For instance, a tumor cell and a LEC compose two distinct environments with different signaling pathways and molecules active; upregulating PDPN in these distinct signaling milieus would likely have diverse outcomes.

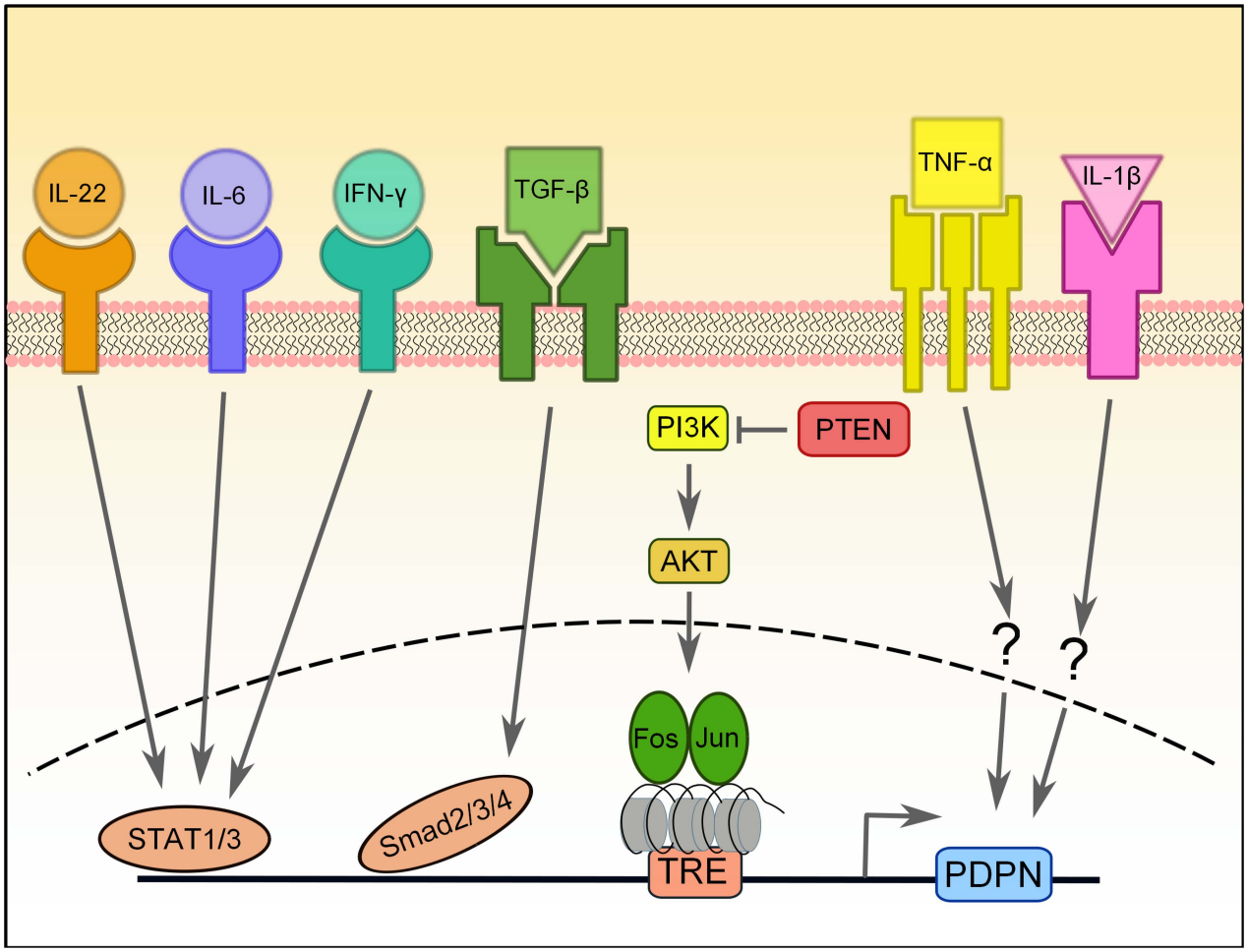

FIGURE 2 | Transcriptional regulation of PDPN expression. PDPN expression can be upregulated by a number of pro-inflammatory cytokines, including IL-22, IL-6, IFN-γ, TGF-β, IL-1β, and TNF-α, but the signaling pathways involved are largely unknown. PDPN upregulation induced by TGF-β requires Smad2/3 and 4 activity, while upregulation induced by IFN-ψ depends on STAT1 and STAT3 and that of IL-6 and IL-22 depends on STAT3. The PI3K-AKT-AP-1 pathway can also induce PDPN expression in brain tumors that have lost the negative regulation normally provided by PTEN. AP-1, a transcription factor comprised of Fos and Jun proteins, binds to the tetradecanoylphorbol acetate-responsive element (TRE) in the promoter of PDPN, which is heavily methylated.

PDPN FUNCTIONS IN THE IMMUNE SYSTEM

While PDPN is a well-established marker for LECs (Wetterwald et al., 1996), FRCs (Farr et al., 1992b), and follicular dendritic cells (FDCs) (Yu et al., 2007) of lymphoid organs, until very recently, no particular function had been ascribed to PDPN in these immune cell populations. Recently, a PDPN-cre mouse was generated, which will be a useful tool in targeting PDPN-expressing stromal cells (Onder et al., 2011). Our lab recently demonstrated that PDPN on FRCs and LECs interacts with CLEC-2 on DCs to promote DC motility from peripheral sites to LNs and within the T cell zone (Acton et al., 2012). We found that murine DCs expressed CLEC-2 and that their migration to draining LNs was impaired when CLEC-2 was deleted. Conversely, siRNA knock down or genetic deletion of PDPN also resulted in impaired DC migration *in vivo* and impeded motility along the FRC network *in vitro*. Furthermore, the interaction between PDPN and CLEC-2 was sufficient to induce protrusion formation in a 3D tissue engineered model. Therefore, both CLEC-2 on DCs and PDPN on stromal cells are necessary for migratory DCs to efficiently reach LNs and initiate immune responses (Acton et al., 2012).

Podoplanin signaling has intrinsic effects on the proliferation, migration, and tube formation of LECs. Navarro et al. (2008, 2010) demonstrated that knocking down PDPN expression *in vitro* inhibited the ability of LECs to properly polarize toward a wound and migrate to close the wound. Reduced PDPN levels also decreased capillary formation when the cells were plated in a deformable 3D matrix (Navarro et al., 2008). These effects were mediated by decreased RhoA activity and increased Cdc42 activity in cells lacking PDPN (Navarro et al., 2010). The mechanism underlying this effect was further investigated by Osada et al. (2012), who found that when LECs were incubated with WT but not CLEC-$2^{-/-}$ platelets, the migration, proliferation, and *in vitro* tube formation of LECs was inhibited. This inhibition was mediated at least in part by BMP9 released in granules

from the platelets upon contact with the LECs (Osada et al., 2012). In contrast, Bertozzi et al. (2010) found that co-culture of platelets with LECs did not affect their viability or proliferation. More work is necessary to determine whether CLEC-2 signals from platelets or other cells provide important signals to LECs *in vivo*.

In addition to its high expression on stromal cells, several recent reports have described PDPN expression on hematopoietic cells, including subsets of T cells and macrophages (Hou et al., 2010; Peters et al., 2011; Kerrigan et al., 2012). Interestingly, in these cases, as in those from cancer studies, PDPN expression is usually correlated with inflammatory or disease settings. In experimental autoimmune encephalomyelitis (EAE), ectopic germinal centers form in the CNS and are believed to accelerate inflammation and disease progression (Weyand et al., 2001). T_H17 cells are particularly important for the formation of these ectopic germinal centers and EAE progression (Jäger et al., 2009). PDPN expression has been reported in ectopic lymphoid tissues in instances of chronic inflammation and cancer (Peduto et al., 2009; Shields et al., 2010; Link et al., 2011), but only on FRC-like stromal cells. Recently, Peters et al. (2011) found that T_H17 cells generated *in vitro* and those found in inflamed CNS tissue of mice with EAE express PDPN (**Table 1**). Administration of a PDPN blocking antibody to mice with EAE did not attentuate disease severity, but significantly reduced the number of ectopic germinal centers induced by T_H17-mediated disease. While the mechanism of PDPN function in T cells is not yet clear, it likely plays an important role in regulating T cell physiology in inflamed tissues.

Podoplanin expression has been observed on some macrophage subsets (**Table 1**). It was first found on F4/80$^+$ macrophages in the red pulp of the spleen. These PDPN$^+$ macrophages exhibited marked phagocytic potential and elevated numbers in mice following systemic zymosan treatment (Hou et al., 2010). PDPN is also expressed by inflammatory macrophages such as thioglycollate-elicited peritoneal macrophages and LPS-treated RAW264.7 cells (Kerrigan et al., 2012). These studies showed that expression of PDPN by macrophages was sufficient to induce CLEC-2-mediated aggregation of platelets *in vitro*. While the *in vivo* functions of PDPN expression by hematopoietic cells have not been fully elucidated, interesting implications abound given what is known about PDPN function in cancer and autoimmunity.

PDPN FUNCTIONS IN CANCER

The setting in which PDPN has been most extensively studied is cancer. Given that it is a specific marker of lymphatic vessels, and that increased lymphangiogenesis is often correlated with poor prognosis in cancer patients, the numbers of PDPN$^+$ vessels in a tumor is often used as a diagnostic marker (Breiteneder-Geleff et al., 1997; Ji, 2006; Swartz and Lund, 2012). Additionally, PDPN is upregulated on tumor cells themselves in several cancer types, including squamous cell carcinoma of the lung, head, and neck (Kato et al., 2005; Martín-Villar et al., 2005; Schacht et al., 2005; Wicki et al., 2006), malignant mesothelioma (Kimura and Kimura, 2005; Ordóñez, 2005), and brain tumors (Mishima et al., 2006; Shibahara et al., 2006). PDPN is often expressed at the leading invasive edge of tumors and appears to play a role in EMT, invasion, and metastasis (Martín-Villar et al., 2006; Wicki et al., 2006). Interactions between CLEC-2 and PDPN in tumors also likely play a role in tumor progression and metastasis due to platelets interacting with tumor cells (Lowe et al., 2012). However, the exact mechanism of PDPN action in tumor cells is still unclear; in some cases, PDPN expression mediates the downregulation of E-cadherin and promotes EMT (Martín-Villar et al., 2006), while in others, PDPN expression enhances tumorigenesis and metastasis in the absence of EMT (Wicki et al., 2006). *In vitro* studies have provided compelling evidence that forced expression of PDPN in cells that normally lack this protein results in a more mesenchymal phenotype, actin-rich filopodia, and increased migration and invasion, as discussed above (Martín-Villar et al., 2005, 2006; Wicki et al., 2006).

Interestingly, PDPN is also upregulated by cancer-associated fibroblasts (CAFs) in the stroma surrounding various tumors, including adenocarcinomas and colorectal cancers (Kitano et al., 2010). There is a wealth of data on the tumor-promoting effects of CAFs, which has been reviewed elsewhere (Kalluri and Zeisberg, 2006; Gaggioli et al., 2007), but only recently have specific functions for PDPN on CAFs been examined. Generally, the expression of PDPN on CAFs is associated with poor prognosis: for example, one study found that invasive adenocarcinomas in the lung had PDPN$^+$ fibroblasts, while non-invasive cases were all negative for PDPN staining (Kawase et al., 2008). Further studies from this group have examined the mechanism by which PDPN enhances the tumor-promoting effects of CAFs. They found that fibroblasts isolated from the vascular adventitia (VAFs) were better at promoting tumor growth than fibroblasts isolated from human lungs. One of the most differentially expressed genes in these cells was PDPN, and knockdown of PDPN in the VAFs abrogated their tumor-promoting effects (Hoshino et al., 2011). Further studies indicated that this activity may be due in part to increased RhoA activity in the PDPN$^+$ fibroblasts (Ito et al., 2012).

While these studies illustrate that PDPN expression in CAFs is linked to poor prognosis for patients, it is important to keep in mind that the effect of PDPN$^+$ CAFs likely depends on the type of tumor cells and the tissue from which the CAFs originate. In fact, one study of colorectal CAFs found that PDPN expression was correlated with a better prognosis (Yamanashi et al., 2009). Knockdown of PDPN in CAFs resulted in enhanced cancer cell migration in a transwell assay. Furthermore, PDPN expression was seen in stroma surrounding the tumors in many areas except at the invasive front (Yamanashi et al., 2009). Thus, it was postulated that PDPN expressing stroma could act as a physical barrier to tumor cell invasion into surrounding tissues. In fact, this theory has been presented elsewhere and for other mucins (Zimmer et al., 1999). The negative charge of the many sialic acids on these proteins acts to repel other molecules such as complement (Michalek et al., 1988; Meri and Pangburn, 1990) and can affect cell adhesion (Taylor and Drickamer, 2007). Whether these properties play a role in PDPN function has not been definitively examined but it is an attractive hypothesis, given that PDPN is expressed on the apical surface of many cells that have contact with proteinase-rich fluids (i.e., lymph).

While it is clear that PDPN plays an important role in tumor progression and metastasis, more mechanistic studies are needed to fully elucidate the function of this molecule. Furthermore, a genetic dissection of PDPN function in malignant cells versus in the surrounding tumor stroma will significantly advance our understanding of this molecule in cancer.

CONCLUSION

Emerging studies of PDPN suggest that this molecule plays diverse roles throughout the body. It is involved in the development of the heart, lung, and lymphatic system as well as driving inflammatory diseases and metastasis. The majority of mechanistic data available on the cellular functions of PDPN come from studies of cancer progression and metastasis. Overexpression of PDPN in various cell lines results in increased motility and a mesenchymal phenotype *in vitro* and increased metastasis *in vivo*. These changes occur through the interaction of PDPN with ERM proteins and subsequent modulation of the Rho proteins and actin cytoskeleton. While these studies are indispensable to our understanding of how PDPN functions, it is also critical to examine PDPN in physiological settings, which we have begun to do only recently. Studies of PDPN on LECs and FRCs have indicated that it plays a critical role in mediating interactions with platelets and DCs; however these studies have largely focused on the effects of CLEC-2 engagement of PDPN rather than downstream effects in the PDPN-expressing cell. Furthermore, recent studies of PDPN expression by leukocytes have demonstrated that PDPN expression has intrinsic effects on these cells as well as tumor cells.

There are still many unknowns about PDPN biology that remain to be answered, but there are three pressing questions in the field: (1) What signaling pathways does endogenously-expressed PDPN employ? It is possible that expression of PDPN in leukocytes leads to similar downstream changes as in tumor cells; however, it is likely that PDPN interacts with different molecules and signaling pathways in stromal cells and leukocytes than in malignant cancer cells. (2) What are the effects of CLEC-2 engagement of PDPN? This interaction has been almost exclusively studied with respect to signaling downstream of CLEC-2. However, in nearly every instance where PDPN is expressed, whether by FRCs or cancer cells, there will be CLEC-2^+ cells in the nearby environment, including DCs or platelets. Given that overexpression of PDPN has striking intrinsic effects on various cells, it stands to reason that there could be some effect on PDPN signaling when it is bound by CLEC-2. (3) What are the consequences of deleting PDPN from cells that endogenously express it? This question has been partially answered by studies of the developing heart, lungs, and lymphatic system, but research has been limited by the lack of a conditional PDPN knockout mouse. Once this tool is generated, we will be able to more closely examine the effects of PDPN in adult animals and in specific tissues or cells. These studies will provide critical insight into whether PDPN is necessary only during embryonic development or into adulthood for proper development and maintenance of organs. Furthermore, we can study how the deletion of PDPN in macrophages or T cells affects disease progression. A better understanding of these open questions will lead to great insights in the diverse fields of development, cellular interactions in the immune system, and cancer progression and metastasis.

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Follicular dendritic cells in health and disease

Mohey Eldin M. El Shikh* and Costantino Pitzalis

Centre for Experimental Medicine and Rheumatology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Kang Liu, Columbia University, USA
Marie Kosco-Vilbois, NovImmune SA, Switzerland

****Correspondence:***
Mohey Eldin M. El Shikh, Centre for Experimental Medicine and Rheumatology, 2nd floor, John Vane Science Centre, William Harvey Research Institute, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK.
e-mail: m.elshikh@qmul.ac.uk; memelshikh@gmail.com

Follicular dendritic cells (FDCs) are unique immune cells that contribute to the regulation of humoral immune responses. These cells are located in the B-cell follicles of secondary lymphoid tissues where they trap and retain antigens (Ags) in the form of highly immunogenic immune complexes (ICs) consisting of Ag plus specific antibody (Ab) and/or complement proteins. FDCs multimerize Ags and present them polyvalently to B-cells in periodically arranged arrays that extensively crosslink the B-cell receptors for Ag (BCRs). FDC-FcγRIIB mediates IC periodicity, and FDC-Ag presentation combined with other soluble and membrane bound signals contributed by FDCs, like FDC-BAFF, -IL-6, and -C4bBP, are essential for the induction of the germinal center (GC) reaction, the maintenance of serological memory, and the remarkable ability of FDC-Ags to induce specific Ab responses in the absence of cognate T-cell help. On the other hand, FDCs play a negative role in several disease conditions including chronic inflammatory diseases, autoimmune diseases, HIV/AIDS, prion diseases, and follicular lymphomas. Compared to other accessory immune cells, FDCs have received little attention, and their functions have not been fully elucidated. This review gives an overview of FDC structure, and recapitulates our current knowledge on the immunoregulatory functions of FDCs in health and disease. A better understanding of FDCs should permit better regulation of Ab responses to suit the therapeutic manipulation of regulated and dysregulated immune responses.

Keywords: follicular dendritic cell (FDC), antigen presentation, B-cells, germinal center reaction, T-cell-independent, Fc receptors, complement, autoimmunity

INTRODUCTION

In 1968, Andras K. Szakal and Michael G. Hanna, Jr. (Szakal and Hanna, Jr., 1968) from the Oak Ridge National Laboratory, Tennessee, USA; and Gustav J. Nossal et al. (Nossal et al., 1968) from the Walter and Eliza Hall Institute in Melbourne, Australia, published the first electron micrographs and description of FDCs. A number of names have been used, but a committee on nomenclature recommended the name "Follicular Dendritic Cell" and the abbreviation "FDC," and this has been generally adopted (Tew et al., 1982).

The characteristics that distinguish FDCs from other cells of the immune system are their ability to retain antigen (Ag)-antibody (Ab)-complement complexes (i.e., immune complexes, ICs) long-term on their surfaces and their follicular localization. Unlike other immune accessory cells, FDCs lack phagocytic activity, lysosomes, lysozyme, and Birbeck granules. FDCs localized to the follicles of secondary lymphoid tissues form interactive networks or reticula of non-mobile Ag-bearing cells. Immobile FDCs in these FDC-reticula engage the mobile B and T-cells and other mobile cells trafficking through the follicles (Szakal et al., 1985; El Shikh et al., 2010).

FDCs in germinal center (GC) light zones of B-cell follicles (**Figures 1** and **2**) retain ICs via the complement receptors (CR1/CR2—CD35/CD21), the low affinity immunoglobulin gamma Fc region receptor II-B (FcγRIIB/CD32) and the low affinity immunoglobulin epsilon Fc region receptor II (FcεRII/CD23). In addition, FDC dendrites bear complement 3 (C3) fragments (iC3b, C3d, and C3dg) attached to the ICs and adjacent cell membranes via ester linkages at the FDC-B-cell interface. Engagement of BCRs with FDC-retained Ags is critical for GC development, B-cell survival, Ig class switching, production of B memory cells, somatic hypermutation (SHM), selection of somatically mutated B-cells with high affinity receptors, affinity maturation, induction of secondary Ab responses, and regulation of serum IgG and IgE levels. Moreover, FDCs exert a pivotal role in organizing the lymphoid micro-architecture, and abrogation of FDC networks as a consequence of ablation of Lymphotoxin (LT) trimers and Tumor Necrosis Factor-α (TNF-α), either through homologous recombination or by pharmacological means, abolishes the sophisticated lymphoid architecture compartmentalization (Kosco-Vilbois, 2003; Allen and Cyster, 2008; El Shikh et al., 2009b, 2010).

FDCs multimerize intact monomeric proteins and present Ags in ICs to B-cells with regular periodicity *in vivo* and *in vitro*. ICs trapped via FDC-FcγRIIB in the absence of complement are displayed in a periodic manner with 200–500 Å spacing between epitopes (Szakal et al., 1985; Sukumar et al., 2006b, 2008). In contrast to proteolysis and the MHC-restricted peptide presentation by DCs to T-cells, and similar to T-cell-independent (TI) type 2 Ags and higher order protein arrays, FDCs display native monomeric

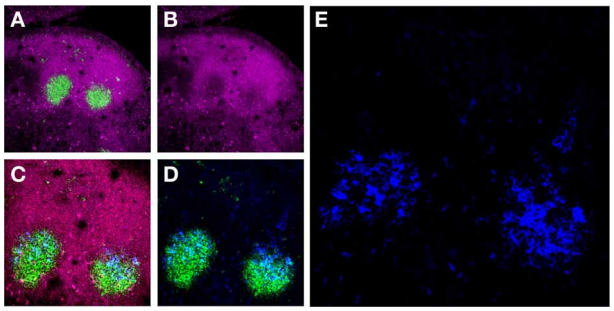

FIGURE 1 | Murine FDCs in lymph node (LN) GCs. (A) Activated GC B-cells labeled with the mAb GL7 (green) are surrounded by a mantle of CD45Rhi (magenta) resting B-cells in two adjacent secondary B-cell follicles in the draining LNs of an immunized mouse. **(B)** These GC B-cells downregulate CD45R expression upon activation. **(C** and **D)** The activated GC B-cells are intimately associated with FDC Ag retaining reticulum (blue). **(E)** High magnification of the FDC reticulum retaining AMCA-conjugated ovalbumin (blue).

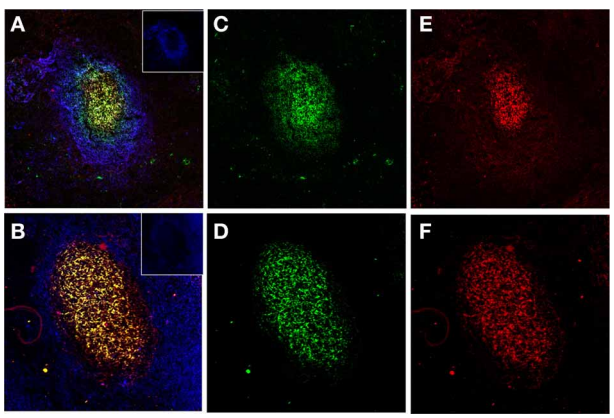

FIGURE 2 | Human FDCs in tonsillar sections trapping IgG/C3-opsonized Ags in ICs. (A and **B)** Two B-cell follicles surrounded by resting IgD+ mantle B-cells (blue, insets in the right upper corner). The overlay of FDCs in the follicles (labeled green with CNA.42 mAb) and the FDC-trapped ICs (labeled red with anti-human IgG in the upper panel and anti-C3 in the lower panel) is orange-yellow. **(C** and **D)** FDCs labeled green with the FDC-specific mAb CNA.42. **(E)** Human IgG in the FDC-trapped ICs labeled red with anti-human IgG. **(F)** Human C3 in FDC-trapped ICs labeled red with anti-human C3.

proteins as multimerized ICs with repeating immunogenic epitopes thus allowing extensive BCR cross-linking and induction of rapid B-cell activation, proliferation, GC formation, and Ig secretion to T-cell-dependent (TD) Ags in a TI manner. In addition to this FcγRIIB-dependent Ag presentation, FDC-derived B-cell Activating Factor of the TNF family (BAFF) and C4b-binding protein (C4bBP) are critical for TI B-cell activation (El Shikh et al., 2009a, 2010).

Engagement of BCRs by free Ag can induce B-cell activation; however, an immunogen is converted into ICs as soon as Ab is produced in a primary response, and instantaneously in recall responses by Ab persisting from previous immunizations. IgG-ICs coligate BCR with FcγRIIB and inhibit B-cell activation via the tyrosine-based inhibitory motif (ITIM). In contrast, IgG-ICs trapped by FDC-FcγRIIB do not engage B-cell-FcγRIIB and consequently ITIM-mediated signaling is minimized (Tew et al., 1976, 2001; Qin et al., 2000; Aydar et al., 2003, 2004b; El Shikh et al., 2006).

Not only FDCs provide the necessary Ag for B-cell stimulation but they orchestrate the removal of self-Ags released from apoptotic cells in the GC environment. FDCs secrete the bridging factor Mfge8, which crosslinks apoptotic cells in the GCs and the phagocytic "tingible body macrophages" (TBMs). This promotes selective debris removal and engulfment of dead cells. Mice lacking LT or LT receptors, which are devoid of FDCs, or lacking the bridging protein Mfge8 itself, are significantly susceptible to autoimmunity suggesting the role of FDCs in protection against autoimmunity by expediting the removal of potentially self-reactive debris from the GCs (Kranich et al., 2008).

Beyond their immunoregulatory functions, FDCs are critically involved in the pathogenesis of several diseases. FDCs are important HIV reservoir where FDC-trapped HIV persists for long periods and can reignite infection and perpetuate the disease (Smith et al., 2001). Moreover, FDCs are the essential sites of prion replication in lymphoid tissues (McCulloch et al., 2011), and they provide a microenvironment that supports B-cell lymphomas and establish a protective sanctuary for malignant cells from otherwise effective drugs (Lee et al., 2012).

In addition, several lines of evidences support the critical role of FDCs in the pathogenesis of autoimmune diseases. Chronic inflammatory autoimmune disorders frequently display follicles with IC-bearing FDCs, autoreactive GCs, and ongoing affinity maturation (Aloisi and Pujol-Borrell, 2006; Manzo et al., 2010). Moreover, interference with FDC reticula attenuates autoreactive GC formation, reduces pathogenic auto-Ab titers and memory B lymphocytes and ameliorates arthritis (Gray and Skarvall, 1988; Gray, 1993; Barrington et al., 2002; Anolik et al., 2008; Victoratos and Kollias, 2009; Vinuesa et al., 2009; Manzo et al., 2010).

In this review we address the current knowledge about the structure and function of FDCs and their role in immune regulation in health and disease.

FDC MORPHOLOGY, DEVELOPMENT, Ag RETENTION, AND ISOLATION

FDC MORPHOLOGY

Light and electron microscopy studies of FDCs have revealed structural details that help clarify FDC functions. FDCs are slightly larger than lymphocytes and possess many fine dendritic processes. These dendrites extend and intimately interact with neighboring cells, creating a unique microenvironment. This intimate interaction with surrounding lymphocytes appears to be important for FDCs to provide potent signals that promote humoral immune responses. Scanning electron microscopy (SEM) has identified two morphological types of FDCs: one having filiform or finger-like processes, and one with "beaded" dendrites. The transition from one form of FDC into another appears to be related to the formation of a specialized Ag delivery system. The resulting "beads" are called "iccosomes" to denote that they consist of immune complex-coated bodies

(Schnizlein et al., 1985; Szakal et al., 1985; Sukumar et al., 2006b, 2008; El Shikh et al., 2007a).

FDCs possess a scanty cytoplasm with few mitochondria, rough endoplasmic reticulum, a Golgi apparatus, and vesicles. They have irregular, sometimes bilobed, euchromatic nuclei containing distinct nucleoli. The dendritic processes of FDCs appear in two general forms: some are attenuated, with folds and intermittent thickenings that form a variety of differently shaped cytoplasmic extensions, while others form more uniform, highly convoluted, labyrinthine configurations. These dendritic processes interdigitate with one another and form a vast network or reticulum on which ICs are trapped (Schnizlein et al., 1985; Szakal et al., 1985; Sukumar et al., 2006b, 2008; El Shikh et al., 2007a).

FDC ORIGIN, DEVELOPMENT, AND LYMPHOID TISSUE ORGANIZATION

The cellular origin of FDCs is still controversial and various cell types have been proposed as a possible origin of FDCs. FDCs, at least in their mature form, are stationary, however, the possibility of an inflow of earlier progenitors of FDCs from other sites could not be fully excluded (Kasajima-Akatsuka and Maeda, 2006).

At present there are data supporting a haematopoietic origin and more data supporting a stromal cell origin. FDCs are radiation resistant making it difficult to study development using adoptive transfer models. Information available on FDCs is based primarily on studies using humans and rodents. However, FDCs are present and functional in birds and if FDCs are defined broadly as cells with the ability to trap ICs, they appear to exist in all jawed vertebrates, including amphibians, reptiles, and fish (El Shikh et al., 2009b).

Using mice homozygous for the SCID mutation, which lack T, B lymphocytes, and FDCs, demonstrated that after reconstitution with bone marrow from donor mice, the FDCs of the reconstituted mice expressed the donor phenotype. These authors concluded that FDC precursors came from bone marrow (Kapasi et al., 1993a,b, 1994; Szakal et al., 1995a,b; Kapasi et al., 1998).

On the other hand, stromal cells expressing FDC markers have been described in several independent studies, and seem to be present in the splenic white pulp before the development of functionally and ultrastructurally recognizable FDC. Using an *in silico* subtraction approach, gene expression of FDCs was determined and compared with that of follicular stromal cells microdissected from the spleen of SCID mice and a remarkably close relationship in gene expression patterns was found (Wilke et al., 2010). However, one of the major limitations in the study of FDC origin is the paucity in markers specific for the various stages of FDC maturation that would allow for discriminating FDC precursors from B-cells as well as from other stromal cells (Aguzzi and Krautler, 2010; Wilke et al., 2010).

Recent transcriptome analysis showed that FDCs express many mesenchyme-associated genes suggesting that FDCs are specialized mesenchymal cell population within the GCs of lymphoid tissues (Mabbott et al., 2011). It was also suggested that cytokines from lymphocytes and macrophages involved in inflammatory process may be responsible for differentiating stromal cells into a FDC phenotype (Cho et al., 2012a).

Another recent study has suggested a mechanism of FDC development that involves both resident and migratory cells. Specifically, it was proposed that a FDC is generated by a cell fusion event between a stromal cell and a migratory $CD35^{+}B220^{+}$ precursor cell, which is consistent with several observations of binucleate FDCs (Murakami et al., 2007; Allen and Cyster, 2008). Moreover, differentiation of FDCs as a specialized form of myofibroblasts that derive from bone marrow stromal cell progenitors has been also suggested (Munoz-Fernandez et al., 2006; Sipos and Muzes, 2011).

TNF and the related molecule LT are essential for FDC development, and mice deficient in these cytokines, their receptors, or associated downstream signaling molecules fail to properly develop FDCs and GCs in secondary lymphoid organs. Through irradiation chimera and adoptive transfer experiments, it was established that TNF and LT were required on lymphocytes, specifically B-cells for normal FDC development. The differential role of soluble and membrane bound TNF in FDC development has been also investigated with more significant role of soluble TNF in FDC development in primary follicles and the membrane-bound TNF form in FDCs of the GCs (Allen and Cyster, 2008; Tumanov et al., 2010).

FDCs help maintain primary follicles as a B-cell exclusive niche and they act to retain and promote the survival of GC B-cells within GCs. Within two days of FDC ablation, primary B-cell follicles lose their homogeneity and become disorganized bands of cells around T zones. Ablation of FDCs during the GC response causes rapid GC B-cell dispersal, death, and disappearance of the GCs (Wang et al., 2011).

The cardinal feature of FDCs is the surface retention of native Ags for extended periods of time and presentation of these Ags together with costimulatory signals to B-cells during normal and abnormal immune responses. This unique property of Ag retention and presentation by FDCs: (1) occurs in different sites of secondary lymphoid tissues, including the spleen, LNs, and mucosa-associated lymphoid tissues (MALT); (2) can be induced in tertiary lymphoid tissues in different organs as a consequence of chronic inflammatory and autoimmune reactions; (3) depends on Ag retention on FDCs, which is directly associated with different Ag transport mechanisms in secondary and tertiary lymphoid tissues; (4) can be induced in cells of haematopoietic and stromal origin under physiological and pathological conditions *in vivo* and *in vitro*. Consequently, the origin of FDCs may be differentially regulated in different anatomical sites, under different sets of microenvironmental conditions, and by the cells and routes that deliver the Ag to the FDC reticulum. Our knowledge is still expanding in the field of FDC biology and more needs to be investigated in the processes of FDC differentiation and the mechanisms of Ag transport to their reticula in different anatomical locations before a final conclusion can be drawn on their origin.

Ag TRANSPORT TO THE FDC RETICULUM

A notable feature of the FDC network is that it is located centrally in the follicle and typically does not extend to the subcapsular sinus (SCS), interfollicular regions or T-cell zone. The logic for this separation of FDCs from the sites of earliest Ag capture has not been defined but it prompted studies on the mechanisms

of Ag transport to the FDC reticulum dating back to the early 1980s.

Perhaps being centred in the follicle and not in substantial contact with macrophages, DCs, or circulatory fluids provide a protected environment in which opsonized Ags can be displayed for long periods without being proteolysed or removed by phagocytic cells. However, the physical isolation of FDCs in follicles necessitates mechanisms for Ag to travel from the first point of capture to the FDC (Cyster, 2010).

In the early 1980s, elegant light and electron microscopic studies indicated that as early as 1 min after Ag injection in passively immune mice, localization of Ag occurs at distinct sites in the SCS and subjacent areas of the cortex on the afferent side. At these sites, between 1 min and 24 h, the Ag forms light microscopically identifiable trails which reaches progressively deeper into the cortex with time toward individual follicular regions. By 24 h this apparent migration of Ag is complete, and the Ag is localized in follicles. Electron microscopy indicated that the apparent migration of ICs was mediated by a group of cells observed in the migration path that had ICs sequestered on their surface or in plasma membrane infoldings. These Ag transporting cells (ATC) were relatively large non-phagocytic cells, with lobated or irregular euchromatic nuclei and cell processes of various complexities. ATCs observed in or near the SCS appeared to be less differentiated, whereas those located deeper in the cortex, appeared more differentiated, interdigitated with Ag-retaining dendritic cells, and shared morphologic characteristics with FDC (Szakal et al., 1983).

More recently, it was reported that large MW proteins (>60 kDa) injected into passively immune mice form ICs that activate complement, resulting in covalent attachment of C3 (C3-IC). SCS macrophages (SCSM) have a low rate of Ag internalization and degradation (Cyster, 2010), and the C3-coated ICs are shuttled via the SCSM-CR3 onto naïve B-cell-CR1/2-CD32 that migrate to the FDC reticulum and off-load the ICs to the FDC-CR1/2-CD32. In the absence of pre-existing specific Abs, innate recognition proteins such as natural IgM; C-type lectins such as MBL and ficolins; pentraxins, including C-reactive protein; and other complement activators could effectively bind the foreign protein and activate complement, then the C3-coated ICs follow the SCSM-CR3/naïve B-cell-CR1/2-CD32/FDC-CR1/2-CD32 transport mechanism. C3-coated smaller MW proteins access the B-cell follicles via the follicular conduits that intersect with FDCs, providing a direct connection for C3-coated Ags to bind to the FDC surface via CR1/2-CD32. DCs residing in the LN medulla may also capture C3-opsonized Ags and hand off the complexes to FDCs in the B-cell follicle (Gonzalez et al., 2011).

It has been also reported that SCSM themselves transport ICs directly to FDCs after immunization with Ag in adjuvant (Cyster, 2010), and the strategic positioning of FDCs around follicular conduits in the B-cell follicles of the LNs gives them direct access to capture soluble Ags draining from subcutaneous sites even in the absence of Ag-specific Abs (Bajenoff and Germain, 2009).

Both marginal zone (MZ) B and B-1-cells help reorganization of FDC networks (Ferguson et al., 2004; Lopes-Carvalho et al., 2005) and B-1-cells promote the development of FDCs reticula (Wen et al., 2005). The continuous shuttling of MZ-B-cells to B-cell follicles has been recently documented and the potential interaction between MZ-B-cells and Ags in ICs on FDCs has been proposed (Cinamon et al., 2008; MacLennan, 2008). In the spleen, MZ-B-cells that express relatively high levels of CD21 and CD35 pick up C3-ICs from the sinus and deliver the complexes to FDCs as they migrate though the follicles (Cinamon et al., 2008; MacLennan, 2008; Gonzalez et al., 2011). Natural and specific Abs differentially regulates Ag transport in the spleen. Particulate proteins expressed on virus-like particles were transported efficiently to murine splenic FDCs in the absence of prior immunity via natural IgM Abs and complement, whereas soluble proteins required Ag-specific IgM or IgG Abs (Link et al., 2012).

FDC ISOLATION AND *in vitro* GC REACTIONS

Major challenges delayed the systematic analysis of FDC functions in health and disease. Retrieval and characterization of FDC-retained Ags, isolation of the Ag-retaining FDCs, detection of picogram quantities of Ags in GCs, and the lack of *in vitro* models for GC reactions represented major technical difficulties in investigating the biology of FDCs. The current development of FDC isolation techniques with >90% purity (Sukumar et al., 2006b), and methods for setting up GC reactions *in vitro* (El Shikh et al., 2007b, 2009a; Wu et al., 2008, 2009); where the influence of Ags, ICs, FDC-, T-cell-, and B-cell-regulatory molecules can be manipulated at will; provide a pathway to analyse the molecular interaction between the cellular and molecular components of the GC reaction. Based on the lack of CD45 expression, a recent study described a novel method for FDC isolation from the spleen of naive mice by flow cytometry. The isolated FDCs, which accounted for ∼0.2% of the spleen cells of naive mice, were $CD45^-$, FDC-$M2^+$, and ICAM-1^+, and supported the survival and LPS-induced proliferation of B-cells via FDC-BAFF-dependent pathway (Usui et al., 2012).

FDCs are fragile cells and are tightly associated with B-cells which hamper the process of FDC isolation. Several FDC lines were established to overcome these problems, however, as these cells are maintained over several weeks in culture, their phenotype no longer reflects the *in vivo* situation (Aguzzi and Krautler, 2010; Wilke et al., 2010). FDCs require constitutive LT-R-mediated stimulation from surrounding lymphocytes to maintain their maturation status. In the absence of this stimulation FDC rapidly dedifferentiate (Mabbott et al., 2011).

Artificial engineering of lymphoid tissue equivalents is an emerging field that aims to provide models for therapeutic applications of vaccines, and drugs used in the treatment of chronic inflammation, autoimmunity, and cancer. Being the hotspot of Ab production, more work on developing *in vitro* GC reactions is critical for these artificial lymphoid tissues (Tan and Watanabe, 2010).

T-CELL-DEPENDENT (TD) AND INDEPENDENT (TI) ANTIGENS, THE GERMINAL CENTER REACTION (GCR), AND THE IMMUNOREGULATORY FUNCTIONS OF FDCs

FDCs critically regulate Ag presentation to B-cells in the GCR. In the following section we will introduce the types of Ags and the

GCR before we discuss the specific immunoregulatory functions of the FDCs.

TD AND TI Ags

Long before T and B lymphocytes were discovered, it was known that certain Ags of bacterial origin were fundamentally different from protein Ags. Lipopolysaccharide (LPS), for example, was found to be immunogenic at very low concentrations and even 1000 molecules could induce a detectable immune response dominated by 19S Abs (IgM) (Moller, 1965; Landy and Baker, 1966).

With regard to their capacity for Ab-induction, Ags are classified as T-cell dependent (TD) or T-cell independent (TI). In the immune responses to TD Ags, provision of cognate T-cell help to B lymphocytes is essential for B-cell proliferation, immunoglobulin (Ig) secretion, class switch recombination (CSR), affinity maturation, and memory B-cell generation. On the contrary, TI Ags induce B-cell activation and Ab production without T-cell help. TI Ags are further divided into TI type 1 Ags (TI-1), which are polyclonal B lymphocyte activators, and TI type 2 (TI-2) Ags which are classically polysaccharides fit for extensive crosslinking of multiple BCRs.

Protein Ags without repetitive epitopes require cognate T-cell help to induce high affinity B-cell immunity. These TD Ags are processed and presented by Ag presenting cells (APCs) which activate Ag-specific helper T (Th) cells in the T-cell zones of secondary lymphoid tissue. Activated Th cells expand and differentiate into effector Th cells that regulate the development of Ag primed B-cells. Under Th cell control, these primed B-cells can switch Ig isotype, terminally commit to the plasma cell pathway or enter the GCR to experience cycles of SHM, affinity maturation, selection of high affinity B-cells, and memory B-cell generation. Ag-specific Th cells contribute to the GCRs where they critically regulate the affinity-based selection of memory B-cells. A long-lived memory Th cell compartment is established in the immune responses to TD Ags, where Ag rechallenge induces exaggerated cellular expansion in the memory B and T-cell compartments (Parker, 1993; McHeyzer-Williams et al., 2003; Jeurissen et al., 2004).

A complex mixture of TD Ags (peptides and proteins) and TI Ags (glycolipids and complex polysaccharides) are present in microorganisms (Leyva-Cobian et al., 1997). Pathogen-associated TI-1 Ags are polyclonal B-cell activators most of them are derived from components of the cell membranes, the cytosol, or excretion/secretion products of bacteria, viruses, and parasites. The Abs secreted by B-cells stimulated with polyclonal activators are non-specific and recognize heterologous as well as homologous Ags. The molecular mechanisms triggered by polyclonal activators derived from microorganisms have not been elucidated completely; however, several lines of evidences support that these molecules are able to activate B-cells through TLRs. For example, DNA or oligodeoxynucleotides containing CpG motifs [CpG-oligodeoxynucleotide (ODN)] act via the intracellular receptor TLR-9. Conversely, LPS in complexes with the LPS-binding protein (LPSBP) is captured by CD14 which then associates with the TLR-4-myeloid differentiation protein 2 complex on B-cells. This initiates signaling pathways, which involve MyD88, and also leads to the activation of NF-κB (Baumgarth, 2000; Montes et al., 2007). Induction of TI GCRs by certain pathogen-associated Ags can promote expansion of B-cell clones expressing V regions selected over evolutionary time to recognize common multivalent structures on the surface of such pathogens. Because SHM does not take place efficiently in TI GCRs, the specificity of the selected V regions would not be altered, and such a process would not pose a threat to self tolerance (Manser, 2004).

TI-2 Ags are classically large MW polysaccharides displaying repeating immunogenic epitopes with poor biodegradability, and complement fixation ability. These Ags can crosslink BCRs and stimulate Ab production by mature B-cell in athymic nude mice lacking MHC class II restricted T-cell help [detailed in (Dintzis et al., 1976, 1982; Brunswick et al., 1988, 1992; Mond et al., 1995; Sulzer and Perelson, 1997; de Vinuesa et al., 2000; Vos et al., 2000; Lentz and Manser, 2001; Zubler, 2001)].

In spite of their chemical diversity, structural analysis of classic TI-2 Ags has shown that all share a minimal MW of 100 kDa and repetitive epitopes that are expressed on a stable backbone with a two-dimensional epitope spacing of 95–675 Å. An extended length of the backbone of 460 nm, carrying 48 epitopes engaging 10–50 mIg receptors forming a small number of highly crosslinked clusters of mIg molecules with 14-fold reduction in the diffusion coefficient of the bound mIg receptors is critical for delivering the BCR-mediated signal in TI-2 responses (Vos et al., 2000).

In view of recent studies, the operational characteristics of TI-2 Ags have been extended beyond classic polysaccharides. Reports on the requirements for generation of Ab responses to repetitive determinants on polymers and higher order structures such as viral capsid proteins indicated that high MW arrays of Ag are efficient in eliciting Ab responses independent of T-cell help, whereas their less ordered counterparts are less immunogenic and require T-cell help (Dintzis et al., 1983; Bachmann and Zinkernagel, 1997). Data from several viral models including, Coxsackie, influenza, foot-and-mouth disease, vesicular stomatitis, and Sindbis viruses, indicate that virus-specific IgM responses are mounted when athymic nude, TCRα- TCRβ- TCRαβ- and TCRαβγδ-knockout mice are infected. All of the antiviral TI Ab responses reported so far are directed to viral Ags repetitively displayed in the virions (Szomolanyi-Tsuda and Welsh, 1998). Moreover, Abs bound to strictly ordered, but not to irregularly arranged, viral Ags dramatically enhanced the induction of anti-Abs after a single immunization and without using adjuvants (Fehr et al., 1997). Similar to TI-2 Ags, and higher order protein arrays on viral capsids, the ability of FDCs to retain ICs in a periodic manner allows multimerization of monomeric proteins that generally express only a single copy of each antigenic determinant and facilitates the multivalent presentation of these determinants in an array fit for crosslinking of multiple BCRs and induction of Ab responses in the absence of T-cell help (El Shikh et al., 2009a, 2010).

A clinically important group among the TI-2 Ags are the bacterial capsular polysaccharides. Capsular polysaccharides of *Streptococcus pneumoniae*, *Haemophilus influenzae,* and *Neisseria meningitidis* are responsible for the bacterial virulence; and Abs

to capsular polysaccharides provides protection against invasive infections with these bacteria. Maturation of the immune response to TI-2 Ags in humans is delayed. The delay in Ab formation to encapsulated bacteria renders infants and young children highly susceptible to infections with encapsulated bacteria, especially from the age of 4 to 6 months on, when the placenta-derived maternal IgG is metabolized. Therefore, children younger than 2 years of age are more at risk for invasive infections caused by encapsulated microorganisms (Jeurissen et al., 2004).

TI B-cell activation is further discussed in the immunoregulatory functions of FDCs.

THE GCR

GCs are highly structured tissue microdomains in activated B-cell follicles wherein B lymphocytes undergo clonal expansion, CSR, Ab gene diversification, and affinity maturation.

GCs were first observed in 1884 by Flemming and his associates who concluded that GCs were one of the major sources of lymphocytes throughout the body. Though the name given to the sites of intense proliferation in the follicles is still used, we now know that the conclusion that the cells originate/germinate within the GC is not true. The term "reaction" in GCR is used to stress the reactive nature of the GC response, i.e., the development (and decline) of a GC as a result of antigenic stimulation (Nieuwenhuis and Opstelten, 1984).

GCRs differ slightly from organ to organ and from species to species. GCs also form in atypical sites (referred to as tertiary lymphoid tissues) such as in the stomach during *Helicobacter pylori* infections, the eye during Chlamydia infections, blood vessels in atherosclerosis, joints in rheumatoid arthritis, the thyroid in Hashimoto's thyroiditis, and the lung in the chronic inflammatory diseases of the respiratory system (Kosco-Vilbois et al., 1997). GC formation is restricted to homoiothermic animals. Associated with the ability to produce GCs in their lymphoid tissues, birds, and mammals produce Abs of heterogeneous affinity, increasing in the secondary as compared to the primary response. The characteristic fast rise of serum Ab after a secondary injection of Ag may provide a survival advantage in the defence against pathogens that multiply more rapidly at the higher body temperature (Thorbecke et al., 1994; Manser, 2004). Furthermore, aging is associated with defective GCRs associated with abnormal functions of the GC cellular components (Kosco et al., 1989; Szakal et al., 1990, 1992, 2002; Smith et al., 1991; Tew et al., 1993; Thorbecke et al., 1994; Zheng et al., 1997; Aydar et al., 2002, 2003, 2004a).

GCs arise in primary lymphoid follicles of secondary lymphoid tissues. The sites that become involved are those receiving the antigenic stimulus via drainage of lymph, blood, or specialized cells in the epithelium called "M" cells. The GCR is a complex process involving numerous cellular and cell surface components together with multiple signaling pathways (Nieuwenhuis and Opstelten, 1984; MacLennan, 1994; Kosco-Vilbois et al., 1997; Cozine et al., 2005; Vinuesa and Cyster, 2011).

The primary B-cell follicle contains a mixture of recirculating naïve and memory B-cells. Upon B-cell activation in the T-cell zone, a few committed Ag specific B-cells migrate into the follicle and expand clonally. Several studies indicated that low numbers of Ag-specific B-cells can be seen within the follicles in the primary response as early as 36 h post-immunization. Three to four Ag-specific B-cells colonize a follicle and go through repeated rapid cell cycles increasing some 4000-fold in around three days. In doing this, they fill the spaces between the network of FDCs. Within 72–84 h of exponential growth the cells differentiate to form a mature GC chracterized by the presence of a light zone and a dark zone. The proliferating cells move to one pole of the GC and become centroblasts. Centroblasts produce non-proliferating centrocytes that are thought to migrate to the light zone of the GC, which is rich in Ag-trapping FDCs and T follicular helper (Tfh) cells. Small, non-cycling follicular B-cells are then moved closer together, forming the follicular mantle. The light zone contains a rich network of FDCs that have the capacity to take up Ag and hold it on their surface for periods of more than a year. The Ag is held as an IC in a native unprocessed form; but the Ag may be taken up from FDC by B-cells, which can process and present it to T-cells. The open structure of GCs allows high-affinity Ag-specific B-cells to be recruited to an ongoing GCR. This enhances competition for the FDC-bound Ags and ensures that rare high-affinity B-cells can participate in Ab responses (MacLennan, 1994; Kosco-Vilbois et al., 1997; MacLennan et al., 2000; McHeyzer-Williams, 2003; Natkunam, 2007; Schwickert et al., 2007).

One of the hallmarks of the GCR is the introduction of somatic mutations into IgV genes of GC centroblasts, mainly single base pair mutations, followed by an efficient selection procedure that ensures selection of Abs with higher affinity (Song et al., 1998; McHeyzer-Williams et al., 2000; Chan and Brink, 2012). SHM adds as many as one mutation per cell per division which provides a substrate for selection of higher affinity and more diverse populations of cells. SHM can result in increased affinity of the BCR for Ag, a critical event in affinity maturation and the production of high-affinity Abs. Conversely, SHM can cause loss of affinity, or altered specificity with the acquisition of autoreactivity. Centroblasts continually give rise to non-proliferating centrocytes, which enter the FDC network where they are subjected to selection. The selection is based on the ability of a centrocyte first to bind Ag held on FDC, and second to present this in a processed form to CD4 T-effector cells, which are sited toward the outer edge of the FDC network. Cells that fail to undergo positive selection die by apoptosis, the default pathway for centrocytes that do not receive selection signals. Selection eliminates those cells that have lost Ag-binding activity, or have acquired autoreactivity (MacLennan et al., 2000; McHeyzer-Williams, 2003; Tarlinton, 2008; Vinuesa and Cyster, 2011; Shlomchik and Weisel, 2012b).

Upon exiting the GCR, selected B-cells can differentiate into two long-lived compartments; memory B-cells, and bone marrow-resident plasma cells. Both cell populations participate in sustaining high-titred Ab levels. Memory B-cells recirculate in the periphery, where they can survive for decades in humans. Upon re-encounter with the Ag for which they are specific, memory B-cells can rapidly proliferate and differentiate into plasma cells secreting high affinity Abs. on the other hand, bone marrow-resident long-lived plasma cells secrete Abs for long periods of time (MacLennan et al., 2000; McHeyzer-Williams, 2003; Kallies

et al., 2004; Tarlinton, 2008; Elgueta et al., 2010; Good-Jacobson and Shlomchik, 2010; Vinuesa and Cyster, 2011; McHeyzer-Williams et al., 2012; Peperzak et al., 2012; Shlomchik and Weisel, 2012a).

GCs persist for about 3 weeks following immunization (MacLennan, 1994), however, in mice immunized with protein Ag in adjuvant (Shlomchik and Weisel, 2012b) and TI GC responses to multimerized protein Ags on FDCs (El Shikh et al., 2009a) the GC response can persist detectably for more that 8 weeks. Moreover, immunization with viruses induces long-term GCs associated with proliferating Ag-specific B-cells (Tarlinton, 1998) and continuous influx of T-cells (Vinuesa et al., 2010). With the demise of the GCR, the dendritic processes of the FDC form tightly folded convolutions of membrane where the Ag is retained extracellularly forming depots of FDC-retained Ags that may contribute to the recall responses (Sukumar et al., 2008; El Shikh et al., 2010).

The contribution of T-cells to the GCR has been studied extensively as T-cell help to B-cells is a fundamental aspect of adaptive immunity and the generation of immunological memory. Tfh cells differentiate in response to immunization or infection and provide help to GC B-cells. Tfh cells, initially described in 2000–2001, are $CD4^+$ T-cells that mature in a Bcl-6-dependent manner and are capable of providing IL-21-dependent help to B-cells for GC formation and switched Ag-specific Ab responses (Vinuesa and Cyster, 2011). This population is characterized by a core set of factors essential for their generation and function; including Bcl-6, IL-21, and CXCR5 expression; CD28 costimulation; ICOS ligation; and SAP-mediated interactions with cognate B-cells (Fazilleau et al., 2009; Crotty, 2011; Nutt and Tarlinton, 2011; Linterman et al., 2012; Weinstein et al., 2012).

The original description of GCs entailed definite T-cell dependency (Nieuwenhuis and Opstelten, 1984), however, recent studies indicated that high-affinity B-cells can be induced to form large GCs in response to (4-hydroxy-3-nitrophenyl) acetyl (NP)-Ficoll in the absence of T-cells (de Vinuesa et al., 2000; Lentz and Manser, 2001). Moreover, FDC-retained TD Ags induce TI B-cell activation and Ig secretion in responses costimulated by FDC-BAFF and C4bBP (El Shikh et al., 2009a). Therefore, T-cells may be required for latter stages of the GCR, but they are dispensable for the induction and initial development of this response (Lentz and Manser, 2001).

Several models have been proposed for the cellular dynamics during the GCR (McHeyzer-Williams et al., 2000; McHeyzer-Williams and McHeyzer-Williams, 2005; Allen et al., 2007; Or-Guil et al., 2007), and although there is no typical GCs in terms of size, recent studies indicated that GCs have typical cellular ratios attained during the established phase of the response (Wittenbrink et al., 2011).

Autoreactive and malignant GC B-cells induce a unique class of disorders because they originate from cells of the immune system that divert from the normal maturation programmes, via genetic rearrangements or somatic mutations. As SHM and gene rearrangements are the physiological landmarks of Ag-driven GC responses, the risk for genetic lesions (and hence autoimmunity and malignant transformation) is exponentially enhanced (Hollowood and Goodlad, 1998; Guzman-Rojas et al., 2002; Vinuesa et al., 2009; Manzo et al., 2010). GC B-cells with low Ag affinity and autoreactivity are eliminated via apoptosis and are rapidly cleared by TBMs. Inefficient clearance of apoptotic cells results in autoimmunity that is thought to be mediated by various intracellular molecules possessing danger-associated molecular patterns (DAMPs), including nuclear self-Ags. DAMPs can be released from apoptotic cells undergoing secondary necrosis due to disruption of the apoptotic cell clearance programmes within the GCs (Rahman, 2011).

Although the enzyme activation-induced deaminase (AID) is essential for creating Ab diversification by causing mutations during the GCR, it also greatly enhances the chance of B-cell lymphoma development. AID increases the mutation rate to one per thousand bases, six orders of magnitude more than spontaneous mutagenesis, and can ultimately lead to mutations of proto-oncogenes and chromosomal translocations resulting in a wide variety of B-cell lymphomas that are thought to derive from GC B-cells based on their carrying somatically mutated V region genes (Peperzak et al., 2012; Shlomchik and Weisel, 2012b).

In tertiary lymphoid tissues, infiltrating B- and T-lymphocytes organize themselves into ectopic follicles and GCs. This has been observed in several autoimmune, chronic inflammatory, and neoplastic diseases. Ectopic GCs are architecturally similar to GCs in conventional secondary lymphoid organs, and comprise proliferating B-cells and networks of FDCs. T-cells are also a regular component of ectopic GCs where they provide cognate T-cell help required for the progression of the GC B-cell response (Aloisi and Pujol-Borrell, 2006; Carragher et al., 2008).

Although some elements of the cellular interactions between cell types within GCs have now been visualized, it is yet to attain the ability to image over extended time periods in order to have clearer image of the GC kinetics.

THE IMMUNOREGULATORY FUNCTIONS OF FDCs

Ag presentation

Studies of TD Ags including ovalbumin, horseradish peroxidase, and human serum albumin indicated that these immunogens are almost instantaneously converted into ICs by Abs persisting from prior immunization(s). Moreover, in primary responses, ICs form as soon as Ab is produced and these ICs are trapped in the FDC reticula. Ag trapping and retention is exquisitely localized to draining lymphoid tissues. Ag injection into a single limb of an immune mouse will be localized to the draining LNs and to a lesser extent in the spleen (Mandel et al., 1980; Donaldson et al., 1986). With time, the Ag persisting on FDCs becomes more and more focused to the lymphoid follicles nearest the site of Ag injection and by 1 year, specific Ab forming cells are almost exclusively confined to the most proximal LN (Donaldson et al., 1986). Remarkably, once Ags are trapped on FDCs, they remain relatively unaffected by a variety of manipulations including gamma-irradiation, stress, and treatment with a number of anticancer drugs. However, cortisone acetate injections result in the loss of a significant portion of persisting Ag although the reason for this remains unclear (El Shikh et al., 2009b).

FDCs trap ICs via Fc and C receptors (Tew et al., 2001; El Shikh et al., 2006). In the spleen, Ag retention requires the presence of complement (Klaus and Humphrey, 1977; Tew et al., 1979),

whereas, in the draining LNs, FDCs are capable of trapping ICs in the absence of complement (Tew et al., 1979; El Shikh et al., 2006). Complement-mediated IC trapping in the draining lymph nodes of FcγRIIB$^{-/-}$ mice is normal compared to WT controls although retention of ICs over time is reduced in FcγRIIB$^{-/-}$ mice suggesting FcγRIIB is important in long-term maintenance of the ICs (Tew et al., 1976). Remarkably, engagement of FDC-FcγRIIB with ICs plays a critical role in activating FDCs. Binding of ICs to FDC-FcγRIIB induces FDC activation which leads to dramatic upregulation of FDC-ICAM-1, -VCAM-1 and -FcγRIIB itself (El Shikh et al., 2006). In addition, FDC-FcγRIIB plays a major role in B-cell activation with Ag-specific and anti-BCR ligation where the high density of FcγRIIB on FDCs binds Ig-Fc in the IC and consequently the ITIM signal delivered via co-ligating BCR and B-cell-FcγRIIB is minimized (Aydar et al., 2003, 2004b).

FDCs multimerize native Ags in a periodic manner as initially discovered using SEM with Ags trapped *in vivo*. Szakal et al. reported patterns of orderly, spiraling, arrangement of ICs made up of alternating light and dark bands (Szakal et al., 1985), and recent studied of ICs trapping by FDCs *in vitro* confirmed Ag periodicity (Sukumar et al., 2008) and indicated that ICs are arranged on FDCs with a 200–500Å spacing between epitopes which correlates well with spacing optimal for BCR crosslinking and activation by TI type 2 Ags (Dintzis et al., 1976, 1983). The periodically arranged ICs interact and "zip" processes together which may help explain IC preservation and long-term retention (Sukumar et al., 2008).

Using two-photon microscopy, it was recently shown that the B-cell encounter with FDC-associated Ag could be detected for >1 week after immunization. B-cell-FDC contact times were often brief but occasionally persisted for >30 min and B-cells sometimes acquired Ag together with FDC surface proteins. These observations establish that FDCs serve as sites of B-cell Ag capture, with their prolonged display time ensuring that even rare B-cells have the chance of Ag encounter (Suzuki et al., 2009).

Persisting Ags on FDCs are critical for SHM which is important for affinity maturation. SHM is a late GC event beginning 9–11 days after primary challenge, and a late specific Ag signal (a week after priming) is delivered by Ags persisting on FDCs to promote SHM and affinity maturation (Wu et al., 2008). Moreover, Ags retained on FDCs are delivered to B-cells in the form of iccosomes, which are highly immunogenic. Iccosomes are readily endocytosed by GC B-cells, which process the Ag and present it to T-cells (Szakal et al., 1988).

Strikingly, FDCs display Ags even in the presence of high levels of specific Ab which might be expected to mask epitopes and thus block successful Ag presentation. However, B-cells cluster with FDCs forming a synapse at the point of FDC-B-cell contact and Ag-specific B-cells recognize FDC-Ag via their BCRs in the synapse even in the presence of high levels of Abs during the GC reaction (El Shikh et al., 2009b).

B-cell costimulation

FDC-derived complement cosignals. In addition to presentation of multimerized Ags in arrays that extensively crosslink BCRs, FDCs provide costimultory signals that regulate Ig secretion in TD and TI Ab responses. FDC-CD21L, -C4bBP, -BAFF, and -IL-6 are major FDC-molecules known to signal B-cells. The important interaction between a complement-derived CD21 ligand on FDCs and CD21 on B-cells in the initiation of IgG responses has been well verified (Qin et al., 1998; Tew et al., 2001; Aydar et al., 2005). Coligation of BCR and CD21 facilitates association of BCR and B-cell co-receptor complex promoting CD19 phosphorylation by a tyrosine kinase associated with BCR (Carter et al., 1997). This cosignal dramatically augments stimulation delivered by engagement of BCR by Ag. Blockade of the CR2 ligand on FDCs by the use of soluble CR2 or use of B-cells from CR2 knockout mice (or B-cells with CR2 blocked) reduced Ab responses 10–1000 folds (Qin et al., 1998; Tew et al., 2001; Aydar et al., 2005). FDCs from C3 knockout mice, which cannot generate the CR2-binding fragments (iC3b, C3d, and C3dg), were unable to provide costimulatory activity (Qin et al., 1998; Fakher et al., 2001).

Not only do FDCs provide C3 (CD21L) as a complement derived cosignal, but FDC-C4bBP has been recently reported to engage B-cell CD40 and serve as a cosignal in FDC-dependent TI responses to polysaccharides. TI polysaccharide Ags fix the alternative complement pathway directly and the classical pathway indirectly by making ICs with specific IgM produced early in the response. In addition to accessory signals delivered by the complement derived CD21L, activated forms of both C3 (C3b) and C4 (C4b) bind C4 binding protein (C4BP), which in humans and likely in rodents, colocalizes with ICs on FDCs. C4bBP in ICs trapped on FDCs has been shown to signal B-cells via intact CD40 in short-lived Ag-specific TI GCs independent of T-cell derived CD40L (CD154). More specifically, in mice with normal FDCs, injection of a blocking mAb, FDC-M2, which recognizes an epitope on C4 bound within ICs, also inhibited TI GC development. C3- and C4-deficient mice showed impaired TI Ab responses consistent with the hypothesis that ICs on FDCs can provide Ag-specific, complement-derived, and CD40-mediated signals to B-cells initiating B-cell proliferation in TI GCs (Gaspal et al., 2006).

FDC-derived cytokines. FDCs produce BAFF (Hase et al., 2004; Magliozzi et al., 2004; Zhang et al., 2005; El Shikh et al., 2009a; Manzo et al., 2010) that has the ability to support TI B-cell activation (Shulga-Morskaya et al., 2004; Groom et al., 2007). FDC-BAFF is critical for FDC-dependent B-cell activation (El Shikh et al., 2009a), and BAFF is directly involved in rescue, activation, and follicular homing of B-cells (Varin et al., 2010). BAFF binds to BAFF-R, TACI (transmembrane activator and calcium-modulator and cyclophilin ligand [CAML] interactor) and, BCMA (B-cell maturation Ag). The functional outcome of BAFF signaling is multifaceted and different receptors mediate different functions. Peripheral B-cell survival, plasma cell survival, MZ-B-cell integrity, GC maintenance, CD21 and CD23 expression, TI B-cell responses and Ig class switching all have been attributed to BAFF (Schneider, 2005).

Recent studies indicated a critical role for IL-6, whose primary source in GCs is FDCs (Kopf et al., 1998), in specific IgG responses and SHM (Wu et al., 2009). IL-6, directly or via IL-17, regulates B-cell activity (Deng et al., 2002), and blocking IL-6 from FDCs inhibits both IgG responses and SHM

(Wu et al., 2009). FDC-BAFF, -IL-6, and -IL-15 can induce T-cell IL-4, IL-5, IFNγ, and TNF-α (Mori et al., 1996; Heijink et al., 2002; Huard et al., 2004; Park et al., 2004), and these cytokines can indirectly promote B-cell activation and Ig class switching.

FDC-B-cell synapse. The integrity of the first BCR-mediated signal depends on extensive crosslinking of BCRs with IC-bearing FDCs which likely entails formation of a properly functioning FDC-B-cell synapse. FDC-CXCL-13 attracts follicular B-cells, and in the spleen MZ-B-cells, and helps organize follicles (Cyster et al., 2000; Estes et al., 2004). Within the follicles, the adhesion molecules ICAM-1 and VCAM-1 are critical for FDC-B-cell synapse formation and activation, and their blockade inhibits FDC-B-cell clustering and B-cell activation (Kosco et al., 1992; Maeda et al., 1995).

T independent B-cell activation

TI type 2 Ags trapped on FDCs can induce GCs in animals lacking cognate T and B-cell interactions. When mice with mutations that inactivate the *TCR Cβ* and *Cδ* genes were immunized with 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll, GCs with peanut agglutinin-binding B-cells were observed in the splenic follicles. These GCs contained mature IC-bearing FDCs and although they are rapidly induced, their duration is short (de Vinuesa et al., 2000; Lentz and Manser, 2001). Nevertheless, the finding that natural and synthetic multivalent Ags can induce Ig secretion in the absence of T-cells does not mean that T-cells do not play a part in TI responses and the involvement of several T-cell cytokines has been established (Vos et al., 2000).

Typically ∼48 h is needed before primed T-cells are able to provide cognate help and it is unlikely that cognate T helper cells are involved in 48 h TI responses. It is important to appreciate that IC activated FDCs produce BAFF and IL-6 and that these cytokines can in turn rapidly induce cytokine production by T-cells (Huard et al., 2001, 2004; Heijink et al., 2002). Owing to their rapid intense cytokine production and their ability to provide B-cell help in a non-MHC-restricted fashion, γδ and NK T-cells could help in the early phases of TI responses. IFN-γ-mediated enhancement of Ig secretion has been demonstrated by *in vitro* addition of γδ T-cell clones to anti-δ-dex stimulated B-cells. Moreover, several reports indicate that pre-incubation of purified B-cells with IL-4, a rapid product of activated NK T-cells, prior to anti-δ-dex stimulation resulted in a 10-fold increase in Ig secretion within 6 h. It has been also shown that proliferation of B-cells stimulated with slg-dependent (anti-μ-dex) or -independent (LPS) polyclonal activators is markedly augmented *in vitro* by addition of purified FDCs in a dose-dependent fashion and cultures containing B-cells from athymic nude mice proliferated normally in the presence of anti-μ-dex plus rlL-4, implying that IL-4 provides adequate help (Burton et al., 1993).

The ability of FDCs to convert TD Ags into TI Ags was recently reported. TD Ags in ICs on FDCs are spaced 200–500 Å apart on the flexible backbone of FDC membranes which is geometrically fit for extensive BCR crosslinking. Moreover, FDCs provide BAFF and C4bBP, which are known to support TI B-cell activation. Nude or athymic mice challenged with ICs produce specific IgM in 48 hr while challenge with free Ag in adjuvant failed to induce IgM even after many weeks. Moreover, the draining lymph nodes of IC-challenged nude mice exhibit well-developed GCs associated with FDC Ag retaining reticula and plasmablasts within 48 h. IgM is the first class of Abs produced during primary Ab responses, and the ability to induce FDC-dependent, TI IgM responses may be critical in host defence in the initial phases of infections before T-cell help is provided or in disease conditions where T-cell insufficiency prevails (El Shikh et al., 2009a, 2010).

Major FDC regulatory and signaling molecules

Table 1 summarizes the currently characterized FDC regulatory and signaling molecules.

FDC activation

Expression of FDC surface and secreted molecules is subject to regulation, and engagement with ICs, TLR ligands, or collagen type 1 induces FDC activation and upregulation of these molecules. In the GCs, FDCs bear high levels of FcγRIIB, ICAM-1, and VCAM-1, and these molecules are involved in converting poorly immunogenic ICs into a highly immunogenic form and facilitate FDC-B-cell interactions. FDC-trapped ICs (El Shikh et al., 2006; Smith and Clatworthy, 2010), TLR ligands (El Shikh et al., 2007b; Garin et al., 2010), and collagen type 1 (El Shikh et al., 2007a) differentially upregulate the FDC accessory molecules ICAM-1, VCAM-1, FcγRIIB, C4bBP, BAFF, IL-1β, IL-10, and IL-6 (El Shikh et al., 2007b; Wu et al., 2009; Garin et al., 2010).

Activated FDCs provide Ags to B-cells in a highly immunogenic form by: (1) multimerizing the Ags thus extensively crosslink multiple BCRs (El Shikh et al., 2009a,b, 2010); (2) minimizing the inhibitory ITIM signaling in B-cells (Aydar et al., 2004b; El Shikh et al., 2006, 2010); and (3) providing B-cell homing, survival, and costimulation signals in efficient FDC-B-cell synapses (El Shikh et al., 2009b, 2010).

A major consequence of activation is a marked increase in accessory activity. Both specific Ab responses and SHM are dramatically enhanced when FDCs are activated by TLR ligands suggesting that adjuvant activity likely involves FDC-activation and not just DC activation for T-cell co-stimulation (El Shikh et al., 2007b; Wu et al., 2008). Moreover, TLR-mediated stimulation of Peyer's patches' FDCs by bacterial products in the gut induces FDC-CXCL13, -BAFF, and -TGF-β1 secretion and enhances IgA production. Via TGF- β1, IgA, and other tissue-protective, anti-inflammatory, and anti-tumor molecules, TLR-stimulated FDCs uniquely regulate mucosal homeostasis and prevent excessive activation that causes inflammation, autoimmunity, or tumor formation in the gut (Suzuki et al., 2010). Recently, the non-toxic CTA1-DD adjuvant hosting the ADP-ribosylating CTA1 subunit from cholera toxin and a dimer of the D fragment from *Staphylococcus aureus* protein A was targeted to the FDC networks and its deposition appeared to be complement-dependent. The adjuvant directly activates complement, enabling binding of the adjuvant to the FDC, which strongly promoted the GC reaction, leading to augmented serum Ab titers and long-term memory development. The mechanism of FDC activation by CTA1-DD may be TLR-dependent or independent and this has to be further verified (Mattsson et al., 2011).

Table 1 | FDC regulatory and signaling molecules.

FDC molecule	Function
BAFF	FDC-BAFF supports TI B-cell activation (El Shikh et al., 2009a), and is required for the proper formation of FDC networks within the GCs (Moisini and Davidson, 2009).
CD21/35 (CR1/2)	CRs are crucial for IC retention especially in the spleen (Tew et al., 1979; Qin et al., 1997, 1998; Carroll, 1998; Barrington et al., 2002). Genetic CD21 deficiency in humans adds to the molecular defects observed in human subjects with hypogammaglobulinemia and severe reduction in memory B-cells (Thiel et al., 2012).
CD21L (iC3b, C3d or C3dg)	Engagement of CD21 in the B-cell co-receptor complex by complement derived FDC-CD21L delivers a critical co-signal. Coligation of BCR and CD21 facilitates association of the two receptors and the cytoplasmic tail of CD19 is phosphorylated by a tyrosine kinase associated with the BCR complex. This co-signal augments stimulation delivered by Ag and blockade of FDC-CD21L reduces B-cell proliferation, activation induced cytidine deaminase, and Ab production 10–1000 fold (Croix et al., 1996; Fischer et al., 1996; Carroll, 1998; Qin et al., 1998; El Shikh et al., 2010).
CD23 (FcεRII)	FDC-FcεRII mediates IC retention, and the regulation of the GC reaction and IgE levels (El Shikh et al., 2009b; Gibb et al., 2010; Chaimowitz et al., 2011).
CD29, CD44	FDCs express CD44 and CD29 and FDC binding to collagen type I *in vitro* induces the regeneration of FDC processes and networks. CD44 also enhances B-cell adherence to FDCs allowing delivery of the FDC-derived B-cell survival signals including 8D6 and BAFF (El Shikh et al., 2007a).
CD32 (FcγRIIB)	FDC-FcγRIIB is critically involved in conversion of poorly immunogenic ICs into a highly immunogenic form, FDC activation, IC periodicity, long-term IC retention, and regulation of serum IgG levels (El Shikh et al., 2009b, 2010).
CD320 (8D6)	The 8D6 molecule inhibits apoptosis and influences both proliferation and Ab secretion by GC B-cells. Moreover, GC B-cells that are induced to differentiate into pre-plasma cells are the most sensitive to the neutralizing effects of anti-8D6 (Zhang et al., 2001; Li et al., 2004; Cho et al., 2008).
CD40	FDCs express CD40 and when incubated with either CD40L trimer or agonistic anti-CD40 Ab, the expression of FDC-CD23 is increased both at the mRNA and protein levels. FDC-CD23 helps regulate IgE levels (Payet-Jamroz et al., 2001; Sukumar et al., 2006a; Gibb et al., 2010).
CD54 (ICAM-1), CD106 (VCAM-1), MadCAM-1	Abs reactive with murine ICAM-1 and/or leukocyte functional Ag-I (LFA-I) interfere with FDC-B-cell clustering resulting in reduced B-cell proliferation. In addition, VLA-4 and VCAM-1 have been observed in GCs and likely also play a role in FDC-B-cell interactions. These adhesion molecules are thought to stabilize the FDC-B-cell synapse and promote interaction of FDC-Ag and FDC-costimulatory molecules with B-cells (Kosco et al., 1992; Maeda et al., 1995; Vinuesa et al., 2010).
CXCL13	CXCL13 is secreted by FDCs and acts as chemoattractant for B-cells via the CXCR5 chemokine receptor. FDC development and expression of this chemokine depend on LTαβ, and TNF-α. The maintenance of lymphoid follicle structure is mediated by a positive feedback loop: CXCL13 stimulates B-cells to express high levels of LTαβ, and TNF-α which stimulates FDCs to produce CXCL13 (Ansel et al., 2000; Cyster et al., 2000; Manzo et al., 2005; Wang et al., 2011).
Fcα/μR	In humans, FDCs are the predominant cell type expressing Fcα/μR which are involved in IC retention (El Shikh et al., 2009b; Honda et al., 2009).
FDC-M1 (Mfge8)	Fat globule epidermal growth factor 8 (Mfge8) "licenses" tingible body macrophages (TBMs) to engulf apoptotic bodies in GCs and helps minimize autoimmunity (Kranich et al., 2008).
FDC-M2 (C4b eptiope)	C4b binding protein (C4bBP) binds C4b and co-localizes with ICs on FDCs. FDC-C4BP has been shown to signal B-cells via CD40, independent of T-cell CD40L (CD154). Injection of mice with FDC-M2 inhibits C4BP development and TI-GC development (Taylor et al., 2002).
Hedgehog (HH) ligand	Sonic Hedgehog (SHH) ligand is produced by FDCs within the GCs of lymphoid follicles, and SHH ligand protects GCer B-cells from apoptosis. GC B-cells express the HH receptors, and their survival is altered after inhibition of the HH signaling pathway (Sacedon et al., 2005; Ok et al., 2012).
IL-15	IL-15 is produced by FDCs and is captured by IL-15R on the surface of FDCs. Surface IL-15 is active and promotes GC-B-cell proliferation (Park et al., 2004), and human primary FDCs *in vitro*. In addition, blocking of FDC IL-15 signaling reduced FDC secretion of CCL-2, CCL-5, CXCL-5 and CXCL-8, suggesting potentially important roles for recruitment of other cellular components required for GC reaction (Gil et al., 2010).
IL-6	FDCs are the source of IL-6 in GCs. Engaging FDC-FcγRIIB by ICs activates FDCs and enhances FDC-IL-6 production. FDC-IL-6 promotes GC development, IgG production, and SHM and terminal B-cell differentiation (Wu et al., 2009).
IL-7	IL-7 has been found in isolated tonsilar FDCs using RT-PCR and cell staining. IL-7 signaling coupled with crosslinking of surface immunoglobulin receptors results in B-cell proliferation (Kroncke et al., 1996).
Notch ligands	Notch ligands, Delta-like1 and Jagged 1, are expressed by FDCs and support GC-B-cell growth and survival (Yoon et al., 2009).

(Continued)

Table 1 | Continued

FDC molecule	Function
Prostaglandins (PGs)	The role of PGs in regulation of the GC reaction has been suggested in the late 90s. TBM were found to be rich in PGs via which they can downregulate the GC reaction (Smith et al., 1998). Recently, FDCs are identified as a source of PGs. Human FDC-HK cell line produce PGE2 that is inhibted by COX-2 depletion (Cho et al., 2011a) and IL-4 via JAK1 and STAT6 signaling pathway (Cho et al., 2011b, 2012b). Human FDC-PGs significantly enhance the expression levels of CD54, CD80, and CD86 on the surface of activated B-cells and augment their Ag presentation activity (Kim et al., 2012b). Using a mouse FDC line, it was also shown that IL-21 induces PGE2 secretion by FDCs (Magari et al., 2011).
TNFα-R and LT-R	FDC development and maturation (Allen and Cyster, 2008)
TNF-α	FDCs produced soluble TNF-α that promotos GC T-cell activation (Thacker et al., 2009).
Toll-like receptors (TLRs)	Dramatic upregulation of FDC-ICAM-1, VCAM-1, and FcγRIIB is observed after injecting LPS into animals expressing WT TLR4 but not in animals with mutated TLR4. Incubation of FDCs with LPS in vitro upregulates FcγRIIB, ICAM-1, and VCAM-1. FDCs express mRNA for TLR2, 3, 4 and 9 and injection of poly I:C brings up FDC-FcγRIIB to levels comparable with LPS (El Shikh et al., 2007b). FDC activation via TLR4 enhances isotype switching, somatic hypermutation, and the production of high-affinity Ig (Deshane and Chaplin, 2010; Garin et al., 2010).
Wnt5a	FDCs secrete Wnt5a and its production is upregulated by polyI:C. FDC-Wnt5a is a GC B-cell survival factor and might be a potential target for the regulation of B-cell immunity (Kim et al., 2012a).

FDCs IN THE PATHOGENESIS OF DISEASES

In addition to their contributions in regulated immunity, FDCs also play important roles in pathological states, including HIV/AIDS, prion diseases, autoimmunity and B-cell lymphomas.

FDCs IN AUTOIMMUNE DISEASES

Autoimmune disorders frequently display follicles with IC-bearing FDCs, autoreactive GCs, and ongoing affinity maturation (Aloisi and Pujol-Borrell, 2006; Manzo et al., 2010). Moreover, FDCs retain ICs for years, and provide constant Ag depot for memory B-cell re-stimulation (Klaus et al., 1980; Tew et al., 1980; MacLennan and Gray, 1986; Gray, 1993; McHeyzer-Williams et al., 1993; Bachmann and Jennings, 2010); and interference with FDC-reticula attenuates autoreactive GC formation, reduces pathogenic auto-Ab titers and memory B lymphocytes and ameliorates arthritis (Anolik et al., 2008; Victoratos and Kollias, 2009; Vinuesa et al., 2009; Manzo et al., 2010). It has been recently demonstrated that FDC follicular units in rheumatoid arthritis synovium invariably express AID and are surrounded by anti-citrullinated protein Ab (ACPA)-producing plasma cells. This was further confirmed by evidence of sustained AID expression, B-cell proliferation, ongoing CSR, and production of human IgG ACPA from GC synovial tissue transplanted into SCID mice, independently of new B-cell influx from the systemic circulation (Humby et al., 2009; Manzo et al., 2010).

Data in various models and human studies suggests the requirement for auto-Ag to sustain the autoimmune response and that auto-Ag withdrawal inhibits it (Bach et al., 1998). FDCs present Ags to B-cells in a highly immunogenic form and membrane-bound Ags seem to be the predominant form of Ag that mediates B-cell activation *in vivo* (Harwood and Batista, 2010).

In contrast to self-Ags of low valency such as small soluble proteins, multivalent Ags crosslink BCRs, maintain downstream signaling, and induce B-cell activation. In addition, tolerized B-cells exhibit enhanced rates of BCR-mediated Ag uptake. However, when the Ag is repetitive, stable, and resistant to BCR-mediated endocytosis, like multimerized FDC-bound Ags, BCRs aggregate and signaling is maintained. This results in clonal expansion and Ab production that is dependent on the multitude of BCR signalosomes activated by extensive crosslinking of BCRs (Hinton et al., 2008). The high levels of FcγRIIB on FDCs protects the immunogenicity of FDC-ICs by minimizing serious inhibition of B-cell activation upon BCR/FcγRIIB crosslinking (Aydar et al., 2003, 2004b; El Shikh et al., 2006, 2009a). In fact, the expression of FcγRIIB is significantly reduced on rheumatoid arthritis memory B-cells and plasmablasts and these alterations on FcγRIIB were associated with high levels of anti-citrullinated vimentin auto-Abs (Catalan et al., 2010).

Not only FDCs present Ags in a periodic multivalent ICs, but also they provide complement-mediated B-cell co-stimulation (Qin et al., 1998; Aydar et al., 2002; Barrington et al., 2002). CR2-mediated complementation of BCR signals can overcome B-cell anergy (Lyubchenko et al., 2007), and activation of the complement system is involved in the pathogenesis of the systemic autoimmune diseases via the classical pathway and the alternative pathways (Chen et al., 2010).

Previous *in vitro* studies indicated that peripheral blood B-cells from rheumatoid arthritis patients as well as healthy controls can be induced *in vitro* to produce anti-CCP Abs using CD40L polyclonal stimulation or in co-cultures with anti-CD3-activated T-cells (Reparon-Schuijt et al., 2001). FDC-C4bBP is a B-cell CD40L on activated FDCs, and infections at the onset of many autoimmune diseases may directly activate FDCs by ligating TLRs, and thus enhance FDC-B-cell interaction (El Shikh et al., 2007b).

The initial Ab needed to trap Ag on FDCs may be induced by molecular mimics or by polyclonal B-cell activation, which may occur as a consequence of infections that herald many autoimmune diseases (Granholm and Cavallo, 1991; Sfriso et al., 2010). However, once the ICs form, they would be trapped by FDCs and

the autoreactive B-cells should be stimulated in a TI manner as we have shown for foreign Ags in the absence of T-cells (El Shikh et al., 2009a, 2010).

Polyvalent presentation of self-proteins in repetitive arrays breaches B-cell tolerance in immune-tolerant animal models (Sauerborn et al., 2010). Aggregated native hIFN-α broke B-cell tolerance in transgenic mice (Hermeling et al., 2006), and virus-like protein-arrayed HEL induced higher titer Ab responses against HEL in tolerant HEL Tg mice (Chackerian et al., 2008; Link and Bachmann, 2010). In certain occasions the high epitope valency rendered Ab responses independent of CD21 (Jegerlehner et al., 2002). Moreover, in humans, active immunization using virus-like particle-based vaccines selectively targeting IL-17A, soluble TNF-α or IL-1β has shown promising results in the protection against autoimmune arthritis and myocarditis in mouse models (Spohn et al., 2005; Rohn et al., 2006; Sonderegger et al., 2006; Spohn et al., 2007, 2008; Kopf et al., 2010).

FDCs IN HIV/AIDS

The contributions of FDCs to HIV pathogenesis include the storage of a large and diverse reservoir of infectious virus, including drug resistant variants, in an activated microenvironment where CD4 T-cells targets are attracted but impaired in their ability to leave thereby increasing the likelihood of their infection. Just as Ags, intact HIV particles are found trapped on FDC dendritic presented in the form of ICs containing Ab and/or complement proteins. FDCs retain virus particles for long periods of time and, in fact, as long as FDCs are present in infected individuals, HIV can be found associated with them (Heath et al., 1995; Masuda et al., 1995; Burton et al., 1997; Smith et al., 2001; Smith-Franklin et al., 2002).

FDCs provide a microenvironment that is highly conducive to HIV transmission. HIV ICs remain infectious for months, and HIV on FDCs is "presented" to T-cells resulting in propagation of infection. FDC-secreted TNF-α increases the rate of HIV transcription by about three-fold in infected T-cells resulting in a significant increase in virus production (Thacker et al., 2009). FDCs also increase the T-cell expression of the HIV coreceptor CXCR4 and these cells become highly susceptible to infection with small quantities of virus that do not infect other cells with lower levels of CXCR4. FDC-produced CXCL13 also contributes to HIV pathogenesis by attracting HIV-susceptible T lymphocytes into the lymphoid follicle (Estes et al., 2004).

FDCs IN PRION DISEASES

FDCs appear to play an important role in prion diseases as evidenced by the finding that in experimental models where FDCs are not present, prion diseases do not typically appear. Prion diseases occur in both animals and humans and are caused by misfolded proteins encoded by the Prnp gene and consist of a normal form (PrPc) and its aberrant counterpart (PrPSc). Without the normal PrPc protein, whose function is still unknown, the diseases are not manifest (Nuvolone et al., 2009; Aguzzi and Krautler, 2010).

Upon exposure to prions, the agents are transported into secondary lymphoid tissues where the proteins accumulate and replicate on FDCs. FDCs is a major source of PrPc and complement proteins play critical role in its retention. PrPc-expressing FDCs are sufficient to sustain prion replication in the spleen (McCulloch et al., 2011).

After prion replication in the secondary lymphoid tissues, the PrPc-PRPSc complexes are transported to the central nervous system where neurodegeneration occurs. The exact mechanism of prion transport from FDCs to peripheral nerves is unknown. Prions could be (1) transported by various cell types leaving GCs toward nerve terminals; (2) incorporated by budding retroviruses; (3) released in FDC-derived iccosomes, or (4) passively diffuse from the site of replication to the site of peripheral innervation (Nuvolone et al., 2009). Recent studies indicate that B-cells interacting with and acquiring surface proteins from FDCs and recirculating between secondary lymphoid tissues via the blood and lymph are involved in the initial propagation of prions from the draining lymphoid tissue to peripheral tissues (Mok et al., 2012). Because FDCs appear to be required for disease pathogenesis in most cases, blocking agents to the LTβR pathway, needed for FDC development and maintenance, results in impaired development of disease suggesting the potential of this approach in therapy (Nuvolone et al., 2009; Aguzzi and Krautler, 2010).

FDCs IN B-CELL LYMPHOMAS

Follicular lymphomas morphologically resemble conventional GCs having B-cells, T-cells, macrophages, and FDCs. Recent studies suggest that the microenvironment in which the malignant cells reside is critical for disease pathogenesis and progression. The malignant cells appear to require the contributions of T-cells, macrophages, and FDCs as evidenced by the observation that in the absence of these cells, the malignant cells are difficult to survive. In addition to providing signals important to tumor cell growth, FDCs also can provide signals that spare malignant B-cells from undergoing apoptosis or programmed cell death. Moreover, FDCs establish a protective sanctuary for malignant cells from otherwise effective chemotherapeutic agents (Asadullah et al., 2000; Li et al., 2004; Kim et al., 2012a; Lee et al., 2012; Ok et al., 2012).

CONCLUDING COMMENTS

FDCs represent a unique accessory immune cell that provides both Ag-driven and co-signals to B-cells. FDC-derived signals contribute to the ability of B-cells to survive, proliferate, differentiate, and produce optimal levels of specific high affinity Abs during the GC reaction. Apart from their immunoregulatory functions, FDCs also contribute to several disease conditions including autoimmune diorders, AIDS, prion diseases, and follicular lymphomas. Further understanding of FDC biology is essential for better control of humoral immunity and paves the way for therapeutic management of FDC-mediated immune disorders.

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Stromal cell contribution to human follicular lymphoma pathogenesis

Frédéric Mourcin*[1,2,3], *Céline Pangault*[1,2,4], *Rada Amin-Ali*[1,2], *Patricia Amé-Thomas*[1,2,4] *and Karin Tarte*[1,2,3,4]

[1] *INSERM, UMR U917, Rennes, France*
[2] *Université Rennes 1, Rennes, France*
[3] *Etablissement Français du Sang, Bretagne, Rennes, France*
[4] *Service ITeCH, Pôle de Biologie, CHU de Rennes, Rennes, France*

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Mark C. Coles, University of York, UK
Laura Santambrogio, Albert Einstein College of Medicine, USA

***Correspondence:**
Karin Tarte, Faculté de Médecine, INSERM, UMR U917, 2 Avenue du Pr Léon Bernard, 35043 Rennes, France.
e-mail: karin.tarte@univ-rennes1.fr

Follicular lymphoma (FL) is the prototypical model of indolent B cell lymphoma displaying a strong dependence on a specialized cell microenvironment mimicking normal germinal center. Within malignant cell niches in invaded lymph nodes and bone marrow, external stimuli provided by infiltrating stromal cells make a pivotal contribution to disease development, progression, and drug resistance. The crosstalk between FL B cells and stromal cells is bidirectional, causing activation of both partners. In agreement, FL stromal cells exhibit specific phenotypic, transcriptomic, and functional properties. This review highlights the critical pathways involved in the direct tumor-promoting activity of stromal cells but also their role in the organization of FL cell niche through the recruitment of accessory immune cells and their polarization to a B cell supportive phenotype. Finally, deciphering the interplay between stromal cells and FL cells provides potential new therapeutic targets with the aim to mobilize malignant cells outside their protective microenvironment and increase their sensitivity to conventional treatment.

Keywords: B cells, bone marrow, lymph nodes, cell interactions, stromal cells

B CELL LYMPHOMA MICROENVIRONMENT

Human mature B cell lymphomas represent a heterogeneous group of neoplasias characterized by recurrent genetic abnormalities and pathway dependencies. Each lymphoma subtype could be assigned to a peculiar stage of normal B cell differentiation, as judged by gene expression profiling and phenotype (Shaffer et al., 2012). In some of them, malignant B cell proliferation, survival, and drug resistance are strongly dependent on a combination of external stimuli delivered by the microenvironment within specific niches in invaded lymph nodes (LN) and bone marrow (BM; Burger et al., 2009). This is particularly true in follicular lymphoma (FL), the most frequent indolent lymphoma, which results from the malignant transformation of germinal center (GC)-derived B cells (**Figure 1**). Whereas over 90% of FL cases display a *BCL2*/*IGH* translocation, this early event, occurring as a mistake of V(D)J recombination in the BM, could be detected at low frequency within recirculating post-GC memory B cells of most healthy individuals (Roulland et al., 2011). These t(14;18)^{pos} cells exhibit additional characteristics that stand as hallmarks of FL cells; i.e., CD10 expression, unleashed AID activity, persistence of surface IgM despite active class-switch recombination on the translocated allele; are thus called follicular-lymphoma like cells. Given that the actual prevalence of FL is around 0.03%, it is clear that FL pathogenesis requires additional oncogenic events as well as a progressive modification of the composition and organization of tumor microenvironment (Bende et al., 2007). Among the recurrent complementary hits identified in FL patients, several alterations target the transcriptional and epigenetic pathways including inactivation of CREBBP/EP300 acetyltransferases, MLL2 methyltransferase, and MEF2B, involved in the recruitment of histone-modifying enzymes, and gain-of-function mutations of *EZH2* polycomb gene (Shaffer et al., 2012). Recently, a frequent inactivation of the soluble inhibitory receptor EPHA7 was also reported (Oricchio et al., 2011). This inactivation hinders the blockade by EPHA7 of the ephrin migratory pathway induced by cell–cell contact.

In agreement, genetic alterations and modification of the microenvironment are not independent transformation mechanisms since several FL-specific genetic alterations are not oncogenic *per se* but favor specific interactions with neighboring cells. Among them, the frequent mutations of *TNFRSF14/HVEM* could play a role in the maintenance of the functional tumor cell niche (Launay et al., 2012; Pasero et al., 2012). In fact, binding of HVEM to its receptor BTLA delivers an inhibitory signal and BTLA is strongly expressed on FL-supportive follicular helper T cells (T_{FH}) within malignant follicles (see below). In addition, nearly all FL-derived immunoglobulin variable regions display unusual sites for N-linked glycosylation, introduced during somatic hypermutation process, and reflecting positive selection associated to lymphomagenesis (Stevenson and Stevenson, 2012). Glycans added to these motifs are atypical in terminating at high mannose that interact with C-type lectins on the surface of surrounding cells and trigger BCR engagement. This functional bridge could mimic for continuous antigen stimulation to promote survival of FL cells (Coelho et al., 2010). About 30% of FL finally transform into aggressive diffuse large B cell lymphomas (DLBCL) that are less dependent on their microenvironment.

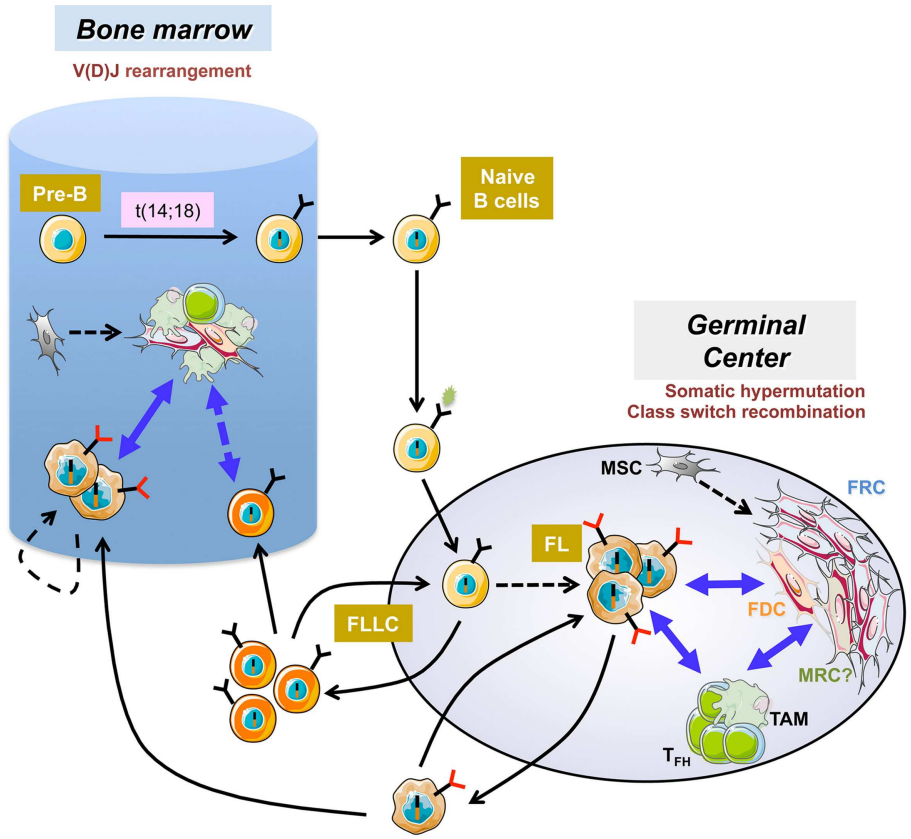

FIGURE 1 | Follicular lymphoma pathogenesis. The first step of follicular lymphoma (FL) development occurs in the bone marrow as a mistake in V(D)J rearrangement and leads to the ectopic expression of the antiapoptotic protein bcl2. After antigen encounter, naive B cells harboring the t(14;18) reach the germinal center where they display a selective growth advantage and could extensively recirculate as atypical IgM^{pos} low affinity memory B cells called follicular-lymphoma like cells (FLLC). Iterative reentries into germinal centers allow the acquisition of additional genetic alterations. The relationship between FLLC and FL is not formerly demonstrated but the hypothesis is that FLLC contain premalignant intermediates that could transform in some patients into FL. FL cells remain strongly dependent on bidirectional crosstalk with heterogeneous stromal cells, including activated fibroblastic reticular cells (FRC), altered follicular dendritic cells (FDC), and perhaps marginal reticular cells (MRC) of unknown origin and infiltrating immune cells including tumor-associated macrophages (TAM) and follicular helper T cells (T_{FH}). FL also developed in the bone marrow where B-cell clones could evolve independently within ectopic specific FL-supportive niches.

Microarray analyses have revealed that the clinical outcome of FL patients is primarily predicted by molecular features of non-malignant cells (Dave et al., 2004). Moreover, immunohistochemical studies have identified a large panel of predictive markers reflecting the number, activation, and/or spatial distribution of infiltrating immune non-B cell subsets, including tumor-associated macrophages (TAM) and $CD4^{pos}$ T cells (Relander et al., 2010). Besides immune cells, three main stromal cell subsets have been described within normal LN: (i) fibroblastic reticular cells (FRC) recruit mature dendritic cells and naïve B and T lymphocytes through the production of CCL19, CCL21, and CXCL12, promote cell–cell interactions within the T cell zone, and are involved in T cell self-tolerance and tissue tropism imprinting; (ii) follicular dendritic cells (FDC) drive CXCL13-dependent B cell attraction within GC where they concentrate unprocessed antigens and promote the selection of high affinity B cells; (iii) marginal reticular cells (MRC) deliver small antigens to cognate B cells through a network of follicular conduits (Mueller and Germain, 2009; Roozendaal and Mebius, 2011). It has long been assumed that some tumor conducive stromal cell niches are shaped within FL LN. In fact, FL LN exhibit a uniform and marked activation of transglutaminase expressing FRC network (Thomazy et al., 2003), whereas FDC display an undifferentiated phenotype (Chang et al., 2003; Jin et al., 2011). MRC have never been evaluated in human lymphomas. More generally a functional characterization of the heterogeneous stromal cell network that is found within malignant follicles is currently lacking. In addition, the *in situ* distribution of lymphoid stromal cell subsets relative to malignant B cells and to the other FL-supporting cell subsets and their activation status in malignant follicles remains unknown.

Follicular lymphoma is generally a disseminated disease and BM is involved in up to 70% of patients at diagnosis (Canioni et al., 2004). BM infiltration is characterized by the ectopic development of heterogeneous lymphoid-like stromal cells of unknown origin that are found admixed with malignant B cells and $CD4^{pos}$ T cells among nodular aggregates (Vega et al., 2002).

Even if BM malignant B cells retain their main follicular features, several morphologic, phenotypic, and genetic differences have been reported among tumor cells found within LN and BM. In particular, BM FL cells are characterized by a lower cytological grade and proliferation (Bognar et al., 2005; Rajnai et al., 2012), and exhibit a distinct transcriptomic profile revealing a downregulation of genes involved in cell proliferation and DNA repair (our unpublished data). In addition, despite a common clonal origin, analysis of intraclonal evolution reveals that a significant part of the BM infiltration evolves independently from the tumor clones detected in the LN indicating that BM provides a specific non-lymphoid malignant cell niche (Bognar et al., 2005; Ruminy et al., 2008). Recently, major differences in the cell composition of BM and LN microenvironments have been highlighted (Rajnai et al., 2012; Wahlin et al., 2012) thus paving the way for dedicated studies evaluating the impact of these variations on the behavior of malignant FL cell clones.

Several experimental limitations have hampered a full understanding of the role of stromal cells in FL pathogenesis: (i) the lack of B cell line reflecting the untransformed indolent stage of FL leading to the use of aggressive GC-lymphoma cell lines; (ii) the limited accessibility of tumor biopsies and the high propensity of primary FL cells to undergo apoptosis *in vitro*; (iii) the impossibility to maintain fully functional human FDC in culture; (iv) the heterogeneity of stromal cell subsets that remain poorly understood in human; (v) the inherent flaws of human FL B cell xenotransplantation into immunocompromised mice that are devoid of structured secondary lymphoid organs; (vi) the lack of validated relevant mouse model of FL. Nevertheless, even if these pitfalls should be carefully kept in mind, some recent studies have provided interesting results about lymphoma-permissive stromal cells, revealing both a direct B cell supportive effect and an indirect activity on the orchestration of the FL cell niche.

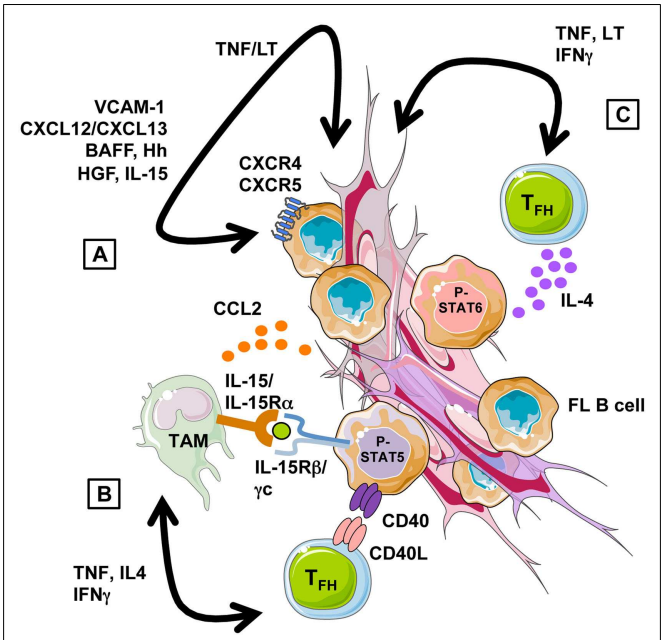

FIGURE 2 | Follicular lymphoma cell niche. (A) Stromal cells recruit and support directly the growth of FL B cells through a combination of adhesion molecules, chemokines, and cytokines. Their supportive properties are strongly influenced by the local cytokine context, in particular by TNF-α and LT-α1β2 that are overexpressed by malignant B cells and promote lymphoid stromal cell differentiation. **(B)** FL-MSC overexpress the chemokine CCL2 that promotes monocyte recruitment and polarization into TAM-like cells. FL-TAM overexpress IL-15 that triggers, in cooperation with TFH-derived CD40L, STAT5-dependent FL B cell activation. **(C)** FL-TFH overexpress TNF-α and LT-α1β2, which favor stromal cell engagement into FRC differentiation, but also IL-4 that drive STAT6-dependent B-cell activation and contribute to the induction of TAM phenotype.

DIRECT PROTUMORAL ACTIVITIES OF STROMAL CELLS

Interplay between lymphoma cells and their microenvironment provides pivotal signals for malignant cell recruitment and growth (**Figure 2**). First, stromal cells have been involved in the homing of FL B cells that display a $CXCR4^{hi}CXCR5^{hi}CCR7^{lo}$ phenotype, resembling to normal GC B cells (Lopez-Giral et al., 2004). We have in particular identified the CXCR4-ligand CXCL12 as the central chemokine in the recruitment of FL cells by both BM and LN-derived stromal cells (Amé-Thomas et al., 2007). Interestingly, CXCL13 has an additive effect with CXCL12 for the migration of FL cells *in vitro* (Husson et al., 2002) and these two chemokines, both produced by FDC *in vivo*, could contribute to the follicular localization of malignant cells. In the Eμ-Myc transgenic mouse model, CCR7 was elegantly reported to drive the specific homing of malignant B cells to the T cell zone where their promote the formation of a lymphoma-supportive stromal cell niche (Rehm et al., 2011). The potential role of CCR7 in human FL is less clear, given its low expression on malignant clones. However, we recently underlined using Affymetrix microarrays that *CCR7* expression is low but upregulated in FL cells compared to normal centroblasts and centrocytes (our unpublished data). The cues that guide normal GC B cell migration have been recently described in mice and involve fine coregulation of CCR7, sphingosine 1-phosphate (S1P) receptor type 2 (SIPR2, also called S1P2), and Epstein-Barr virus-induced gene 2 (EBI2, also known as GPR183) whose ligands are produced by various lymphoid stromal cell subsets. CCR7 is the receptor for CCL19/CCL21 produced by FRC of the T cell zone. SIPR2 belongs to the family receptors for S1P, a lipid signaling molecule presents in a decaying gradient in the follicle. S1PR2 engagement by S1P inhibits cell migration and is involved in the retention of GC B cells. Finally, the EBI2 ligand 7α,25-dihydroxycholesterol is suggested to be present at high levels in outer follicle and interfollicular regions. Briefly, normal antigen-activated B cells upregulate CCR7 and EBI2 and localize to the T-B border zone before migrating to the outer follicle region. By day 4, the downregulation of CCR7 and EBI2, and the upregulation of S1PR2 direct their movement and their confinement to the follicle center, allowing GC formation (Pereira et al., 2010; Green and Cyster, 2012). Interestingly, *S1PR2* is downregulated in FL whereas *EBI2* is similarly expressed in FL cells and normal GC B cells (our unpublished results). How the specific migratory profile of FL B cells compared to their normal counterpart, i.e., similarly high expression of CXCR4 and CXCR5, similarly low expression of EBI2, higher expression of CCR7, and lower expression of S1PR2, could be involved in their dissemination pattern is a fascinating issue. In addition, even if the mechanisms of S1PR2 lymphoma

suppressor function remain to be elucidated, S1PR2-deficient mice develop tumors displaying histologic and molecular features of GC-derived DLBCL and *S1PR2* is aberrantly mutated in 26% of human DLBCL (Cattoretti et al., 2009).

Besides migration, stromal cells provide essential survival factors to FL B cells. Among them, hedgehog (Hh) ligands are secreted by BM and LN stromal cells, including FRC and FDC, and prevent spontaneous normal and malignant GC B cell apoptosis (Sacedon et al., 2005; Dierks et al., 2007). In addition, paracrine Hh signaling induces the upregulation of the drug transporter ATP-binding cassette (ABC) G2 and could be involved in stroma-mediated chemotolerance in indolent lymphomas (Singh et al., 2010). The gain of cell-autonomous activation of Hh pathway in DLBCL compared to FL, likely contributes to stroma-independence in this aggressive disease (Kim et al., 2009). Drug resistance has also been associated to the induction by stromal cells of microRNA-181a in malignant B cells, promoting a downregulation of the proapoptotic protein Bim (Lwin et al., 2010). However, whereas regulation of miRNA expression in lymphoma cells by stromal cells is probably a more common event than previously anticipated (Lin et al., 2011), the mechanisms of this modulation remain unsolved. VLA-4-dependent adhesion to stromal cells also protects FL B cells from apoptosis induced by therapeutic antibodies, in particular the anti-CD20 antibody rituximab (Mraz et al., 2011). Interestingly, the VLA-4-ligand VCAM-1/CD106 is upregulated during lymphoid stroma differentiation with a very strong expression in FDC. BAFF, IL-15, and HGF are also produced by FDC and have been proposed to contribute to the antiapoptotic effect of stromal cells on normal and malignant GC B cell growth (Park et al., 2004; Tjin et al., 2005, 2006; Mueller et al., 2007; Lwin et al., 2009; Epron et al., 2012), whereas the role of FDC-derived Wnt5a and Notch ligands in GC-derived lymphomas has not been explored to date (Yoon et al., 2009; Kim et al., 2012).

Importantly, neoplastic B cell-stroma interaction should really be considered as a bidirectional crosstalk. We have previously demonstrated that human secondary lymphoid organs contain *bona fide* mesenchymal stromal cells (MSC) that could be committed, like BM-MSC, to FRC differentiation in response to a combination of tumor necrosis factor (TNF)-α and lymphotoxin (LT)-α1β2, the two main factors involved in the differentiation and maintenance of secondary lymphoid organs (Amé-Thomas et al., 2007). These FRC-like cells are more powerful than resting MSC to support malignant B cell survival. Moreover, malignant B cells themselves could trigger such FRC engagement, at least in part through the release of high levels of TNF-α and LT-α1β2 (Guilloton et al., 2012). In agreement, MSC obtained from invaded BM of FL patients (FL-MSC) are already committed to a FRC-like differentiation, and support more efficiently the growth of malignant B cells than MSC obtained from BM of healthy donors (HD-MSC). FL-MSC display a specific gene expression profile (Guilloton et al., 2012) and it would be of major importance to identify the mechanisms of their increased direct tumor-promoting capacity.

Finally, the activity of stromal cells on malignant B cells could be modulated depending on the cytokine context. We have demonstrated that human MSC, in particular after commitment to a FRC-like phenotype, could express indoleamine-2,3 dioxygenase in response to interferon (IFN)-γ signaling, leading to an inhibition of normal and malignant B cell proliferation but not survival (Maby-El Hajjami et al., 2009). Of note, *IFNG* is upregulated within FL microenvironment, compared to reactive non-malignant LN. Similarly, mice FRC could express nitric oxide synthase 2 after priming by IFN-γ and could thereafter inhibit T cell proliferation to control T cell expansion (Lukacs-Kornek et al., 2011; Siegert et al., 2011). In addition, it has been suggested that adhesion of lymphoma B cells to BM stroma could induce a p27-dependent reversible cell cycle arrest, promoting cell survival, and drug resistance (Lwin et al., 2007). Finally, stromal cells are well known to secrete tumor-transforming growth factor (TGF)-β that inhibits the proliferation of GC-derived B cell lines in a Smad1-dependent manner (Munoz et al., 2004). Altogether these data demonstrate that stromal cells finely regulate the behavior of lymphoma B cells that, in turn, affect their differentiation and activation.

INDIRECT PROTUMORAL ACTIVITIES OF STROMAL CELLS

Besides this direct lymphoma-supportive activity, stromal cells could also interact with non-malignant tumor-infiltrating cells and orchestrate FL cell niches. We have in particular demonstrated that FL-MSC overexpress CCL2 at both RNA and protein levels compared to HD-MSC (Guilloton et al., 2012). Interestingly CCL2 is detectable at higher levels within FL-invaded BM compared to normal BM. Moreover, CCL2 is upregulated in HD-MSC by coculture with malignant B cells in a TNF-α-dependent manner. Tumor-derived CCL2 recruits monocytes in several malignant models (Roca et al., 2009; Qian et al., 2011). We have demonstrated that CCL2 specifically contributes to monocyte recruitment by FL-MSC that in turn trigger their differentiation toward a proangiogenic and anti-inflammatory TAM-like phenotype (Guilloton et al., 2012). Furthermore, stromal cells and macrophages cooperate to sustain malignant B cell survival and proliferation *in vitro*. The reverse activity of macrophages on MSC remains to be evaluated in this context. The poor prognostic value of a high TAM content in FL patients treated with conventional chemotherapy has been reproducibly documented (Relander et al., 2010). However the precise mechanism of the supportive activity of macrophages toward neoplastic B cells remains unknown. Among the candidates, we recently demonstrated that purified FL-TAM overexpress IL-15 that cooperates with T cell-derived CD40L signal to sustain FL cell growth (Epron et al., 2012). In DLBCL, BAFF was also proposed as a monocyte-derived survival factor (Mueller et al., 2007). Another highly interesting signaling pathway is the expression of C-type lectins by myeloid cells that could crosslink the mannosylated FL BCR independently of antigen specificity (Stevenson and Stevenson, 2012).

Another crucial FL-supportive cell subset is the $CD4^{pos}$ $CXCR5^{hi}ICOS^{hi}PD\text{-}1^{hi}$ T_{FH} compartment. T_{FH} provide survival signals to antigen-selected normal GC B cells and help them to achieve class-switch recombination and terminal differentiation into antibody-secreting plasma cells (Crotty, 2011). In human, T_{FH} are also a major source of CXCL13 within follicles. We have first demonstrated that T_{FH} could be found in very high numbers within the FL cell niche and efficiently support the survival of FL cells *in vitro*, unlike non-TFH $CD4^{pos}$ T cells (Amé-Thomas et al., 2012). In agreement, FL-T_{FH} overexpress several genes directly

involved in B cell activation, in particular *CD40LG* and *IL4*, and we confirmed the importance of the T_{FH}-dependent IL4 centered pathway (Pangault et al., 2010). Interestingly, compared to tonsil-T_{FH}, FL-T_{FH} also overexpress some genes involved in the crosstalk with stromal cells, in particular *TNF* and *LTA*, that could favor the differentiation and maintenance of lymphoid stromal cells and the production of CCL2. Lymphoid stromal cells favor T_{FH} survival *in vitro* (our unpublished results) suggesting another positive loop that sustains lymphomagenesis.

Follicular lymphoma cell niche should be thus considered as a highly intricate network of heterogeneous cell subsets where stromal cells directly promote tumor growth but play also a central role as global organizers of the malignant microenvironment through the regulation of non-B cell recruitment, survival, and polarization.

STROMAL CELLS AS THERAPEUTIC TARGETS IN LYMPHOMA

Given the preeminent role of stromal cells within FL microenvironment, they progressively emerged as promising therapeutic targets in this essentially incurable disease. A first approach is to transiently mobilize malignant B cells outside their protective niches to render them more sensitive to chemo- and immunotherapy. CXCR4 antagonists, such as plerixafor (AMD3100), could be combined with drugs targeting the malignant cells, such as anti-CD20 antibodies, to increase their activity (Hu et al., 2012). Cell-penetrating lipidated peptides targeting CXCR4 intracellular domains also significantly increase the antitumoral activity of rituximab *in vitro* and *in vivo* (O'Callaghan et al., 2012). Anti-VLA-4 antibody natalizumab has been evaluated as an alternative stromal adhesion-disruptive drug (Mraz et al., 2011). Finally lenalidomide, an immunomodulatory drug clinically active in several mature B cell malignancies and under evaluation in FL, could disrupt B cell-stroma interaction through a decrease in both CXCL12 production by stromal cells (Wobus et al., 2012) and RhoH expression in malignant B cells (Troeger et al., 2012). Interestingly, the Btk inhibitor PCI-32765 has been initially developed to target BCR signaling but it also impairs the chemokine-induced adhesion and migration of primary chronic lymphocytic leukemia B cells (de Rooij et al., 2012). Similar results have been obtained with Syk and PI3K inhibitors and a recent study suggests that the Syk-mTOR pathway is involved in FL cell invasion, through the regulation of metalloproteinase-9 (MMP-9) and angiogenesis, as an upstream regulator of vascular endothelial growth factor (VEGF; Fruchon et al., 2012). Collectively, these results demonstrate that these antagonists of BCR-related kinases could also affect lymphoma cell homing and retention within specific niches. Surface molecules, growth factors, or signaling pathways involved in the bidirectional crosstalk between malignant B cells and stromal cells also represent individual attractive therapeutic targets even if the causative lymphoma-driven signals remain to be precisely defined.

CONCLUDING REMARKS

In vitro and *in vivo* studies corroborate the hypothesis that lymphoid BM and LN stromal cells play a central role in the development, growth, progression, and drug resistance in FL. The mechanisms of the crosstalk between malignant B cells and stromal cells are currently the subject of intensive research and several major questions remain unsolved. Whether or not the concept of cancer stem cells applies to this disease and, if true, what are their specific cell niches remains to be explored and could have major therapeutic consequences. Another important issue is the specificity of FL cell niches. Are they shared with premalignant follicular-lymphoma like cells or are they progressively induced by the contact with malignant cells? A detailed comparison of stromal cell niches in BM versus LN and their longitudinal study at diagnosis in the context of minimal residual disease and in relapse would be helpful to define the minimal but essential cell components that support FL cells and rescue them from drug cytotoxicity. It has also to be understood how such a supportive stromal microenvironment is assembled, in particular outside LN. The exact origin of ectopic FRC and FDC, and their relationships with local MSC should be further analyzed. Finally, developing new tools to better understand the functional heterogeneity of FL stroma may provide innovative strategies to disarm the vicious circle where stromal cells support malignant B cells that in turn convert their niche into a fully supportive microenvironment.

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Lymphatic endothelial cells – key players in regulation of tolerance and immunity

Eric F. Tewalt[1,2†], Jarish N. Cohen[1,2†], Sherin J. Rouhani[1,2†] and Victor H. Engelhard[1,2]*

[1] Department of Microbiology, Immunology, and Cancer Biology, University of Virginia School of Medicine, Charlottesville, VA, USA
[2] Carter Immunology Center, University of Virginia School of Medicine, Charlottesville, VA, USA

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Melody A. Swartz, Ecole Polytechnique Fédérale de Lausanne, Switzerland
Theresa T. Lu, Hospital for Special Surgery/Weill Cornell Medical Center, USA
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

***Correspondence:**
Victor H. Engelhard, Department of Microbiology, Immunology, and Cancer Biology, University of Virginia School of Medicine, P.O. Box 801386, Charlottesville, VA 22908-1386, USA. e-mail: vhe@virginia.edu

† Eric F. Tewalt, Jarish N. Cohen, and Sherin J. Rouhani have contributed equally to this work.

The lymphatic vasculature provides routes for dendritic cell and lymphocyte migration into and out of lymph nodes. Lymphatic endothelial cells (LEC) control these processes by expression of CCL21, sphingosine-1-phosphate, and adhesion molecules. LEC express MHC-I and MHC-II, but not costimulatory molecules, and present antigen on MHC-I via both direct and cross-presentation. Whether LEC present to CD4 T cells on MHC-II is unknown. Interestingly, LEC express antigens otherwise restricted to a small number of peripheral tissues in an autoimmune regulatory element-independent manner. Direct presentation of peripheral tissue antigens (PTA) to CD8 T cells results in abortive proliferation and deletion, due to both a lack of costimulation and active PD-L1 engagement. Autoimmunity develops when deletion is subverted, suggesting that LEC presentation of PTA could lead to human disease if PD-1 signaling were impaired by genetic polymorphisms, or aberrant costimulation occurred during inflammation. The expression of additional inhibitory molecules, which are not involved in LEC-mediated deletion, suggests that LEC may have additional immunoregulatory roles. LEC express receptors for several immunomodulatory molecules whose engagement alters their phenotype and function. In this review we describe the role of LEC in distinct anatomical locations in controlling immune cell trafficking, as well as their emerging role in the regulation of T cell tolerance and immunity.

Keywords: lymphatic endothelial cells, tolerance, trafficking, inflammation, antigen presentation

LYMPHATIC ENDOTHELIAL CELLS REGULATE THE TRAFFICKING OF DENDRITIC CELLS AND LYMPHOCYTES BETWEEN TISSUES AND SECONDARY LYMPHOID ORGANS

Lymphatic endothelial cells (LEC) compose the lymphatic vasculature, which maintains tissue fluid balance and transports antigen and dendritic cells (DC) to the lymph node (LN). Lymphatic vasculature in tissues is composed of blind-ended capillary-like structures, termed initial lymphatics (Leak, 1976), which join to form larger collecting lymphatic vessels (Schmid-Schönbein, 1990) and ultimately feed into the LN subcapsular sinus. Within the LN, LEC are localized to the subcapsular, cortical, and medullary sinuses, where they interact with incoming and exiting leukocytes (Grigorova et al., 2010).

Whereas the blood vasculature in peripheral tissues attracts leukocytes to inflamed sites to exert effector functions, the lymphatic vasculature facilitates the induction of immunity and tolerance. DC enter the initial lymphatics through portals in the basement membrane (Lämmermann et al., 2008; Pflicke and Sixt, 2009). T cells are likely to enter in a similar manner. LEC of the initial lymphatics express CCL21-Leu, one of two CCL21 isoforms in mice, in punctate clusters on the abluminal surface (Vassileva et al., 1999; Tal et al., 2011). CCL21-Leu is the primary determinant for DC entry through engagement of CCR7, but it is not expressed by LEC in LN and does not mediate migration to the node itself (Vassileva et al., 1999; Luther et al., 2000; Nakano and Gunn, 2001). Humans express a single CCL21 isoform, which encompasses the functions of both murine isoforms. LEC that form dermal lymphatics also express CXCL12, which mediates DC entry via CXCR4 (Kabashima et al., 2007).

Extravasation of lymphocytes from blood vasculature is highly integrin dependent; however, the requirement for integrin-mediated entry into the initial lymphatics is controversial. Although LEC in the initial lymphatics express ICAM-1, and engagement of immobilized CCL21 promotes DC integrin activation and adhesion to ICAM-1 *in vitro* (Schumann et al., 2010), steady-state migration of DC into LN *in vivo* does not require integrin engagement (Lämmermann et al., 2008). This suggested other adhesion molecules may be involved. Recently, it was discovered that DC migration into lymphatic vessels and into the T cell zone of the LN requires CLEC-2 binding to podoplanin, a glycoprotein expressed by lymphatic vessel and LN-LEC as well as fibroblastic reticular cells (FRC; Acton et al., 2012). Other potential candidates include the scavenger receptor CLEVER-1, which has been implicated in the transmigration of T cells into the lumen of initial lymphatic vessels (Salmi et al., 2004). Thus, LEC-mediated entry into the afferent lymphatics is distinct from blood vascular endothelium-mediated entry of leukocytes into tissues.

Lymphatic endothelial cells also mediate the migration of DC into the LN. Once inside the collecting vessels, DC, and presumably T cells, detach from LEC and rhythmic vessel contractions propel DC toward the LN (Randolph et al., 2005). LN-LEC as well as FRC make CCL19 and CCL21-Ser, which mediate direct entry

into the LN (Vassileva et al., 1999; Luther et al., 2000; Nakano and Gunn, 2001). It has been hypothesized that LEC in the collecting lymphatics also make these chemokines (Randolph et al., 2005). Additionally, LEC in the subcapsular sinus express CCL1, which can facilitate cell entry into LN (Qu et al., 2004; Kabashima et al., 2007). Once in the subcapsular sinus, DC enter the LN cortex immediately, while T cells enter the LN paracortex via medullary lymphatic sinuses (Braun et al., 2011). It is unclear how these different routes of entry are regulated. We have found that medullary and subcapsular LEC differentially express MAdCAM-1 (unpublished). These results suggest that cellular trafficking into and through the lymphatics is based on anatomically and molecularly distinct subpopulations of LEC that have different functional properties.

Lymphatic endothelial cells also control egress of lymphocytes from the LN. Upon LN entry, lymphocytes downregulate CCR7 and exit the LN through cortical and/or medullary lymphatic sinuses (Britschgi et al., 2008; Pham et al., 2008). Lymphocytes adhere to LEC and probe the sinus lumen prior to exit. Candidate molecules for adherence include CLCA1 (Furuya et al., 2010) and mannose receptor (MR; Irjala et al., 2001). Both of these molecules are more highly expressed on LN-LEC than tissue LEC (Irjala et al., 2001; unpublished). The binding partners for CLCA1 include LFA-1 and MAC-1, and *in vitro* studies demonstrated a greater role for CLCA1–LFA-1 interactions than ICAM-1–LFA-1 interactions in lymphocyte adhesion to LEC cell lines (Furuya et al., 2010). Also, MR binds CD62L, and blockade of MR on frozen LN-sections decreased lymphocyte adherence to LEC (Irjala et al., 2001). LEC are also the sole producers of sphingosine-1-phosphate (S1P) in the LN which promotes lymphocyte egress by binding to S1P1 (Pham et al., 2008; Cyster and Schwab, 2012). Lymphocyte migration and adherence to LEC, as well as probing of the cortical sinus lumen, is S1P independent. Instead, S1P is necessary for commitment to lumen entry (Grigorova et al., 2009). Thus, although the role for LEC S1P in mediating lymphocyte egress is well established, the importance of CLCA1 and MR in mediating lymphocyte egress *in vivo* remains to be examined. Collectively, these results establish the importance of chemokine and sphingolipid ligands released by LEC in controlling all phases of DC and/or lymphocyte migration in and around LN. However, the involvement of integrins or other molecules that could mediate additional adhesive interactions remains to be clarified.

LN-LEC FUNCTION AS SPECIALIZED ANTIGEN PRESENTING CELLS

Lymphatic endothelial cells share a number of characteristics with professional antigen presenting cells (APC). LEC in LN, but not those in tissue lymphatics, constitutively express MHC-II molecules (Amatschek et al., 2007; Tripp et al., 2008; unpublished), suggesting there is a functional immunological difference between LEC in these two locations. LEC also endocytose and cross-present MHC-I antigens, although they do so less efficiently than professional APC (Lund et al., 2012). However, LEC do not express costimulatory molecules CD80, CD86, 4-1BBL, or OX40L extracellularly or intracellularly (Tewalt et al., 2012). LEC express CD70 intracellularly but it is unknown whether this is functional. LEC also express ICAM-1 and LFA-3, and LFA-3 can provide costimulation to enhance IL-2 secretion from activated T cells *in vitro* (Nörder et al., 2012). The lack of costimulatory molecule expression on LEC predisposes them to induce tolerance (see below). While professional APC upregulate costimulatory molecules upon toll-like receptor (TLR) stimulation, TLR3 ligation does not substantially upregulate CD80 or CD86 expression on LEC (Fletcher et al., 2010). It is unknown whether LEC can upregulate costimulatory molecules under other inflammatory conditions, enabling them to induce immunogenic responses. Thus, steady-state LN-LEC are semi-professional APC: they express MHC-II, activate naïve T cells and cross-present antigen, but do not constitutively express costimulatory molecules and are not known to induce outcomes other than tolerance.

Although LN-LEC express MHC-II molecules, there is limited information about the functionality of the class II processing pathway and antigen presentation to CD4 T cells. Peptide-pulsed LEC induce proliferation of naïve CD4 cells, indicating the MHC-II molecules are functional (unpublished). LEC endocytose and process exogenous antigens leading to cross-presentation on MHC-I (Lund et al., 2012), but it is not known whether this also leads to presentation on MHC-II. However, cultured human LEC do not induce allogeneic proliferation of CD4 T cells (Nörder et al., 2012). In mice that selectively express β-galactosidase (β-gal) in LEC and FRC, adoptively transferred β-gal specific CD4 T cells proliferate (Onder et al., 2011), but it was not determined whether this was due to direct antigen presentation by LEC and/or FRC, or to antigen endocytosis and presentation by hematopoietic cells. We have found that in mice expressing β-gal under control of the LEC-specific Lyve-1 promoter, proliferation of β-gal specific CD4 cells is due to presentation by hematopoietic cells (unpublished). Thus, LEC can provide antigens to DC for MHC-II presentation, analogous to medullary thymic epithelial cell (mTEC) handoff of antigens to thymic DCs (Koble and Kyewski, 2009). Further work will elucidate whether the failure of LEC to induce CD4 proliferation is due to a defect in MHC-II processing and presentation, active suppression by regulatory T cells or by molecules such as IDO or nitric oxide (NO), or induction of anergy.

LEC AND PTA EXPRESSION

Recently, we and others have shown that multiple subsets of LN stromal cells (LNSC), including LEC, express peripheral tissue antigens (PTA) that are otherwise restricted to one or a few tissues such as skin, pancreas, gut, and central nervous system (Lee et al., 2007; Nichols et al., 2007; Gardner et al., 2008; Cohen et al., 2010; Fletcher et al., 2010). Microarray analysis comparing LN-LEC and LN blood endothelial cells (BEC) identified several additional candidates for LEC-expressed PTA (unpublished). The majority of these PTA were overexpressed in LN-LEC compared to tissue LEC, suggesting the LN microenvironment plays a role in determining PTA expression. Presentation of epitopes derived from PTA by LEC, FRC, and extrathymic autoimmune regulatory element (Aire) expressing cells (eTAC) leads to CD8 T cell abortive proliferation and deletion (Lee et al., 2007; Nichols et al., 2007; Gardner et al., 2008; Cohen et al., 2010; Fletcher et al., 2010). Collectively, these findings suggest that LN-LEC and other PTA-expressing LNSC perform a function in the periphery

analogous to that of mTEC in the thymus in promoting systemic tolerance.

The molecular mechanisms controlling PTA expression by LEC have not yet been established. PTA expression by LEC is not dependent on the Aire, which controls PTA in mTEC and eTAC (Anderson et al., 2002; Gardner et al., 2008; Cohen et al., 2010). One LEC-expressed PTA, Ppy, is regulated by Deaf-1, a member of the SAND transcription factor family that includes Sp100, Aire, and NucP41/75 (Yip et al., 2009). However, Deaf1 has not been shown to regulate other LEC-expressed PTA. Deaf1 and other SAND family members are expressed at comparable levels in all LNSC subsets (Fletcher et al., 2010; unpublished), so it is unclear how Deaf1 would regulate the expression of non-overlapping PTA in different LNSC populations. However, it is also unknown how Aire controls distinct PTA repertoires in mTEC and eTAC. It is possible that the control of non-overlapping PTA repertoires in different cells by the same transcriptional regulator is due to differences in chromosomal positioning and/or epigenetic modifications. Another possibility is that multiple transcription factors play a role in LNSC PTA expression.

CONSEQUENCES OF CD8 ANTIGEN PRESENTATION BY LEC

As mentioned above, despite sharing some characteristics with professional APC, antigen presentation by LEC leads to tolerance. Direct presentation of tyrosinase by LN-LEC induces abortive proliferation and deletion of tyrosinase-specific T cells *in vivo* (Nichols et al., 2007; Cohen et al., 2010; **Figure 1**). Utilizing β-gal driven under control of the LEC specific Lyve-1 promoter, LEC also induce abortive proliferation and deletion of β-gal specific CD8 T cells (unpublished). Presentation of exogenous antigen by LEC was also shown to induce CD8 apoptosis *in vitro* (Lund et al., 2012). In other models, antigen level determines whether CD8 T cells undergo anergy or deletion (Redmond et al., 2005). It remains to be clarified whether LEC can induce outcomes other than deletion.

We have recently elucidated the mechanism by which LEC induce abortive proliferation and deletion of PTA-specific CD8 T cells (Tewalt et al., 2012). LEC-mediated deletion requires both a lack of costimulation and signaling through the PD-L1:PD-1 pathway (**Figure 1**). Lack of costimulation leads to rapid and elevated expression of PD-1 on T cells. Signaling through PD-1 blocks upregulation of IL-2R, which is at least in part responsible for apoptotic death (Tewalt et al., 2012). PD-1 signaling had previously only been associated with downregulation of IL-2 itself (Carter et al., 2002; Chikuma et al., 2009). These results integrate previous demonstrations that tolerance is due either to a lack of costimulation (Harding et al., 1992; Hawiger et al., 2001; Hernandez et al., 2002) or to engagement of inhibitory molecules (Martin-Orozco et al., 2006; Nurieva et al., 2006; Goldberg et al., 2007; Tsushima et al., 2007; Liu et al., 2009; Reynoso et al., 2009), and shows that they are actually interdependent pathways. Importantly, antigen presentation by LEC leads to the development of autoimmune disease when PD-L1 is blocked or exogenous costimulation is provided (Tewalt et al., 2012). Based on previous findings that LEC express multiple PTA (Cohen et al., 2010; Fletcher et al., 2010), this opens the possibility that dysregulation of their tolerance inducing capability might influence the development of some human autoimmune diseases. Finally, LEC express ligands for additional inhibitory pathways, including HVEM:BTLA/CD160, MHC-II:LAG-3, and CD48:2B4. These pathways are known to induce additional forms of tolerance, including anergy and Treg formation (Huang et al., 2004; Grosso et al., 2007; Liu et al., 2009). They are not involved in LEC-mediated abortive proliferation and deletion, but their expression suggests that LEC may have additional immunoregulatory roles under steady-state conditions.

We have also investigated the anatomical basis of CD8 abortive proliferation and deletion. LN-LEC express higher levels of PD-L1 than other LNSC populations or tissue lymphatic LEC (Tewalt et al., 2012; unpublished). The low level of PD-L1 and PTA expression by tissue LEC suggests that they are unlikely to induce tolerance. In addition, medullary and subcapsular sinus LEC express higher levels of PD-L1 than those in the cortical sinus.

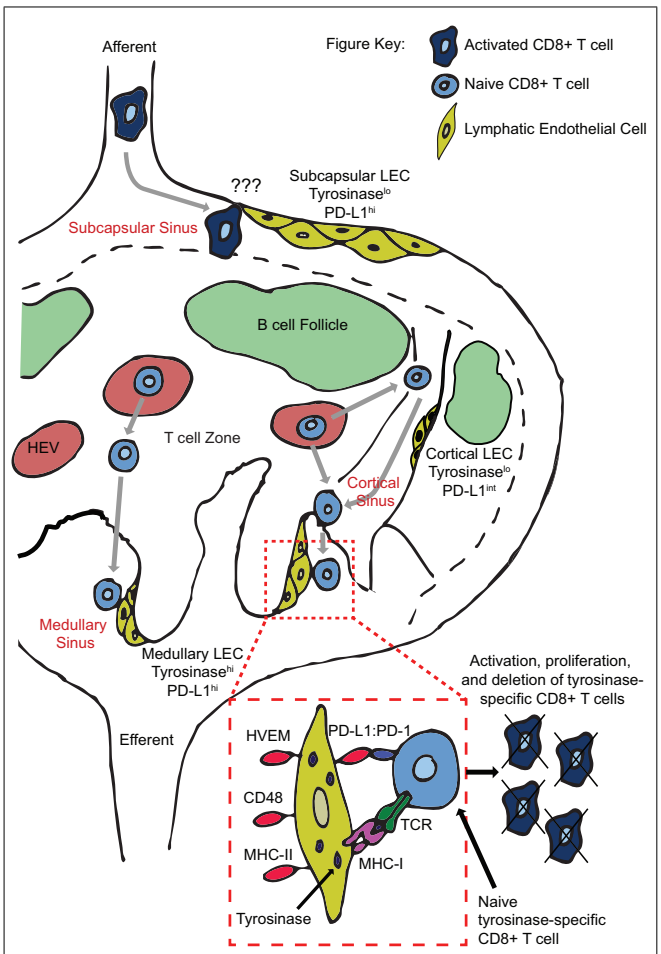

FIGURE 1 | Peripheral tolerance induction by anatomically distinct subsets of lymphatic endothelial cells during T cell trafficking through lymph nodes. Naïve T cells enter the LN through high endothelial venules, and exit via cortical and/or medullary sinuses. Tyrosinase presentation occurs on medullary but not cortical sinus LEC, leading to proliferation and PD-L1 mediated deletion of tyrosinase-specific T cells. Deletion may occur based on engagement of PD-L1^{hi} medullary sinus LEC in the same LN as activation occurs, and/or on PD-L1^{hi} subcapsular sinus LEC in downstream LN.

Importantly, tyrosinase epitope presentation to CD8 T cells is confined to medullary sinus LEC, consistent with their higher expression of tyrosinase message (unpublished; **Figure 1**). This suggests that abortive proliferation and deletion occurs as T cells attempt to exit the LN. Whether the lower level of PD-L1 expressed by cortical sinus LEC is also capable of inducing tolerance to antigens expressed at a higher level than that of tyrosinase remains to be examined. However, the high-level expression of PD-L1 by medullary sinus LEC suggests they may also induce the deletion of egressing T cells activated by other LN-resident tolerogenic APC that express low levels of PD-L1, such as FRC.

OTHER FACETS OF IMMUNE CROSS-TALK BETWEEN LEC AND LEUKOCYTES

Lymphatic endothelial cells express multiple TLR, as well as receptors for inflammatory cytokines (Link et al., 2007; Pegu et al., 2008; Kataru et al., 2011), which enable them to respond to changes in tissue and LN microenvironments. Stimulation of cultured tissue or LN-LEC with TLR agonists, TNFα, IL-1, or infection with cytomegalovirus induces the expression of numerous chemokines (Pegu et al., 2008; Sawa et al., 2008a,b; Fiorentini et al., 2011; Garrafa et al., 2011), but the role of this enhanced expression *in vivo* has not been established. In contrast to the steady-state, DC entry into LN under inflammatory conditions is dependent upon ICAM-1 and VCAM-1, which are also upregulated on LEC by proinflammatory agents (Johnson et al., 2006; Pegu et al., 2008; Sawa et al., 2008a,b; Fiorentini et al., 2011; Garrafa et al., 2011). Inflammation also leads to proliferation and sprouting of LEC, a process known as lymphangiogenesis, by inducing the production of ligands for VegfR2, VegfR3, and the lymphotoxin β receptor (LtβR; Angeli et al., 2006; Furtado et al., 2007; Kim et al., 2009; Flister et al., 2010; Mounzer et al., 2010). Lymphangiogenesis following skin inflammation aids in the resolution of inflammation by increasing lymph flow and cell migration to the draining LN, but lymphangiogenesis following peritoneal inflammation reduces lymphatic drainage (Kataru et al., 2009; Kim et al., 2009). LN lymphangiogenesis has been shown to promote lymphocyte egress during prolonged inflammation (Tan et al., 2012). This suggests that one of the primary functions of LEC exposed to inflammatory agents is to attract a range of innate and adaptive immune cells into lymphatics to broaden and sustain ongoing immune responses.

In addition to enhancing leukocyte migration during inflammation, LEC attenuate T cell responses. TNFα activated LEC downregulate CD86 on DC, impairing their ability to induce T cell proliferation (Podgrabinska et al., 2009). LEC also limit T cell proliferation (Khan et al., 2011; Lukacs-Kornek et al., 2011) through release of NO in response to IFNγ and TNFα (Lukacs-Kornek et al., 2011). However, T cells undergoing LEC-mediated abortive proliferation and deletion produce little to no IFNγ and TNFα (unpublished). Thus, NO is unlikely to participate in LEC-mediated peripheral tolerance, but may limit the size of an immune response. Cortical sinus LEC, which express an intermediate level of PD-L1, upregulate PD-L1 in response to TLR3 ligation and IFNγ to match the high levels seen on medullary and subcapsular sinus LEC (unpublished). This could broaden the anatomical locations in the LN in which T cell tolerance occurs, or provide a means to protect cortical sinus LEC from being destroyed by emigrating effector T cells.

Inflammation modulates the expression of PTA in LEC, but not in a consistent manner. TLR3 ligation causes LEC, as well as FRC, to downregulate proteolipid protein but upregulate α-fetoprotein (Fletcher et al., 2010). However, double-negative LNSC upregulated both PTA, while BEC upregulated only one. The effect of inflammatory signals on other PTA expressed in LEC has not been examined. Downregulation of PTA could provide a means to avoid the induction of autoimmunity resulting from the increased availability of costimulation in an inflamed LN. Conversely, upregulation of PTA, particularly in the context of enhanced expression of PD-L1, could provide a means to enforce tolerance more stringently.

CONCLUDING REMARKS

Recent work has conclusively demonstrated that LEC play a variety of active roles in shaping immune responses and tolerance. LEC guide lymphocyte and DC trafficking into and out of the LN, and inflammation increases their ability to attract cells. LEC also actively enforce CD8 T cell tolerance to PTA through their high-level expression of PD-L1 and lack of costimulatory molecules. It will be immensely interesting to determine the ways in which other inhibitory molecules expressed by LEC control T cell fate. In addition, the general immunoregulatory role of LEC will be more definitively established by understanding their ability to directly induce CD4 tolerance or to serve as a reservoir of PTA for presentation by DC. Furthermore, the identification of a second transcriptional control mechanism, in addition to Aire, will provide the possibility to understand the basis for additional human autoimmune diseases. Finally, LEC represent attractive therapeutic targets to control autoimmunity and prevent transplant rejection or to enhance tumor immunotherapy.

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Tight control – decision-making during T cell–vascular endothelial cell interaction

Sonja Firner, Lucas Onder, Veronika Nindl and Burkhard Ludewig*

Institute of Immunobiology, Kantonsspital St. Gallen, St. Gallen, Switzerland

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Theresa T. Lu, Hospital for Special Surgery/Weill Cornell Medical Center, USA
Jonathan Bramson, McMaster University, Canada

****Correspondence:***
Burkhard Ludewig, Institute of Immunobiology, Kantonsspital St. Gallen, 9007 St. Gallen, Switzerland.
e-mail: burkhard.ludewig@kssg.ch

Vascular endothelial cells (ECs) form the inner layer of blood vessels and exert crucial functions during immune reactions including coagulation, inflammation, and regulation of innate immunity. Importantly, ECs can interact with T cells in an antigen-specific, i.e., T cell receptor-dependent manner. In this review, we will discuss EC actions and reactions during acute inflammation and focus on the interaction of T cells with ECs at two vascular sites: the high endothelial venule (HEV) of lymph nodes, and the vascular lesion during transplant vasculopathy (TV). HEVs are characterized by a highly active endothelium that produces chemoattracting factors and expresses adhesion molecules to facilitate transit of lymphocytes into the lymph node (LN) parenchyma. Yet, T cell–EC interaction at this anatomical location results neither in T cell activation nor tolerization. In contrast, the endothelium at sites of chronic inflammation, such as solid organ transplants, can promote T cell activation by upregulation of major histocompatibility complex (MHC) and costimulatory molecules. Importantly, a major function of ECs in inflamed tissues must be the maintenance of vascular integrity including the efficient attenuation of effector T cells that may damage the vascular bed. Thus, antigen-specific T cell–EC interaction is characterized by a tightly controlled balance between immunological ignorance, immune activation, and tolerization.

Keywords: high endothelial venules, inflammation, transplantation, costimulation

INTRODUCTION

The inner layer of blood vessels, the intima, consists of endothelial cells (ECs) that are attached to the basal membrane. The major function of the endothelium is to control the exchange of gas, metabolites, signal-transmitting molecules, and cells between blood and the tissues. Coping with this range of transport functions requires functional and phenotypical diversity. Hence, ECs can appear as fenestrated endothelium in liver sinusoids that permit free exchange of cells, molecules, and metabolites (Crispe, 2009) or as tight vascular endothelium in the central nervous system that forms a part of the blood–brain barrier (Bechmann et al., 2007). Thus, the particular function of an EC strongly depends on its anatomical location. However, the functional repertoire of ECs can be efficiently modulated by inflammatory stimuli including microbial pathogens or their products or inflammatory mediators derived from other cells.

During an immune reaction, ECs regulate coagulation, react to and secrete acute inflammatory mediators, and coordinate trafficking of leukocytes from the blood stream into the tissue (Danese et al., 2007). Since ECs express not only major histocompatibility complex (MHC) class I and II molecules, but also an array of different costimulatory molecules (Pober and Tellides, 2012), direct and antigen-specific interaction with T cells is possible. Given the diverse phenotypes and functions of ECs together with their functional modulation during inflammatory reactions, it is not surprising that the interaction of T cells with ECs can range from activation to tolerization (Pober and Tellides, 2012). A third interaction pattern is referred to as immunological ignorance (Zecher and Lakkis, 2008). To illustrate these different forms of T cell–EC communication involving MHC–T cell receptor (TCR) contact, we will focus at two distinct vascular sites: the high endothelial venule (HEV) of lymph nodes (LNs), and the vascular lesion during chronic transplant rejection. We propose that the major principle underlying the antigen-specific communication of T cells with ECs is the maintenance of vascular integrity, i.e., the tight control over the exchange of fluids, molecules, and cells between blood and the tissues.

ENDOTHELIAL CELLS DURING ACUTE INFLAMMATION

The principle of tight control over vascular integrity applies as well to the rapid functional adaptation of ECs during acute inflammation. The basal functions of ECs under homeostatic conditions are the regulation of blood flow and vessel permeability (Dejana et al., 2009). A major control mechanism at the resting state is the inhibition of coagulation which is achieved through the expression of an array of inhibitory molecules including thrombomodulin and heparan sulfate proteoglycans (van Hinsbergh, 2012). Blood flow is regulated by nitric oxide synthase 3 (NOS3) in ECs through the production of nitric oxide, a pathway that alters the tone of vascular smooth muscle cells (Gkaliagkousi et al., 2009). Resting ECs do generally not interact with leukocytes or at least minimize the interaction with leukocytes through the low expression of adhesion molecules such as vascular cell-adhesion molecule 1

Abbreviations: FRC, fibroblastic reticular cell; LN, lymph node; mHAg, minor histocompatibility antigen; SLO, secondary lymphoid organ.

(VCAM1) and intercellular adhesion molecule 1 (ICAM1) and the sequestration of adhesion molecules and chemokines in special intracellular storage compartments. However, ECs can react efficiently to perturbations and switch from the resting to an activated state during acute inflammation (Danese et al., 2007; Pober and Sessa, 2007; Lemichez et al., 2010).

Infectious agents can trigger EC activation directly by infection resulting in stimulation of ECs by microbial products sensed via pathogen recognition receptors (Paolillo et al., 2012). Such initial triggers lead to the activation of multiple, partially self-amplifying cascades. For example, the EC growth factor angiopoietin-2 primes ECs to higher responsiveness to tumor necrosis factor leading, in turn, to enhanced leukocyte adhesion (Fiedler et al., 2006). A particular feature of EC activation is the swiftness of their reaction which is achieved through the release of adhesion molecules and inflammatory mediators from their intracellular storage and a rapid change in the gene expression profile (Pober and Sessa, 2007). Further amplification of the initial EC activation is achieved through adherence of platelets. Following contact with activated ECs, platelets release immune-activating factors such as CCL5 (Laubli et al., 2009) which further activate the endothelium and help to recruit immune cells. In addition, platelets interact with the activated endothelium through membrane-bound and soluble CD154, the ligand of CD40, thereby mimicking the interaction of T cells with the endothelium (Henn et al., 1998; Buchner et al., 2003). Importantly, ligation of CD40 on ECs by platelet-derived CD154 promotes tissue factor induction and coagulation (Slupsky et al., 1998).

The rapid local activation of ECs through several cascading systems is most likely a key step during systemic infection and helps to contain the pathogen (Lemichez et al., 2010). However, such powerful activation circuits must be controlled to prevent overshooting clotting reactions, excessive leakage of blood fluids into the tissues, or massive neutrophil degranulation. Indeed, EC activation is restricted by particular regulatory factors such as the Down syndrome critical region gene 1 (DSCR1) which is induced by inflammatory mediators including vascular endothelial growth factor (VEGF) or thrombin (Hesser et al., 2004). Lack of DSCR1 results in elevated ICAM1, VCAM1 and E-selectin expression on ECs and renders ECs more susceptible to apoptosis. Consequently, partially unrestrained EC activation in DSCR1-deficient mice is associated with increased lethality under septic conditions (Minami et al., 2009). Thus, attenuation of EC activation – following a first wave of immune-stimulation – is critical to maintain vascular barrier integrity during acute inflammation. EC-specific mechanisms that maintain barrier integrity include the stabilization of vascular endothelial cadherin function through increased association with p120 catenin subsequently leading to increased resistance against cytokine storm-associated vascular damage (London et al., 2010). A further important property of ECs that most likely improves vascular barrier integrity is their constitutively high resistance to apoptosis, even following exposure to inflammatory stimuli (Bannerman et al., 2001). Taken together, during acute inflammation, ECs can switch rapidly from the resting state into an activated, proinflammatory state that is important for the initiation of the global tissue-defense reaction. However, excessive promotion of the potentially self-promoting inflammatory reactions at the vascular wall must be efficiently attenuated to preserve vascular integrity. We will use the example of chronic transplant rejection to illustrate that the maintenance of vascular integrity through attenuation of endothelial damage by negative immune regulation applies also the antigen-specific interactions between T cells and ECs. Before that, however, we will briefly elude to a third interaction pattern between T cells and ECs, namely attachment and transmigration without cognate or limited MHC–TCR interaction.

EC–T CELL INTERACTION IN HIGH ENDOTHELIAL VENULES

The induction of efficient T cell responses is fostered by the concentration of both antigen and T cells bearing the appropriate TCR in secondary lymphoid organs (SLOs; Junt et al., 2008). To maximize the chance for successful encounter with their antigen, naïve T cells constantly recirculate through different SLOs (Mempel et al., 2006). It is noteworthy that not only the nature of the SLO, e.g., LNs or Peyer's patches (PPs) critically impinge on EC–T cell interaction, also differences between anatomically distinct LNs results in qualitatively different interaction patterns between T cells and EC (Buettner and Bode, 2011). The ability of lymphocytes to enter LNs and PPs depends on the presence of specialized post-capillary venules. These HEVs are formed by specialized ECs that have been described as paracortical, vascular endothelium containing cuboidal ECs (Anderson and Anderson, 1976). HEV ECs develop a polarized organization with luminal adhesion molecules such as ICAM1 which function as anchors for cells circulating in the blood and expressing the appropriate ligands (Blum and Pabst, 2006). Tethering and rolling of lymphocytes on the HEV endothelium is further enhanced in certain anatomical locations through the expression of particular adhesion molecules such as the mucosal addressin cell adhesion molecule-1 (MAdCAM1). Expression of this mucosal addressin on HEVs in the mesenteric LN and PPs mediates the interaction with $\alpha_4\beta_7$ integrin on a subset of lymphocytes and facilitates homing of T cells with a more gut-restricted TCR repertoire (Sigmundsdottir and Butcher, 2008). Furthermore, the endothelium of HEVs produces the constitutive chemokines CCL19, CCL21, CXCL12, and CXCL13. These small chemoattractant cytokines bind to G protein-coupled chemokine receptors on lymphocytes and foster thereby T cell migration, activation, and proliferation (Hayasaka et al., 2010). Hence, the endothelium of HEVs facilitates the highly efficient transit of T cells from the blood stream into the LN or PP parenchyma. In other words, HEV ECs are constantly in close contact with naïve T cells and other migrating hematopoietic cells, a feature that is not shared with other ECs.

Endothelial cells arise from endothelial progenitor cells that are recruited from the mesodermal layer and form the large vasculature of the early mammalian embryo. HEV ECs develop together with the LN when lymphatic endothelial progenitors leave the cardinal vein and form the lymph sac, the primordial tissue of the lymphatic system (van de Pavert and Mebius, 2010; Domigan and Iruela-Arispe, 2012). During LN development, mesenchymal and hematopoietic cell-derived signals initiate chemokine expression and LN growth (van de Pavert and Mebius, 2010). Since lymphotoxin beta receptor (LTβR)-deficient mice completely lack peripheral LNs, it has been suggested that

LTβR-signaling in mesenchymal organizer cells is crucial for LN development (Roozendaal and Mebius, 2011). Indeed, hematopoietic cell-derived lymphotoxin induces the expression of cytokines and chemokines in non-hematopoietic stromal cells (Ansel et al., 2000). Thus, HEV ECs develop in an environment of highly active signal exchange between hematopoietic and non-hematopoietic cells. Hence the extensive interaction of HEV ECs with T cells may function not only via adhesion molecules or chemokine–chemokine receptor pairs, but also via antigen-specific TCR–MHC contact.

Antigen presentation and activation of T cells is a well-controlled process that relies to a large extent on a division of labor between different myeloid cell subsets (Turley et al., 2010). It is possible that a similar specialization in the display of self-antigens for the tolerization of autoreactive T cells can be assigned to different stromal cell subsets. Indeed, several studies suggest that stromal cells such as lymphatic ECs or T cell zone fibroblastic reticular cells (FRC) in LNs express peripheral tissue antigens (PTAs) in order to mediate peripheral tolerance to autoreactive T cells (Gardner et al., 2008; Cohen et al., 2010; Fletcher et al., 2010). However, it is unlikely that HEV ECs can perform a similar task because ubiquitous expression of an antigen in ECs driven by the Tie2 promoter does neither lead to activation nor tolerization of antigen-specific $CD8^+$ T cells (Bolinger et al., 2008). Likewise, FRCs can present viral antigen during systemic infection with the non-cytopathic lymphocytic choriomeningitis virus leading to the elimination of these stromal cells by antiviral $CD8^+$ effector T cells (Mueller et al., 2007; Scandella et al., 2008). However, HEV ECs seem not to be affected by immunopathological $CD8^+$ T cells during this viral infection. On the contrary, stimulation of HEV ECs via LTβR through B cell-derived lymphotoxin was found to be very important for the adaptation of the LN, i.e., for efficient LN remodeling (Kumar et al., 2010). Taken together, in a homeostatic LN, T cells appear not to communicate with HEV ECs in an antigen-specific manner, i.e., the interaction pattern of immunological ignorance is predominant. Whether HEV ECs are specifically protected from immunopathological T cell attack or whether they remain immunologically ignored even during systemic viral infection remains to be determined. Clearly, ECs do not remain immunologically ignored during transplant rejection.

ANTIGEN PRESENTATION BY ECs DURING CHRONIC TRANSPLANT REJECTION

Graft rejection after solid organ transplantation is characterized by the recognition of the donor tissue as foreign and subsequent attack by the host immune system. The immunological reaction can be directed against parenchymal cells or cells of the vascular system. Acute graft rejection (in the absence of immunosuppression) occurs usually 1–2 weeks following transplantation. These grafts characteristically contain dense leukocyte infiltrates in the parenchyma and show extensive vessel thrombosis. Chronic immunological reactions of the host against the graft that may occur despite immunosuppression, can be directed against the parenchyma resulting in progressive fibrotic replacement of graft tissue (Libby and Pober, 2001). However, more frequent is the chronic immune-mediated damage of blood vessels. Despite advances in immunosuppressive therapies for acute allograft rejection, successful long-term survival of transplanted solid organs is still hampered by late graft failure. Chronic graft rejection is caused to a large extent by host-anti-graft immune responses against the graft vasculature leading to transplant vasculopathy (TV; Cailhier et al., 2006). Since ECs of the transplanted organ are the first graft cells encountered by the host immune system and ECs are preserved in long-term allografts (Al-Lamki et al., 2008), it is most likely that T cell responses against ECs crucially contribute to the process of chronic vascular rejection (Libby and Pober, 2001).

It has been demonstrated that ECs can act as antigen-presenting cells (APC) to $CD8^+$ T cells mainly via the direct pathway (i.e., recognition of allo-MHC complexes). However, *in vitro* experiments suggest that ECs directly stimulate mainly pre-activated memory but not naïve $CD8^+$ T cells (Dengler and Pober, 2000). EC-specific $CD8^+$ T cells have been shown to exist *in vivo* and are able to mediate significant EC damage in human graft-versus-host disease (Biedermann et al., 2002). Furthermore, it has been demonstrated in a transgenic mouse model that MHC class I expression on non-hematopoietic cells of the graft is sufficient to initiate $CD8^+$ T cell activation and acute allograft rejection (Kreisel et al., 2002). These results from a TCR-transgenic system have been interpreted as evidence for the direct activation of $CD8^+$ T cells by ECs outside of SLOs. However, direct recognition of allo-MHC complexes by the highly frequent alloreactive T cells can only occur under conditions of MHC disparity, i.e., in allogeneic mixed-lymphocytes reactions *in vitro* or following transplantation of MHC mismatched organs.

Whereas T cell precursor frequencies against the "major" alloantigens, i.e., directly recognized MHC molecules, are in a range of 0.1–10%, T cell precursor frequencies against minor histocompatibility antigens (mHAg) are low (Heeger, 2003). It is noteworthy that due to the almost complete MHC matching procedures in transplantation medicine (Cecka, 2010), transplant rejection is mainly driven by T cell reactions against mHAg (Spencer et al., 2010). Hence, EC–T cell interaction during TV is characterized by low T cell precursor frequencies, whereby the T cells most likely recognize antigen presented by ECs. To model this situation experimentally, expression of a model antigen can be directed to vascular ECs using the Tie2 promoter (Tie2-LacZ mice) (Schlaeger et al., 1997). Using this EC-specific mHAg expression system in combination of mHAg-specific TCR transgenic T cells, it could be shown that mhAg presentation by EC does neither precipitate T cell activation nor tolerization (Bolinger et al., 2008), i.e., tolerizing effects on $CD8^+$ T cells were not observed, although resting mhAg-presenting ECs in Tie2-LacZ mice provided signal 1 (i.e., antigen) in the absence of signal 2 (i.e., costimulation). Hence, in the absence of appropriate stimulation, naïve $CD8^+$ T cells ignore their antigen presented solely on ECs (**Figure 1A**). In principle, it is possible that ECs possess an impaired capacity to present immunodominant peptides (Kummer et al., 2005) and therefore fail to interact with naive $CD8^+$ T cells. However, once appropriately activated, T cells can form invadosome-like protrusions that permit probing of the MHC:peptide complexes expressed on ECs (Carman et al., 2007; Sage et al., 2012).

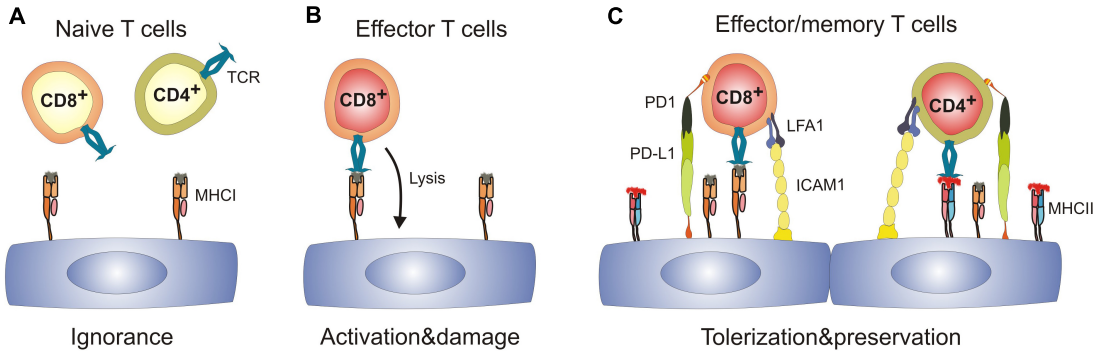

FIGURE 1 | Spectrum of antigen-specific T cell–EC interactions. **(A)** Naïve T cells immunologically ignore resting ECs which express MHC class I, but are MHC II^{low} or MHC II^{-}. **(B)** Effector CD8^{+} T cells can recognize cognate antigen on ECs leading to EC activation and may potentially damage the vascular bed. **(C)** Activated ECs upregulate expression of MHC II, adhesion molecules and ligands of T cell co-inhibitory molecules. Engagement of co-inhibitory molecules can lead to tolerization of both CD8^{+} and CD4^{+} T cells and hence preserves the integrity of the EC layer. ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; MHC I/II, major histocompatibility complex I/II; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; TCR, T cell receptor.

The presence of SLOs is critical for the generation of transplant-specific T cells (Lakkis et al., 2000). Furthermore, priming of mHAg-specific CD8^{+} T cells has been shown to be strictly dependent on cross-presenting CD11c^{+} DCs (Bolinger et al., 2008; Wang et al., 2011). In addition, other myeloid cells can enter the graft to sample antigen and return to the local LN to initiate T cell responses (Celli et al., 2011). Thus, ECs in transplanted organs expressing a particular antigen can become targets for CD8^{+} effector T cells (**Figure 1B**) once professional APCs have presented the peptide within SLOs. As a consequence, grafts can develop a vascular inflammatory disease with neointima formation and vascular occlusion, the pathological signs of chronic vascular rejection (Bolinger et al., 2010).

However, antigen recognition on ECs does not necessarily lead to aggression. ECs could negatively regulate immune responses by utilizing co-inhibitory receptors such as Herpes simplex entry mediator (HVEM; Murphy and Murphy, 2010). Clearly, programmed cell death ligand-1 (PD-L1) expression on mHAg-presenting ECs is strongly upregulated during inflammation (Bolinger et al., 2010). Importantly, PD-L1 expression on ECs is regulated to a large extent via the IFN-γ receptor (Grabie et al., 2007; Bolinger et al., 2010) and the efficacy of PD-1-dependent CD8^{+} T cell down-tuning correlates with the levels of systemic IFN-γ (Bolinger et al., 2010). As a consequence, upregulation of negative regulatory factors such as PD-L1 on ECs provides a potent negative feedback for EC-specific CD8^{+} T cells and thereby reduces vascular pathology (**Figure 1C;** Bolinger et al., 2010). Importantly, this mechanism may not only operate in chronic transplant rejection, but may also limit endothelial destruction and, thus fatal parenchymal damage during viral infection (Iwai et al., 2003; Barber et al., 2006). Taken together, expression of co-inhibitory molecules on ECs during inflammatory processes appears to be a central regulatory step in the control of EC-specific CD8^{+} T cell responses and hence, in the promotion of shielding tissues from T cell-mediated damage.

CONCLUDING REMARKS

Maintenance of vascular integrity during inflammation, i.e., securing the barrier function of the endothelium, represents an important challenge for the cooperation between the immune and the vascular system. Tight control over the exchange of fluids, molecules, and cells between blood and tissues during antigen-specific EC–T cell interaction is achieved through different mechanisms. Importantly, naïve T cells ignore their cognate antigen on ECs and only adequately activated T cells can recognize their antigen on ECs and subsequently exert their effector function. Since recognition of tissues by CD8^{+} effector T cells can precipitate severe immunopathological consequences, potent tissue-protective mechanisms must be activated during the antigen-specific interaction of these cell types. Hence, the ligation of PD-1 or the HVEM-receptor BTLA on EC-specific effector T cells represents an attractive therapeutic target to avoid excessive EC damage during inflammation. Furthermore, cell type-specific signal transduction pathways downstream of the IFN-γ receptor in ECs (Miura et al., 2006) may harbor specific targets that could permit stimulation of peripheral inhibitory signals. Clearly, further research is warranted to better understand how proinflammatory stimuli can be translated locally into anti-inflammatory signals for the benefit of vascular and tissue integrity.

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Stromal cells control soluble material and cellular transport in lymph nodes

Marc Bajénoff*[1,2,3]

[1] Centre d'Immunologie de Marseille-Luminy, Parc Scientifique et Technologique de Marseille Luminy, Aix-Marseille University, UM2, Marseille, France
[2] Institut National de la Santé et de la Recherche Médicale, U1104, Marseille, France
[3] Centre National de la Recherche Scientifique, UMR 7280, Marseille, France

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Michael Sixt, Institute of Science and Technology Austria, Austria
Paul Kaye, University of York, UK

****Correspondence:***
Marc Bajénoff, Centre d'Immunologie de Marseille-Luminy, Parc Scientifique et Technologique de Marseille Luminy, Aix-Marseille University, UM2, Case 906, 13288 Marseille, France.
e-mail: bajenoff@ciml.univ-mrs.fr

Lymphocytes continuously patrol the secondary lymphoid organs (SLOs) of mammals in search for their cognate antigens. SLOs are composed of leucocytes (~95%) and lymphoid stromal cells (~5%) that form the structural framework of these organs. These sessile cells have been considered for decades as inert elements of the immune system. This simplistic view has dramatically changed in recent years, when it was discovered that these architectural cells are endowed with immuno-regulatory functions. Lymph nodes (LNs) are located at the interface between the blood and lymphatic systems, thus allowing tissue-derived antigen/antigen presenting cells (APCs) to gather with blood-derived lymphocytes. As a typical LN contains ~10 million of tightly packed cells, this accumulation of immune cells and information is probably not sufficient to foster the rare cellular interactions mandatory to the initiation of adaptative immune responses. Herein, I review some of the physico-chemical elements of stromal cells that are used to transport and guide immune cells and soluble molecules within LNs.

Keywords: stroma, lymph node, soluble material transport, cellular migration

TRANSPORTATION OF IMMUNE INFORMATIONS

Lymphatics continuously transport soluble and particulate Ags from peripheral tissues to draining lymph nodes (LNs; Young, 1999; Willard-Mack, 2006). This lymphatic content reflects the immunological status of peripheral tissues and is constantly deciphered by antigen presenting cells (APCs) and lymphocytes within LNs. Afferent lymphatics discharge their content in the LN subcapsular sinus (SCS), a hollow tubular structure that surrounds the LN, thus preventing free diffusion of the lymphatic content to the underlying parenchyma (Forkert et al., 1977; van Ewijk et al., 1988; Willard-Mack, 2006). The vast majority of APCs and lymphocytes reside in the enclosed LN parenchyma from which free soluble particles are excluded. This structural confinement raises a critical question: how is soluble and particulate material transported from the SCS throughout the parenchyma?

SCS MACROPHAGES

The floor of the SCS is composed of a layer of sinus endothelial cells and a layer of specialized fibroblasts (Forkert et al., 1977; Farr et al., 1980). The integrity of the floor of the SCS is a subject of conflicting reports. Ultrastructural studies demonstrate pores in the floor of the SCS by electron microscopy (Forkert et al., 1977; van Ewijk et al., 1988) while others argue against such evidences (Farr et al., 1980). Despite the putative existence of pores in the floor of the SCS, there is evidence that penetration of particulate material from lymph into LN cortex is limited (Gretz et al., 1997). SCS is populated by a subpopulation of SCS macrophages that extend cytoplasmic protrusions to the underlying B cell follicle. Intravital imaging of the SCS in live animals demonstrated macrophage capture of particulate antigen and transfer to Ag-specific B cells via these protrusions (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007; **Figure 1**, item 1). Further experiments demonstrated that complement receptors 1 and 2 expression on B cells is important for the capture and delivery of immune complexes from SCS macrophages to germinal centers (GCs) and follicular dendritic cells (FDCs; Phan et al., 2007, 2009). Therefore, SCS macrophages act as Ag-bridging channels between the impermeant SCS and B cell follicles.

THE CONDUIT SYSTEM

The SCS is a shielded unit that prevents the free diffusion of particulate Ags and soluble material >70 kDa to the parenchyma (Gretz et al., 2000). Tissue-derived cells such as dendritic cells (DCs) can actively cross the layer of SCS-lining cells (Braun et al., 2011) whereas chemokines, interleukins (ILs), and small Ags can diffuse to the parenchyma via a dedicated network of pipes named conduits (Sainte-Marie and Peng, 1986). Conduits originate between the SCS-lining cells (Gretz et al., 1997) and are found throughout the paracortex, mainly within the T cell zone. These reticular fibers are composed of several layers of extracellular matrix molecules precisely assembled around a core of collagen fibers (refer to Sixt et al., 2005; Roozendaal et al., 2008 for an extensive description of the conduits composition). Conduits are produced and ensheathed by Fibroblastic reticular cells (FRCs; Gretz et al., 1997; Katakai et al., 2004b; **Figure 1**, item 2a) and as a result, most of the conduit system is shielded from lymphoid and myeloid cells within the T cell zone. Despite its physical enclosure, many immune cell types capture soluble material from the lymphatic content conveyed by the conduit network.

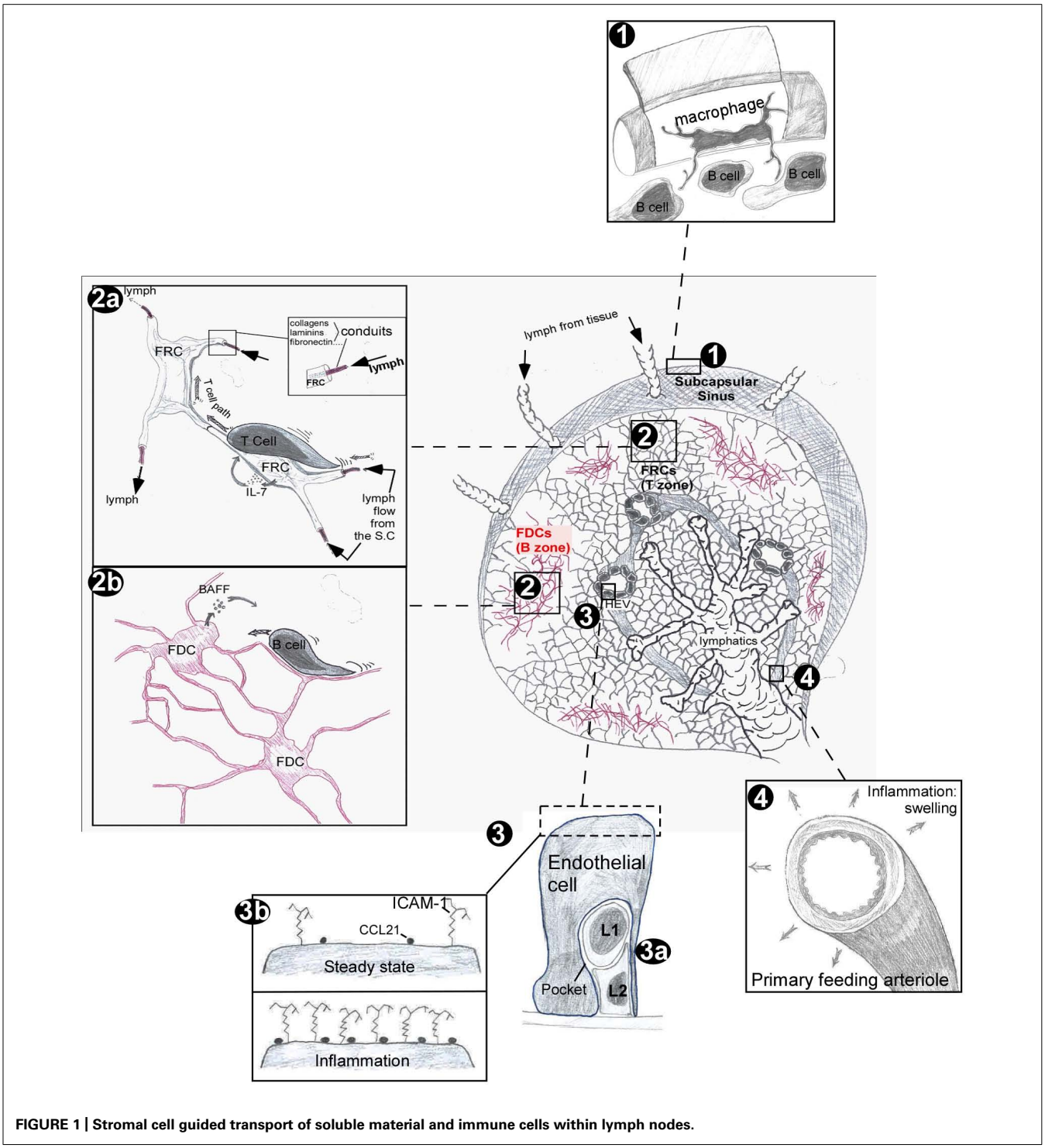

FIGURE 1 | Stromal cell guided transport of soluble material and immune cells within lymph nodes.

High endothelial venules

High endothelial venules (HEVs) are the gateways intravascular lymphocytes use to immigrate into the LN parenchyma from the blood circulation (Cho and De Bruyn, 1986; Girard and Springer, 1995). Lymphocyte recognition of HEVs involves a multistep adhesion cascade in which primary adhesive interactions (rolling) are followed by firm arrest (sticking) in response to chemokines (Sasaki et al., 1996). The endothelial cells of HEVs secrete and present chemokines such as CCL21 on their luminal surface (Gunn et al., 1998). As most of the tubules of the conduit system end in HEVs, the luminal surface of these structures is also rapidly decorated by lymph borne chemokines transported via the conduit system (Gretz et al., 2000; Baekkevold et al., 2001; Palframan et al., 2001). Therefore, the conduit system acts as a remote

messenger system able to modulate lymphocytes trafficking across LN HEVs.

Resident dendritic cells

Lymph nodes contain an important population of resident DCs that settle on the FRC network (Sixt et al., 2005; Bajenoff et al., 2006) and are capable of taking up and processing soluble antigens transported within the conduits (Sixt et al., 2005).

In steady state conditions, these Ag-loaded DCs present peptide/MHC complexes to T cells in absence of co-stimulatory molecules and should hence promote peripheral tolerance (Probst et al., 2003, 2005). Upon infection, inflammatory stimuli, soluble Ag, and tissue-derived DCs loaded with pathogen peptide/MHC complexes are drained to the proximal LN (Itano et al., 2003). In these conditions, resident DCs probably present tissue-derived Ags in a pro-inflammatory environment susceptible to initiate adaptive immune responses (Itano et al., 2003).

The important meshwork of resident DCs may represent a very efficient way to "deploy" the antigenic repertoire conveyed by the conduit system. Such widespread antigenic representation may ensure an optimal scanning of lymphocytes during their journey in the LNs, both in steady state and inflammatory conditions.

B cells and follicle dendritic cells

Although the conduit system is synthesized by T cell zone FRCs, sparse conduits are present in B cell follicles. Like their T cell counterparts, follicular conduits convey soluble (but not particulate) material from the SCS throughout B cell follicles. Using two-photon (2P) technology, Roozendaal et al. (2009) observed that conduits deliver small antigens and chemokines such as CXCL13 to B cells that directly contact the conduits. Another study demonstrated that the conduit system is also used to deliver soluble Ag to FDCs (Bajenoff and Germain, 2009).

Efficiency of the transport

Subcutaneous injection of fluorescent tracers demonstrated that the transportation of soluble material from the peripheral tissue to the parenchyma of the draining LN occurs within minutes (Itano et al., 2003; Roozendaal et al., 2009). The efficacy of the conduit system is quite surprising given (i) the numerous resident DC processes supposedly stuck in narrow conduits, (ii) the complex 3D branching pattern of the conduit system, and (iii) the absence of identified lymph propelling system. Further experiments will be required to understand the fine details that control lymph propulsion within these micropipes.

TRANSPORTATION OF CELLS

CONTROL OF LYMPHOCYTE FLUX

Steady state

Millions of lymphocytes enter and exit LNs each day, accessing the parenchyma via HEVs and egressing via efferent lymphatics. Despite this high rate of cellular flux, the number of lymphocytes present in a resting LN is extraordinary stable over time. The control of lymphocyte trafficking is mediated by the endothelial cells of HEVs that harbor typical cobblestone shapes with numerous embedded lymphocytes (Girard and Springer, 1995). Recent evidences revealed that these T and B cells are frequently packed together underneath the endothelial cell inside "pockets" composed of 4–5 lymphocytes (**Figure 1**, item 3a). These pockets function as waiting areas that hold and grant lymphocytes access to LN parenchyma in proportion to the rate of lymphocyte egress from the LN, enabling the LN to maintain a constant cellularity while supporting the extensive cellular trafficking necessary for repertoire scanning (Mionnet et al., 2011).

Inflammation

Lymph nodes are highly vascularized structures that, upon inflammation, can remodel and expand their primary feed arterioles by 50%, leading to a four- to fivefold increase in the rate of naive lymphocyte flow rate through the draining LNs (**Figure 1**, item 4; Soderberg et al., 2005). At the same time, the pro-inflammatory mediators released from the inflammatory site are transported via the conduits to the HEVs of the draining LNs (Baekkevold et al., 2001). IL-6 increases intercellular adhesion molecule-1 (ICAM-1) expression on HEVs, thereby promoting lymphocyte adherence to HEVs of the draining LN. This phenomenon may also apply to IL-8 and tumor necrosis factor (TNF-α) that have been shown to rapidly increase T cell entry into the draining LN (Larsen et al., 1989; McLachlan et al., 2003). In addition, memory and effector T cells that lack CD62L expression rapidly gain entry into inflamed LNs through expression of CXCR3 and its interactions with CXCL9 deposited on the luminal surface of inflamed HEVs (Wurtz et al., 2004). Finally, temperatures ranging from 38–40 °C act directly on lymphocytes to enhance CD62L-dependent homing across HEVs while also increasing the expression of CCL21 and ICAM-1 on the surface of HEVs (**Figure 1**, item 3b; Chen et al., 2006).

Altogether, these results present HEVs as gatekeepers in charge of modulating lymphocyte trafficking to LNs, both at steady state and during inflammation.

CONTROL OF LYMPHOCYTES MOTILITY AND TERRITORIALITY

Within SLOs, T and B cells are highly mobile and segregate in distinct geographical areas populated by different stromal cells (Miller et al., 2002). FRCs reside in the T cell zone while FDCs populate B cell follicles (Gretz et al., 1997; Allen and Cyster, 2008; Mueller and Germain, 2009). Both stromal cell populations form dense, intermingled 3D networks in their respective areas (Schneider and Tschopp, 2003; Bajenoff et al., 2006; Munoz-Fernandez et al., 2006; Link et al., 2007; Allen and Cyster, 2008).

Fibroblastic reticular cells

Fibroblastic reticular cells are fibroblast-like cells that reside in the T cell area of LNs and spleen. FRCs produce and enwrap the conduit system, forming a rigid cellular network embedded amongst motile lymphocytes (Anderson and Anderson, 1976; Gretz et al., 1997; Sixt et al., 2005). Intravital two-photon (2P) imaging experiments have revealed that the FRC network supports and guides T and B cell motility in the T cell area (Bajenoff et al., 2006), dictating the apparent characteristic random migratory behavior of these cells. Lymphocytes follow the supporting fibers of the FRC as they migrate in the T cell zone that is itself defined by the extent of this network (**Figure 1**, item 2a).

The molecular cues that drive lymphocyte locomotion on FRCs have partially been deciphered. FRCs secrete the homeostatic

chemokine CCL21 that stick to collagen IV and glycosaminoglycans (GAGs) present on the surface of FRCs (Ansel et al., 2000; Sixt et al., 2005; Link et al., 2007; Yang et al., 2007). CCR7, the receptor for CCL21, is expressed by many cell types, including DCs, T and B lymphocytes (Yanagihara et al., 1998; Forster et al., 1999). Both molecules are critical for the proper delimitation of the T/B cell boundary within SLOs as evidenced by the inability of T and B cell areas to properly segregate in the SLOs of CCL21 and CCR7 deficient mice (Nakano et al., 1998; Forster et al., 1999). Three recent dynamic imaging studies demonstrated that CCR7/CCR7-L signaling pathway is a key modulator of T cell locomotion (Asperti-Boursin et al., 2007; Okada and Cyster, 2007; Worbs et al., 2007). These studies concluded that the effect of CCR7 or CCR7-ligand deficiency could account for ∼40% of the Gi-dependent motility of T cells in LNs.

Altogether, these results indicate that the random locomotion of T cells in LNs is physically and chemically guided by FRCs while CCR7/CCR7-ligands modulate the velocity of T cells. The exact set of molecular/chemical cues that regulate T cell migration remains to be determined.

Follicular dendritic cells and follicular stromal cells

Follicular dendritic cells present native antigens in the form of immune complexes on their surface and are critical for the maintenance of B cell follicle integrity (Cyster et al., 2000; Allen and Cyster, 2008; Wang et al., 2011). Recent evidence indicate that FDCs arise from ubiquitous perivascular precursors (preFDC) expressing platelet-derived growth factor receptor β (PDGFRβ; Krautler et al., 2012). During immune responses, FDCs organize the development of GCs in which mature B lymphocytes rapidly proliferate, differentiate, mutate their antibodies through somatic hypermutation, and class switch their antibodies (MacLennan, 1994; Allen et al., 2007a; Allen and Cyster, 2008; Wang et al., 2011). Intravital 2P experiments have revealed that B cells migrate on the thin and intermingled processes of radio-resistant stromal cells populating B cell follicles, suggesting that FDCs are the counterparts of FRCs in the B cell follicles (Bajenoff et al., 2006; **Figure 1**, item 2b).

Follicular dendritic cells are defined by their capacity to trap and retain immune-complexes and their expression of various markers such as CD21/35 (complement receptors 1 and 2) and C4 complement fraction (Cyster et al., 2000). FDCs also express BP3, a glycosylphosphatidyl-anchored membrane protein (McNagny et al., 1991) of unknown function. Surprisingly, BP3 staining in B cell follicle highlights a non-FDC network, suggesting the existence of a second follicular stromal cell network (McNagny et al., 1991; Allen and Cyster, 2008). Therefore, it is likely that both FDCs and these radio-resistant follicular stromal cells support B cell migration in primary B cell follicles. Further experiments will be required to address this issue.

Follicular dendritic cells and follicular stromal cells are an important source of CXCL13 in follicles and this chemokine is known to promote B cell migration *in vitro* and organize B cell follicle formation *in vivo* (Legler et al., 1998; Ansel et al., 2000; Saez de Guinoa et al., 2011). Two-photon microscopy analysis of GC B cell motility showed that it was reduced in the absence of CXCL13 suggesting that this chemokine may also promote B cell motility in primary follicles (Allen et al., 2007b).

OPEN QUESTIONS

Control of lymphocyte trafficking on FRC and FDC networks

T and B cells actively migrate on stromal cell networks, adapting their paths to the 3D processes of these supporting cells. Such stochastic behavior may ensure that a given lymphocyte will eventually visit its entire territory before leaving the LN. However, LNs are densely packed organs in which extracellular space is limited, if not absent. Therefore, wandering lymphocytes should constantly "bump" to each other during their random migration. Interestingly, we consistently observed that T cells never turned back in the middle of a FRC fiber but always changed direction at FRCs intersections (personal observation). It would then be interesting to determine how lymphocytes move as a population and whether they constantly bump and squeeze on each other or line up along stromal cells during their migration.

Stromal cell behavior in inflamed LNs

Lymph nodes draining an inflamed tissue rapidly enlarge in response to the massive influx of naive cells and the proliferation of the activated ones, probably inducing a tremendous and rapid remodeling of the various stromal cell subsets that should not only continue to fulfill their steady state duties but also create new microenvironments necessary for the development of the immune response (e.g., GCs, medullary cords, etc.; Katakai et al., 2004a,b; Allen et al., 2007a; Allen and Cyster, 2008). So far, we do not understand how LN stromal cells manage these rapid structural changes and cellular demands.

Are inflamed stromal cells able to stretch? Fibroblastic reticular cells and FDCs form 3D substrata for lymphocytes. Upon inflammation, these networks should accommodate the massive influx of lymphocytes and continue to generate cellular roads for them. FRCs and FDCs express contractile molecules normally restricted to smooth muscles (desmin, smooth muscle actin, etc.) and myofibroblasts, a subset of activated fibroblasts capable of speeding wound repair by contracting the edges of the wound (Sixt et al., 2005; McAnulty, 2007). FRCs are also endowed with contractile properties as evidenced by their capacity to induce wrinkles on deformable collagen-coated silicone substrate (Link et al., 2007). As FRCs are attached to collagen-rich conduits, these properties may allow them to stretch in order to increase their surface and accommodate the massive influx of T cells consecutive to inflammation. Their contractile properties may also be used to shrink the conduits upon the completion of the immune response in order to restore the original size of the LN. The calculation of FRC and FDC densities as well as the precise measurement of their dimensions in resting and inflamed LNs may test these hypotheses.

Origin of additional stromal cells in inflamed LNs. Inflamed LNs can triple their size in few days and undergo a tremendous enlargement in chronically infected mice (Webster et al., 2006; Ruddle and Akirav, 2009). It is thus likely that FRC and FDC networks incorporate new stromal cells in order to sustain this remodeling. The origin of lymphoid stromal cells

remains elusive, though there are growing evidences that they are of mesenchymal origin (Munoz-Fernandez et al., 2006; Mabbott et al., 2011). In addition, the SLOs of irradiated hosts reconstituted with syngeneic bone marrow cells possess lymphoid stromal cells of host origin (Humphrey et al., 1984; Bajenoff et al., 2006). These observations led to the conclusion that adult lymphoid stromal cells do not originate from bone marrow mesenchymal cells at steady state. However, these conclusions should be interpreted with caution. Bone marrow hematopoietic stem cells only engraft when adoptively transferred in an irradiated host, demonstrating that the destruction of pre-existing hematopoietic cells/progenitors is a prerequisite for the engraftment of hematopoietic progenitors. Stromal cells and their progenitors are radio-resistant. Therefore, if grafted bone marrow cells contain mesenchymal stromal cell progenitors, these cells will fail to engraft, even when adoptively transferred in an irradiated host. In absence of prior stromal cell destruction, any adoptive transfer of stromal cell progenitor is probably destined to fail. The determination of the origin of stromal cells in resting and inflamed LNs will probably require the creation of new animal models that are currently critically lacking in the field of stromal cell biology.

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Optical projection tomography reveals dynamics of HEV growth after immunization with protein plus CFA and features shared with HEVs in acute autoinflammatory lymphadenopathy

Varsha Kumar[1], Susan Chyou[1], Jens V. Stein[2] and Theresa T. Lu[1,3]*

[1] *Autoimmunity and Inflammation Program and Pediatric Rheumatology, Hospital for Special Surgery, New York, NY, USA*
[2] *Theodor Kocher Institute, University of Bern, Bern, Switzerland*
[3] *Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY, USA*

Edited by:
Burkhard Ludewig, Cantonal Hospital St. Gallen, Switzerland

Reviewed by:
Masayuki Miyasaka, Osaka University Graduate School of Medicine, Japan
Nancy Ruddle, Yale University School of Medicine, USA
Elke Scandella, Cantonal Hospital St. Gallen, Switzerland

***Correspondence:**
Theresa T. Lu, Autoimmunity and Inflammation Program and Pediatric Rheumatology, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021, USA.
e-mail: lut@hss.edu

The vascular–stromal compartment of lymph nodes is important for lymph node function, and high endothelial venules (HEVs) play a critical role in controlling the entry of recirculating lymphocytes. In autoimmune and autoinflammatory diseases, lymph node swelling is often accompanied by apparent HEV expansion and, potentially, targeting HEV expansion could be used therapeutically to limit autoimmunity. In previous studies using mostly flow cytometry analysis, we defined three differentially regulated phases of lymph node vascular–stromal growth: initiation, expansion, and the re-establishment of vascular quiescence and stabilization. In this study, we use optical projection tomography to better understand the morphologic aspects of HEV growth upon immunization with ovalbumin/CFA (OVA/CFA). We find HEV elongation as well as modest arborization during the initiation phase, increased arborization during the expansion phase, and, finally, vessel narrowing during the re-establishment of vascular quiescence and stabilization. We also examine acutely enlarged autoinflammatory lymph nodes induced by regulatory T cell depletion and show that HEVs are expanded and morphologically similar to the expanded HEVs in OVA/CFA-stimulated lymph nodes. These results reinforce the idea of differentially regulated, distinct phases of vascular–stromal growth after immunization and suggest that insights gained from studying immunization-induced lymph node vascular growth may help to understand how the lymph node vascular–stromal compartment could be therapeutically targeted in autoimmune and autoinflammatory diseases.

Keywords: lymph node, high endothelial venules, stromal, angiogenesis, optical projection tomography, autoimmunity, autoinflammation, regulatory T cells

INTRODUCTION

Lymph nodes are sites of immune responses, and the lymph node blood vessels are an integral part of immune function. In addition to the delivery of oxygen and micronutrients, the lymph node blood vessels deliver lymphocytes and other immune cells to the lymph node parenchyma, where these cells can interact with antigen and one another to generate an adaptive immune response. Recirculating lymphocytes enter the lymph node parenchyma at specialized postcapillary venules characterized by cuboidal, rather than flat, endothelial cells that are known as high endothelial venules (HEVs). The HEV endothelial cells display chemokines and adhesion molecules that promote the extravasation of lymphocytes. Peripheral lymph node HEVs are identified in part by their expression of peripheral node addressin (PNAd) which binds L-selectin on recirculating lymphocytes and thus allowing lymphocyte entry (von Andrian and Mempel, 2003; Benahmed et al., 2012). The HEVs and the vessels in general are suspended within a conduit network consisting of collagen-rich fibrils ensheathed by fibroblastic reticular cells (FRCs). The conduit network is functionally connected to the blood vessels, with FRCs or related pericyte-like cells ensheathing the vessel walls (Gretz et al., 1997; Malhotra et al., 2012). Disrupting trafficking across HEVs disrupts lymphocyte accumulation in lymph nodes and delays immune responses (Arbones et al., 1994; Steeber et al., 1996; Xu et al., 1996). Thus, insight into the events and regulation of HEV alterations during immune responses may lead to new ways by which to therapeutically manipulate immunity.

Lymph node enlargement induced by immunization or infection is associated with vascular expansion (Burwell, 1962; Herman et al., 1972; Anderson et al., 1975; Mebius et al., 1990; Webster et al., 2006; Kumar et al., 2010). Studies in recent years have begun to delineate the mechanisms that regulate the growth of HEV and other portions of the vasculature (Soderberg et al., 2005; Angeli et al., 2006; Liao and Ruddle, 2006; Webster et al., 2006; Kumar et al., 2010; Kataru et al., 2011). From our studies of lymph node vascular growth after immunization, we have identified at least three phases based on the vascular characteristics and regulatory mechanisms. During the initiation phase from day 0 to day 2, there is rapid upregulation of endothelial cell proliferation which is independent of T and B cells, a modest increase in HEV endothelial

cell numbers, but no expansion of non-HEV blood endothelial cells. The initiation phase appears to involve at least a tripartite interaction whereby dendritic or other $CD11c^+$ cells stimulate FRCs to upregulate VEGF, which then promotes endothelial cell proliferation. The initiation phase is followed by an expansion phase from day 2 to about day 7 after ovalbumin/CFA (OVA/CFA), and it is characterized by continued upregulated proliferation, expansion of HEV, non-HEV blood, and lymphatic endothelial cell numbers, expansion of FRC numbers, and dependence of the proliferation and expansion on T and B cells (Webster et al., 2006; Tzeng et al., 2010; Chyou et al., 2011; Benahmed et al., 2012). After expansion, the vessels become more quiescent, with reduced endothelial cell proliferation and HEV trafficking efficiency. Stimulating the lymph node transiently with bone marrow-derived dendritic cells leads to cessation of vascular expansion at this stage, but a chronic stimulus such as with OVA/CFA leads to continued expansion as the proliferation rate slowly drops. Re-establishment of vessel stabilization accompanies the quiescence, with FRCs more tightly organized around HEVs. The quiescence and stabilization are mediated by $CD11c^{hi}$ presumed dendritic cells, as depletion of $CD11c^{hi}$ cells during this phase results in increased endothelial cell proliferation, disorganization of the FRC sheath around the vessels, and increased vascular permeability (Tzeng et al., 2010).

In autoimmune and autoinflammatory disorders such as systemic lupus erythematosus and systemic onset juvenile arthritis, lymph nodes can be hypertrophic (Fox and Rosahn, 1943; Shapira et al., 1996; Schneider and Laxer, 1998; Calguneri et al., 2003; Livingston et al., 2011). Apparent blood vascular proliferation is one of the characteristics of these enlarged lymph nodes (Benahmed et al., 2012). We recently showed in a chronic lupus model that the expanded lymph node vasculature is in a state of re-established quiescence as seen by a low endothelial cell proliferation rate, reduced HEV trafficking efficiency, and the accumulation of $CD11c^{hi}$ cells (Chyou et al., 2012). Among other things, these results suggested that insights gained from studying lymph nodes stimulated by model immunization strategies may be applicable to vascular regulation in chronic autoimmune models and, potentially, in human disease. While the lymph nodes in the chronic lupus model steadily enlarged over weeks (Chyou et al., 2012), lymph nodes in human lupus and systemic onset juvenile arthritis can also acutely enlarge (Fox and Rosahn, 1943; Eisner et al., 1996; Grom and Passo, 1996). Whether acute lymphadenopathy associated with autoimmunity or autoinflammation can be accompanied by an expanded vasculature and whether this expansion shares features with acute vascular expansion induced by OVA/CFA immunization is unknown.

Blood vessel expansion in immune-stimulated lymph nodes was demonstrated decades ago using techniques such as resin casting and microangiography (Burwell, 1962; Herman et al., 1972; Anderson et al., 1975; Steeber et al., 1987). Optical projection tomography (OPT) is an imaging technique developed 10 years ago whereby the specimen is rotated while a fixed detector obtains light or fluorescent images through the course of the 360° rotation, allowing a three-dimensional rendering of the specimen. The apparatus for OPT data capture displays many similarities to a micro-computer tomography (CT) scanner: a stepper motor is used to rotate the specimen to precisely controlled angles, a 2D array detector (CCD) is used to capture the signal, and photon sources are included for illumination. OPT generally employs relatively low numerical aperture optics, which provide the large depth of field and working distance needed to analyze thick samples. The use of visible light and image-forming optics is one of the important advantages of OPT – it is capable of being used in two different modalities: transmission OPT (tOPT), and emission OPT (eOPT). The use of optics in OPT allows a sharp image to be focused onto the CCD, yielding images that can be considered good approximations to projections for computational purposes. OPT is ideal for specimens 1–10 mm in size (Sharpe et al., 2002) and has been used in studies of organ development during embryogenesis (Davies and Armstrong, 2006). Recently, OPT was used to study HEV growth associated with virus-induced lymph node hypertrophy (Kumar et al., 2010).

In this current study, we use OPT to characterize the morphologic aspects of HEV growth upon immunization with OVA/CFA and compare that with the HEV alterations that occur in an acute autoimmune and autoinflammatory model induced by depletion of regulatory T cells. The results show (1) distinct morphologic alterations during the different phases of vascular growth after immunization, (2) that HEVs in acutely enlarged autoinflammatory lymph nodes are expanded, and (3) that there are morphologic similarities between expanded HEVs in the two models. These results reinforce the idea of distinct phases of lymph node vascular growth after immunization and raise the possibility that insights gained from studying lymph nodes stimulated by OVA/CFA and other experimental immunization strategies may be useful in understanding and treating autoimmune and autoinflammatory diseases.

MATERIALS AND METHODS

MICE

C57Bl/6 mice between 6 and 12 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME, USA), Taconic Farms (Hudson, NY, USA) or National Cancer Institute (Frederick, MD, USA). Foxp3-DTR mice were originally generated as described (Kim et al., 2007). All procedures were performed in accordance with the Institutional Animal Use and Care Committee of the Hospital for Special Surgery.

MOUSE TREATMENTS AND IMMUNIZATIONS

For immunization with OVA in complete Freund's adjuvant (OVA/CFA), OVA was emulsified in CFA at final concentration of 1 mg/ml OVA, and mice received hind footpad injection of 20 μl of OVA/CFA as described (Webster et al., 2006).

Foxp3-DTR mice express simian diphtheria toxin (DT) receptor that allows for depletion of $Foxp3^+$ regulatory T cells upon DT administration (Kim et al., 2007; Chinen et al., 2010). For regulatory T cell depletion, wild-type control or Foxp3-DTR mice were injected intraperitoneally with 1.5 μg of DT (Calbiochem, San Diego, CA, USA or List Biological Laboratory, Campbell, CA, USA) on days 0, 1, 4, and 7 and examined on day 8. $CD4^+$, $CD25^+$, $Foxp3^+$ regulatory T cells in lymph nodes were depleted by at least 12-fold by day 8.

FLOW CYTOMETRY ANALYSIS

For flow cytometric analysis of endothelial cells and FRCs, we generated single cell suspensions from lymph nodes and stained cells as previously described (Webster et al., 2006). Briefly, lymph nodes were digested with collagenase type II (Worthington, Lakewood, NJ, USA) and stained with antibodies for flow cytometry. Antibodies to the following antigens were used: CD45 (BD Biosciences, San Jose, CA, USA), CD31 (BD Biosciences), PNAd (clone MECA-79, BD Biosciences), gp38 (Biolegend, San Diego, CA, USA or Developmental Studies Hybridoma Bank, Iowa City, IA, USA). The unconjugated anti-PNAd is detected using fluorophore-conjugated anti-rat IgM (Jackson ImmunoResearch, West Grove, PA, USA). Cells were analyzed using a FACS CANTOS (BD Biosciences) and CellQuest Pro (BD Biosciences) software.

OPTICAL PROJECTION TOMOGRAPHY

MECA-79 conjugation was performed according to vendor instructions using Alexa-568 conjugation kit (Invitrogen, Carlsbad, CA, USA) and visualization of the HEVs by OPT (eOPT modality) was performed essentially as described in Kumar et al. (2010). To label the HEVs, 15 μg of Alexa-568-conjugated MECA-79 was injected intravenously into the mouse at 20 min prior to lymph node excision. Excised lymph nodes were cleaned of fat, fixed in 1% ultrapure paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 4 h at 4°C and then washed in PBS. The lymph nodes were embedded in 1% ultrapure agarose (Invitrogen) and then dehydrated in 100% methanol overnight. The lymph nodes were then immersed in BABB solution (benzyl alcohol + benzyl benzoate at 1:2 ratio) and OPT scanning was performed according to the manufacturer's instructions (Bioptonics). Filter sets were: exciter 425/40, emitter LP475 for autofluorescent signal and exciter 545/30, emitter 617/75 for red fluorescent signal. Raw data were converted into 3D voxel datasets using NRecon software from Bioptonics. Reconstructed virtual xyz data sets were exported as .TIFF or .bmp files and analyzed with IMARIS (Bitplane) for isosurface calculation of total lymph node volume. IMARIS reconstructions were carefully adjusted to fit original NRecon reconstructions. The total HEV length and thickness, branching points, and individual/average segment lengths were obtained by using the IMARIS Filament tracer module (Bitplane). Segments were defined as an HEV section between 2 branching points.

RESULTS

OPT CHARACTERIZATION OF HEV GROWTH AFTER IMMUNIZATION WITH OVA/CFA

We used OPT to examine the morphologic alterations that occur as HEVs expand after immunization with OVA/CFA. The HEVs were labeled prior to imaging with intravenously injected fluorophore-tagged MECA-79, allowing us to visualize HEVs that express the PNAd epitope recognized by MECA-79 on the luminal surface. We examined the nodes at days 2, 4, 7, 14, and 21 to examine the morphologic alterations that occur during initiation, expansion, and the re-establishment of quiescence and stabilization. Popliteal lymph node volume was increased by day 2 after footpad immunization with OVA/CFA (**Figures 1A,B**), consistent with the rapid increase in lymph node cellularity observed previously (Webster et al., 2006; Tzeng et al., 2010; Chyou et al., 2011). Total HEV length doubled (**Figure 1C**). There was a modest increase in segment numbers as well as branch points (**Figures 1D,E**), suggesting that some of the HEV growth was attributable to the generation of new branches. The average segment length also increased, with the proportion of the longer segments increasing and segments over 200 μm doubling (**Figures 1F,H**). This suggested that HEV elongation contributed to HEV growth at day 2. HEV width was reduced at day 2 (**Figures 1G,I**), suggesting vasoconstriction or vessel stretching.

Lymph node volume and total HEV length increased further by day 4 (**Figures 1B,C**). Segment numbers and branch points showed continued increases while segment length did not increase from day 2 levels (**Figures 1D–F,H**). This suggested that the continued HEV growth at day 4 was mainly due to vessel arborization. Vessel width was restored to day 0 levels, further supporting the idea of HEV expansion (**Figures 1G,I**). At day 7, at a point when endothelial cell numbers are still expanding after OVA/CFA immunization (Tzeng et al., 2010), the combination of high segment numbers and branch points along with a reduction in segment length relative to that of day 4 (**Figures 1D–F,H**) suggested continued arborization and limited segment elongation. After day 7, when we have previously observed the re-establishment of vascular quiescence and stabilization (Tzeng et al., 2010), total HEV length, segment numbers, branch points, and segment length remained constant (**Figures 1C–F,H**) while vessel width decreased (**Figures 1G,I**), suggesting cessation of HEV growth and the process of vessel narrowing. Together, these results suggested that there is an initial HEV elongation and modest arborization in the first 2 days that is followed by increased HEV arborization and, after day 7, vessel narrowing.

THE HEVs IN ACUTE AUTOINFLAMMATORY LYMPHADENOPATHY RESEMBLE HEVs AT DAYS 4–7 AFTER IMMUNIZATION WITH OVA/CFA

We asked whether we could observe apparent vascular expansion in acutely enlarged lymph nodes associated with an autoimmune and autoinflammatory model and whether the expanded vasculature shared features with that induced by OVA/CFA immunization. Depletion of regulatory T cells in Foxp3-DTR mice by DT injection results in an acute autoimmune and inflammatory phenotype characterized by rampant inflammation, T cell activation, splenomegaly, lymphadenopathy, and autoantibody production by day 8 and death starting at day 10 (Kim et al., 2007; Chinen et al., 2010). As it is unknown whether the peripheral lymphadenopathy is directly driven by antigen-specific autoimmune responses or by the generalized autoinflammation, we will refer to the lymphadenopathy here as "autoinflammatory" in origin. We treated (control) wild-type mice and Foxp3-DTR mice with DT and examined lymph nodes at day 8. OPT analyses showed that lymph node volume, total HEV length, number of segments, number of branch points, and segment length were all increased in DT-treated Foxp3-DTR mice relative to wild-type mice (**Figures 2A–F,H**). Vessel width was similar in the WT and Foxp3-DTR mice (**Figures 2G,I**). The magnitude of the alterations in the Foxp3-DTR mice was similar to that at days 4–7 after OVA/CFA immunization (compare **Figures 1A–I** with **Figures 2A–I**). These results suggested

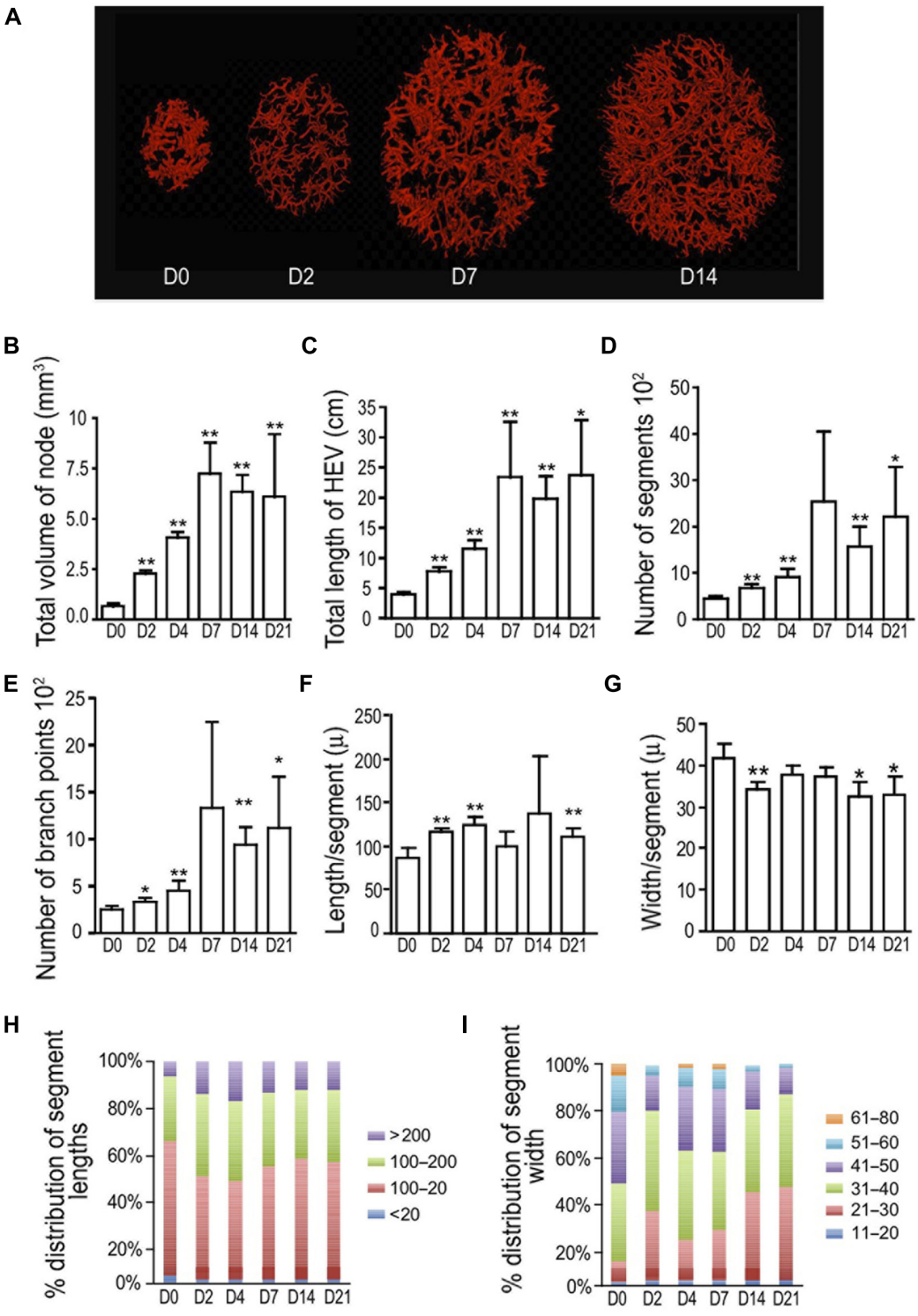

FIGURE 1 | Initial HEV lengthening is followed by branching and then narrowing after OVA/CFA. Mice were immunized in the footpad with OVA/CFA on day 0 and draining popliteal nodes were prepared for OPT imaging of PNAd^{+} vessels on indicated days. **(A)** Representative images from OPT scanning. **(B)** Total lymph node volume. **(C)** Total length of HEV per lymph node. **(D)** Number of HEV segments per lymph node. **(E)** Number of branch points per lymph node. **(F)** Average segment length. **(G)** Average vessel width. **(H)** Relative distribution of segment lengths. Lengths were divided into discrete "bins" and the percent of segments in each bin was calculated and graphed. **(I)** Relative distribution of segment width. Widths were divided into discrete "bins" and the percent of segments in each bin was calculated and graphed. For **(B–G)**, $*p < 0.05$ and $**p < 0.01$ relative to day 0 measurement using *t*-test. Error bars represent standard deviations. $n = 3$–5 lymph nodes for each condition.

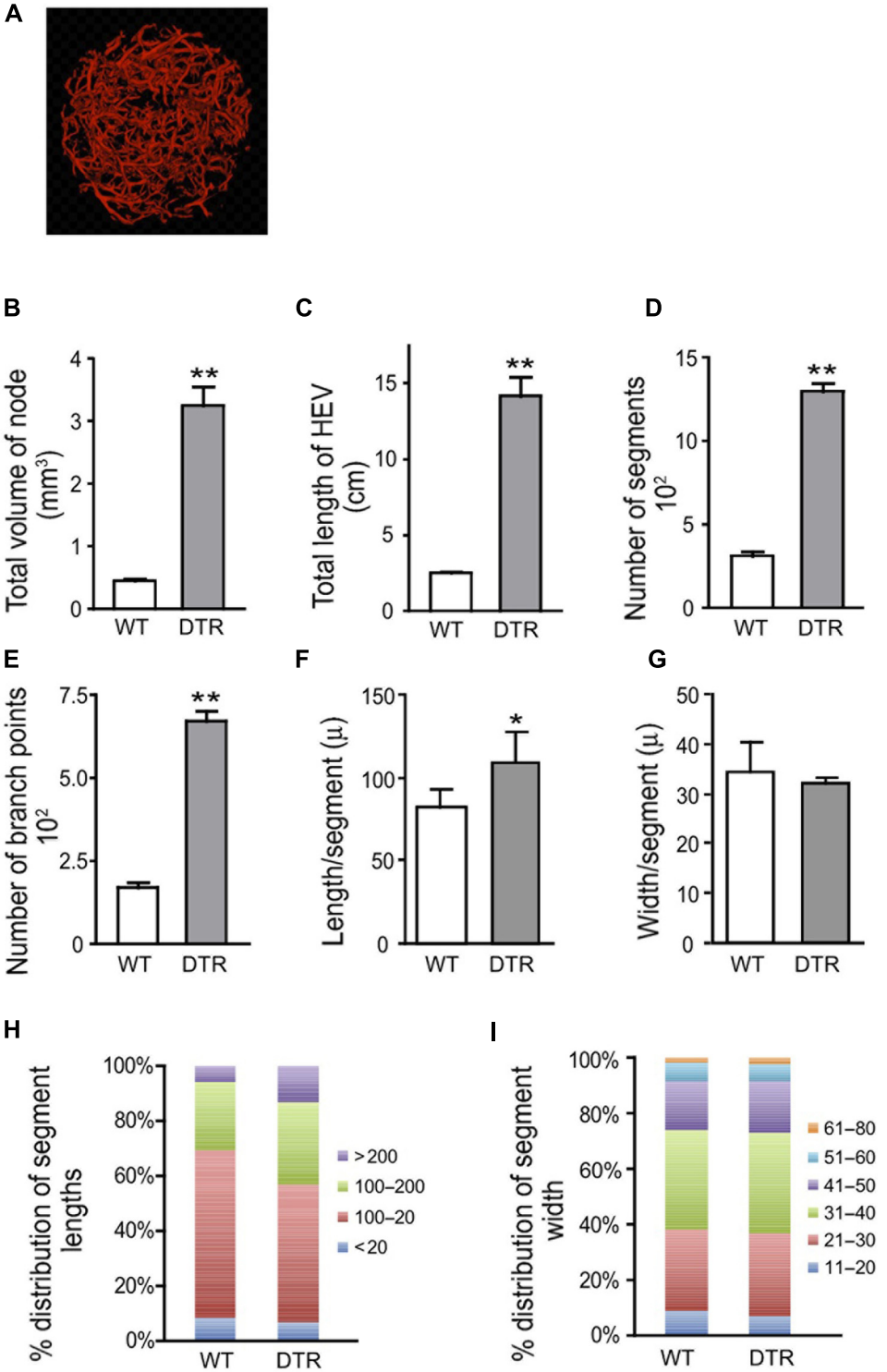

FIGURE 2 | High endothelial venule (HEV) growth in Foxp3-DTR mice is similar to that induced by OVA/CFA immunization. Wild-type (WT) and Foxp3-DTR (DTR) mice were injected with DT to deplete regulatory T cells as per Section "Materials and Methods" and popliteal lymph nodes were prepared for OPT imaging of PNAd$^+$ vessels. **(A)** Representative image of Foxp3-DTR lymph node from OPT scanning. Scale is same as that of **Figure 1A** to allow comparisons to nodes stimulated with OVA/CFA. **(B)** Total lymph node volume. **(C)** Total length of HEV per lymph node. **(D)** Number of HEV segments per lymph node. **(E)** Number of branch points per lymph node. **(F)** Average segment length. **(G)** Average vessel width. **(H)** Relative distribution of segment lengths. Lengths were divided into discrete "bins" and the percent of segments in each bin was calculated and graphed. **(I)** Relative distribution of segment width. Widths were divided into discrete "bins" and the percent of segments in each bin was calculated and graphed. For **(B–G)**, $*p < 0.05$ and $**p < 0.01$ relative to WT mice measurement using t-test. Error bars represent standard deviations. $n = 4$ lymph nodes for each condition.

that the HEVs in the acutely enlarged autoinflammatory lymph nodes were expanded and shared morphologic features with the expanded HEVs of lymph nodes acutely stimulated with OVA/CFA immunization.

To further characterize vascular–stromal alterations in addition to HEV morphologic characteristics in the regulatory T cell-depleted mice, we enumerated endothelial cells and FRCs in the brachial nodes of the DT-treated Foxp3-DTR mice by flow cytometry. Lymph node cellularity was increased by four- to fivefold in the Foxp3-DTR brachial nodes (**Figure 3A**), a number fairly consistent with the six- to sevenfold increase in volume seen by OPT in the popliteal nodes (**Figure 2B**). As we have done previously, we gated as shown in **Figure 3B** to derive counts for endothelial cell subpopulations. $CD45^{-}CD31^{+}$ cells are "total endothelial cells" that can be subsetted into $PNAd^{+}$ "HEV endothelial cells" and $PNAd^{-}$ "non-HEV mixed" lymphatic and blood endothelial cells. The non-HEV mixed endothelial cells can be further subsetted into $gp38^{+}$ "lymphatic endothelial cells" and $gp38^{-}$ "non-HEV blood endothelial cells." The three- to fourfold increase in HEV endothelial cell numbers in the Foxp3-DTR mice (**Figure 3C**) was consistent with the four- to fivefold increase in total HEV length observed in popliteal nodes using OPT (**Figure 2C**). In contrast to the HEV endothelial cells, the number of non-HEV blood endothelial cells was not increased in the Foxp3-DTR mice ($p = 0.68$, t-test, $n = 3$ per condition; **Figure 3C**). Lymphatic endothelial cells and ($CD45^{-}CD31^{-}gp38^{+}$) FRCs showed a suggestion toward increased numbers in the Foxp3-DTR mice (**Figure 3C,D**), but the differences did not reach statistical significance ($p = 0.12$ for lymphatic endothelial cells; $p = 0.14$ for FRCs). These flow cytometry results corroborated the OPT results showing HEV expansion in the autoinflammatory lymph nodes and indicated that, at the time point examined, HEVs but not other

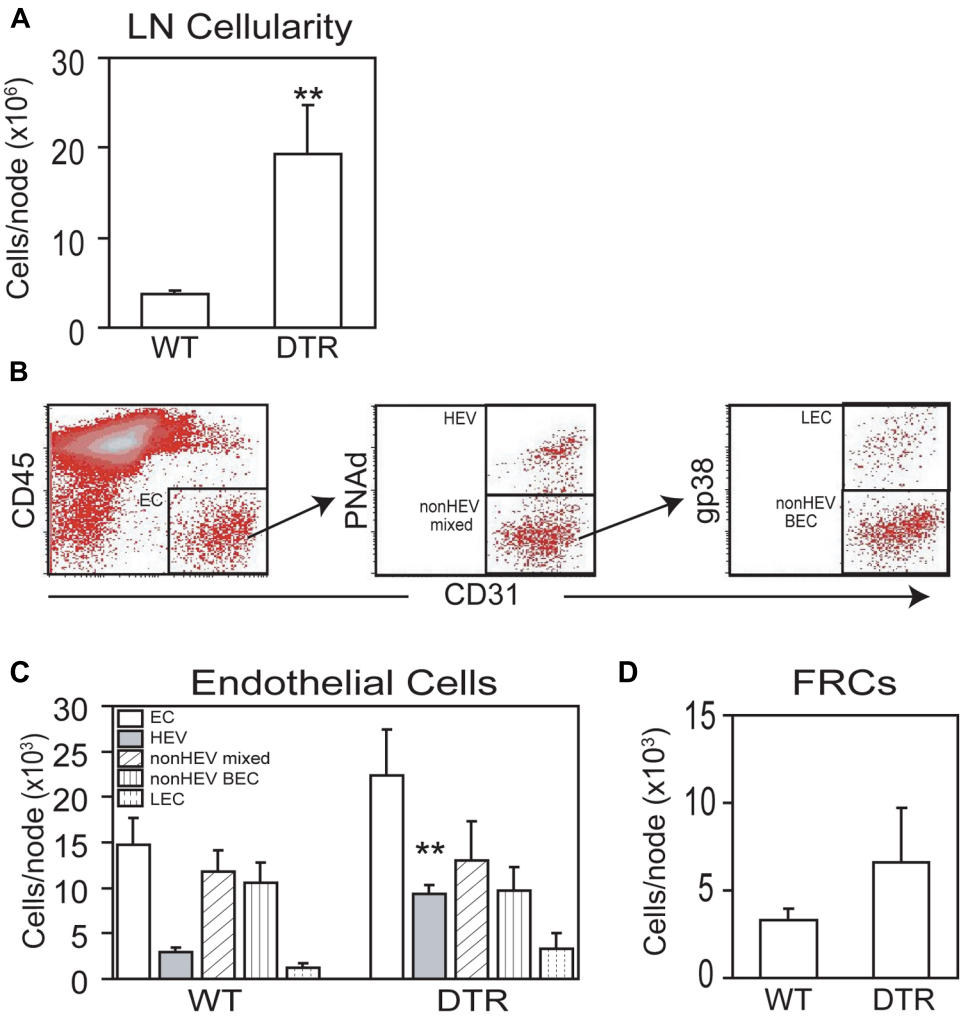

FIGURE 3 | Diphtheria toxin-treated Foxp3-DTR mice also shows more generalized lymph node vascular–stromal growth. Wild-type (WT) and Foxp3-DTR (DTR) mice were injected with DT as per Section "Materials and Methods" and brachial lymph nodes were analyzed by flow cytometry. **(A)** Lymph node cellularity. **(B)** Flow cytometry plots to demonstrate gating of endothelial cell subpopulations. **(C)** Enumeration of endothelial cell subpopulations. **(D)** Enumeration of $gp38^{+}$ FRCs per lymph node. For **(A,C,D)**, $*p < 0.05$, t-test, $n = 3$ mice per condition. For **(C)**, t-test is relative to the same endothelial cell subset in WT mice.

subpopulations of the vascular–stromal compartment examined were expanded.

DISCUSSION

In this study, we characterized the morphologic features of HEV expansion after OVA/CFA immunization and examined the HEVs in a model of acute autoinflammatory lymphadenopathy. We used OPT, which detects changes in HEVs expressing luminal PNAd. Because PNAd can be expressed both luminally and abluminally and it is possible for some HEVs to express only abluminal PNAd (Hemmerich et al., 2001; Drayton et al., 2003; Liao and Ruddle, 2006), OPT may miss remodeling involving cells expressing only abluminal PNAd. Nevertheless, the results reinforce the concept that there are distinct phases of lymph node vascular growth induced after immunization and demonstrate that acute autoinflammatory lymphadenopathy can be associated with HEV expansion. The HEV expansion with autoinflammation and morphologic similarities with HEVs in OVA/CFA-stimulated lymph nodes raise the possibility that insights gained from studying lymph nodes responding to exogenous stimuli may help to better understand vascular–stromal regulation in autoimmune and autoinflammatory diseases.

The initial elongation of HEV after OVA/CFA immunization followed by dominance of arborization corresponds with the findings of Anderson et al. (1975) who used intra-arterial Alcian blue dye and thick sections to study the lymph node vasculature. The initial segment elongation at day 2 could be due to either vessel stretching, as vessel width was reduced at this time point, or to the addition of $PNAd^+$ endothelial cells to existing segments. In support of the latter, flow cytometric analysis showed a doubling of $PNAd^+$ endothelial cell numbers at day 2 (Chyou et al., 2011). However, as flow cytometry is likely able to detect cells expressing only abluminal PNAd as well as cells expressing luminal PNAd, we cannot rule out that the additional $PNAd^+$ cells detected by flow cytometry are cells expressing only abluminal PNAd and that elongation seen by OPT may simply reflect stretching. The subsequent increased arborization and expansion seen at days 4–7 corresponds well in time to the large increase in $PNAd^+$ endothelial cell numbers that is seen during the expansion phase (Chyou et al., 2011). With OVA/CFA, endothelial cell numbers plateau at day 8 (Tzeng et al., 2010), similar to the plateau of HEV expansion seen with OPT at day 7. Given that the expansion of endothelial cell numbers is dependent primarily on B cells (Chyou et al., 2011), the dependence of branching expansion on B cells seen in the viral infection-induced model (Kumar et al., 2010) fits well with the idea that the increased arborization is the morphologic correlate of the increase in endothelial cell numbers seen during the lymphocyte-dependent vascular–stromal expansion phase. During the re-establishment of quiescence and stabilization after expansion, vessels become less leaky and FRCs reorganize more tightly around the vessels (Tzeng et al., 2010). The narrowing of vessels observed after day 7, then, may be the morphologic correlate of vascular stabilization. We have identified a role for $CD11c^{hi}$ presumed dendritic cells in mediating the reduced permeability and reorganization of FRCs (Tzeng et al., 2010), and it will be of interest in future studies to understand whether the HEV narrowing is also dependent on $CD11c^{hi}$ cells. Therefore, new data presented here add to our model of multiple phases of vascular expansion following peripheral immunization. Taking these data along with our previous studies, we propose that vascular expansion is comprised of (1) a $CD11c^+$ cell-dependent, lymphocyte-independent initiation phase that is characterized by upregulation of vascular–stromal proliferation and a modest increase in HEV endothelial cell numbers resulting in HEV elongation and modestly increased arborization, (2) a lymphocyte-dependent phase characterized by continued vascular–stromal proliferation, generalized vascular–stromal expansion, and HEV growth that is mainly due to arborization, and (3) a $CD11c^{hi}$ cell-dependent re-establishment of quiescence and stabilization that involves the re-accumulation of FRCs around vessels and consequent vessel narrowing.

Similar to the enlarged lymph nodes induced by immunization with OVA/CFA, enlarged lymph nodes in the DT-treated Foxp3-DTR mice also showed HEV expansion, lengthening, and branching. The magnitude of these changes at day 8 after the first injection of DT is similar to the changes at days 4–7 after OVA/CFA and this slight delay may be attributable to time required for regulatory T cell depletion. Consistent with the idea that these lymph nodes at day 8 are similar to a relatively early time point after exogenous immunization is the lack of non-HEV blood endothelial cell expansion, as HEV endothelial cells expand more rapidly than non-HEV blood endothelial cells after immunization with OVA/CFA or bone marrow-derived dendritic cells (Tzeng et al., 2010; Chyou et al., 2011). The death of animals by day 10 after regulatory T cell depletion (Kim et al., 2007) precludes satisfactory longer term analysis of the vascular–stromal growth to understand whether non-HEV endothelial cells and the rest of the vascular–stromal compartment also later expand, but it will be of interest to further examine the early events of HEV growth in the Foxp3-DTR mice to understand whether initial stages of HEV expansion is similar to that induced by OVA/CFA. Interestingly, HEV expansion at days 4–7 in the OVA/CFA model is similar to that at day 8 after LCMV infection in terms of total HEV length and segment numbers (Kumar et al., 2010), and the two models share a dependence on B cells (Kumar et al., 2010; Chyou et al., 2011) and multiple models share sensitivity to lymphotoxin beta receptor blockade (Liao and Ruddle, 2006; Kumar et al., 2010; Benahmed and Lu, unpublished observations) for induced HEV expansion. However, in the LCMV model, HEV branching precedes elongation (Kumar et al., 2010), suggesting stimulus-dependent differences in the initiation of HEV growth. The morphologic similarities of the expanded HEVs in the Foxp3-DTR mice with that after acute OVA/CFA immunization or viral infection (Kumar et al., 2010) suggests the possibility of at least some shared regulatory mechanisms.

We used regulatory T cell depletion as a means to induce a model of autoimmunity and autoinflammation, but it is possible that the associated lymph node vascular expansion reflects direct regulatory T cell activity on the vascular–stromal compartment. This would be consistent with studies that have implicated regulatory T cells in directly limiting endothelial cell activation (He et al., 2010; Matrougui et al., 2011). However, we have shown that $CD11c^+$ cells are important for the initiation of lymph node

vascular growth (Webster et al., 2006; Tzeng et al., 2010; Chyou et al., 2011). Regulatory T cell depletion results in the accumulation and activation of dendritic and other $CD11c^+$ cells (Kim et al., 2007; Lund et al., 2008), raising the possibility that regulatory T cell depletion resulted in lymph node vascular expansion at least in part by promoting $CD11c^+$ cell activation and accumulation.

Lymphadenopathy in diseases such as lupus and systemic onset juvenile arthritis can occur acutely with disease flare. Our results here suggest that acutely enlarged lymph nodes in the setting of an autoimmune and autoinflammatory model can have an expanded HEV compartment and that these HEVs share some similarities with those seen after immunization with exogenous stimuli. These results suggest the possibility that insights gleaned from vascular–stromal regulation in immunization models may be instructive for understanding how to therapeutically control immunity in autoimmune and autoinflammatory diseases.

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Positive and negative regulation of T cell responses by fibroblastic reticular cells within paracortical regions of lymph nodes

Stefanie Siegert and Sanjiv A. Luther*

Department of Biochemistry, University of Lausanne, Epalinges, Switzerland

Edited by:
Paul Kaye, University of York, UK

Reviewed by:
Masaaki Murakami, Osaka University, Japan
Xinjian Chen, University of Utah, USA

***Correspondence:**
Sanjiv A. Luther, Department of Biochemistry, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland.
e-mail: sanjiv.luther@unil.ch

Fibroblastic reticular cells (FRC) form the structural backbone of the T cell rich zones in secondary lymphoid organs (SLO), but also actively influence the adaptive immune response. They provide a guidance path for immigrating T lymphocytes and dendritic cells (DC) and are the main local source of the cytokines CCL19, CCL21, and IL-7, all of which are thought to positively regulate T cell homeostasis and T cell interactions with DC. Recently, FRC in lymph nodes (LN) were also described to negatively regulate T cell responses in two distinct ways. During homeostasis they express and present a range of peripheral tissue antigens, thereby participating in peripheral tolerance induction of self-reactive $CD8^+$ T cells. During acute inflammation T cells responding to foreign antigens presented on DC very quickly release pro-inflammatory cytokines such as interferon γ. These cytokines are sensed by FRC which transiently produce nitric oxide (NO) gas dampening the proliferation of neighboring T cells in a non-cognate fashion. In summary, we propose a model in which FRC engage in a bidirectional crosstalk with both DC and T cells to increase the efficiency of the T cell response. However, during an acute response, FRC limit excessive expansion and inflammatory activity of antigen-specific T cells. This negative feedback loop may help to maintain tissue integrity and function during rapid organ growth.

Keywords: lymph node stromal cells, immune tolerance, suppression, mesenchymal stem cells, T lymphocyte activation, fibroblastic reticular cell (FRC), inducible nitric oxide synthase, cyclooxygenase 2

Secondary lymphoid organs (SLO), such as lymph nodes (LN) and spleen, are essential for efficient initiation of adaptive immune responses. Their structure is thought to optimize antigen concentration and presentation to naive recirculating lymphocytes and allow for rapid selection and proliferation of antigen-specific T and B lymphocytes, as well as their differentiation into effector cells. To make these processes more efficient SLO are compartmentalized into functionally distinct microenvironments: the B zone (follicle or outer cortex), T zone (LN paracortex or splenic periarteriolar lymphoid sheath), and entry and exit zones. Three-dimensional networks of specialized radio-resistant fibroblasts (CD45- Ter119- CD31-) are responsible for generating and maintaining these B- and T-cell rich compartments. On the one hand, follicular dendritic cells (FDC; CD21/35+ FDC-M1/2+) inside B zones constitutively produce the chemokine CXCL13 to attract and retain B cells. On the other hand, fibroblastic reticular cells (FRC; CD21/35- gp38+) inside T zones constitutively express the chemokines CCL19 and CCL21 to attract and retain T cells and antigen-presenting dendritic cells (DC) [reviewed in (Cyster, 2005; Junt et al., 2008; Mueller and Germain, 2009; Turley et al., 2010)]. While our understanding of the origin and differentiation of these fibroblast subsets is still very limited (Cyster et al., 2000; Koning and Mebius, 2011), much progress has been made in defining novel roles of FRC in adaptive immunity.

Lymphocytes constantly recirculate between SLO. Once inside the SLO they continue migrating using a random walk to search their cognate antigen. T lymphocytes use the FRC network as physical guidance during this migration. B cells often move first across the T zone by following the FRC network before switching to the connecting FDC network when entering the B zone (Bajenoff et al., 2006). Finally, immigrating and tissue-resident DC associate with the T zone FRC network as well in order to present antigens to recirculating T cells (Lindquist et al., 2004; Bajenoff et al., 2006). It is within this FRC-rich T zone microenvironment where DC and T cells interact to find rare antigen-specific T cells and where some key decisions are being taken: Is a T cell response or rather tolerance induced? If there is a T cell response induced, what will be the type and magnitude? Where will the effector T cells be directed (reviewed in Haring et al., 2006; Junt et al., 2008; Mora et al., 2008; Mueller and Germain, 2009)? In this review we will discuss the recent findings which suggest that T zone FRC are not simple scaffolding cells, but actively influence these critical decisions. A major focus will be on the surprising finding that FRC not only enhance but may also suppress adaptive immunity.

FRC AS POSITIVE REGULATORS OF THE T CELL RESPONSE

T zone FRC are thought to orchestrate and enhance productive T-DC encounters in SLO by various means (**Figure 1A**). First,

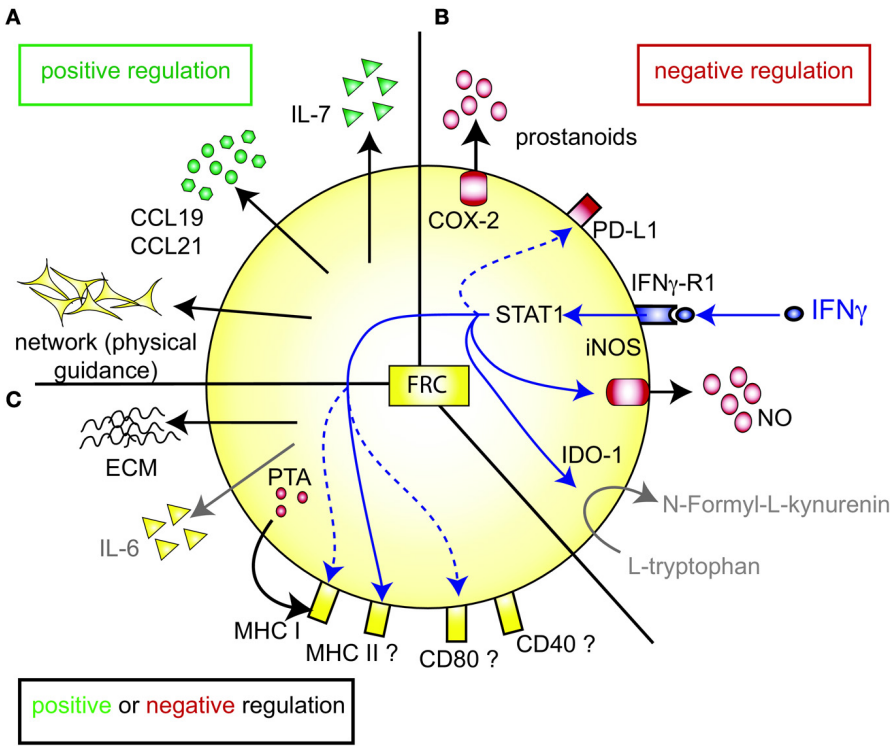

FIGURE 1 | FRC-expressed molecules that positively or negatively regulate the T cell response. (A) FRC are thought to positively regulate T cell immunity in several ways. Throughout the T zone of SLO FRC form a three-dimensional network that serves as a scaffold for DC adhesion and T cell migration, thereby increasing DC-T interaction. In addition, FRC constitutively produce CCL19 and CCL21 that retain T cells in the T zone while increasing their motility. FRC also constitutively produce IL-7 that promotes T cell survival. CCL19, CCL21, and IL-7 may all serve as co-stimulatory signals in case of weak T cell receptor triggering. **(B)** FRC express several molecules that may negatively regulate the immune response: COX-2 and PD-L1 are constitutively expressed, while iNOS and IDO-1 are only expressed after induction by IFNγ. iNOS expression in FRC is also induced by other pro-inflammatory cytokines, such as TNFα, IL-1β and IFNα (not shown). PD-L1 expression on FRC is increased by LCMV infection, IFNγ or TLR3 stimulation (Mueller et al., 2007; Fletcher et al., 2010; Ng et al., 2012). While blocking iNOS and COX improves T cell proliferation in the presence of FRC, no impact of FRC-derived PD-L1, IDO-1, CD80/86, or IL-2 on T cell proliferation has been shown. It is unclear whether FRC can express L-arginase, but arginase inhibitors showed no effect *in vitro* (Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). **(C)** FRC produce several factors which may regulate adaptive immunity in a positive or negative way. They secrete extracellular matrix (ECM) proteins some of which can bind IL-7 and CCL21 and thereby regulate cytokine availability and localization (Katakai et al., 2004b; Förster et al., 2008; Huang and Luther, 2012). ECM molecules may also directly modulate T cell and DC behavior. Microarray data suggest that FRC express constitutively IL-6, which is both anti- and pro-inflammatory depending on the context. FRC express MHC I and low levels of MHC II, CD80, and CD40. IFNγ or TLR3 stimulation enhances the expression of MHC I, MHC II and CD80. In addition, LCMV infection or inflammation evoked by LPS injection *in vivo* can induce MHC II expression on FRC. LN FRC express PTA, which are presented in the context of MHC I, leading to T cell proliferation followed possibly by clonal deletion of self-reactive T cells. Therefore, in certain settings FRC may act as APC.

the FRC network acts as a 3D "road system" for T cells forcing them to pass by antigen-presenting DC attached to FRC, presumably enhancing the frequency of encounters between these two cell types (Katakai et al., 2004a; Lindquist et al., 2004; Sixt et al., 2005; Bajenoff et al., 2006; Mueller and Germain, 2009). This view was recently challenged by computer simulations of T cell trafficking within the LN FRC network which showed that mechanical guidance cues only enhance these encounters when FRC provoke T cell streams, or when FRC also provide motility factors such as the two CCR7 ligands CCL19 and CCL21 (Graw and Regoes, 2012). Second, FRC are the major constitutive source of CCL19 and CCL21, which bring T cells and DC into close proximity (Luther et al., 2000; Link et al., 2007). These chemokines also enhance the motility and survival of T cells (Stachowiak et al., 2006; Asperti-Boursin et al., 2007; Link et al., 2007; Okada and Cyster, 2007; Worbs et al., 2007) as well as the maturation, dendrite formation and antigen uptake by DC (Yanagawa and Onoé, 2002, 2003; Marsland et al., 2005). *In vitro* both CCL19 and CCL21 can act as co-stimulatory signals enhancing T cell priming, especially in settings with sub-optimal T cell stimulation (Flanagan et al., 2004; Friedman et al., 2006; Gollmer et al., 2009). *In vivo* the importance of CCR7 for mounting efficient T cell responses varies greatly depending on the model antigen or pathogen used [reviewed in (Förster et al., 2008; Junt et al., 2008)]. On the ligand side, CCL19 was shown by two laboratories to be dispensable for T cell activation *in vivo* (Saeki et al., 1999; Britschgi et al., 2010), with one report claiming the opposite (Robbiani et al., 2000). Currently, little is known about the *in vivo* role of FRC-derived CCL21, however, the lack of CCL21 expression in HEV of human LN suggests a key role for it in lymphocyte transmigration across HEV (Carlsen et al., 2005). Third, FRC are the major source of constitutive IL-7 expression

in LN and thereby regulate the fitness, survival and homeostasis of naive recirculating T cells (Link et al., 2007; Hara et al., 2012; Huang and Luther, 2012). Adding or blocking IL-7 *in vitro* showed little effect on T cell receptor (TCR)-dependant T cell activation besides the improved viability of T cells and DC in presence of IL-7. However, IL-7 appears to be important *in vivo* for effective interactions between DC and T cells by enhancing TCR signaling and boosting primary antigen-specific T cell expansion (Saini et al., 2009; Mackall et al., 2011; Pellegrini et al., 2011; Huang and Luther, 2012). Administration of IL-7 *in vivo* also augments the effector function and memory formation of T cells (Pellegrini et al., 2011). Not surprisingly, IL-7 is regarded as an attractive adjuvant, which is currently being investigated in several clinical trials (Mackall et al., 2011; Huang and Luther, 2012). In conclusion, LN FRC positively regulate T cell migration and homeostasis. However, the evidence that FRC also augment T cell responses, namely T cell priming as well as effector and memory differentiation, is weaker and more indirect as it is often based on *in vitro* assays or on recombinant proteins expressed non-exclusively by FRC (**Figure 1A**).

FRC AS NEGATIVE REGULATORS OF THE T CELL RESPONSE

Paradoxically, recent studies have proposed that FRC also negatively regulate T cells, both during homeostasis and immune responses. On one hand, FRC induce T cell tolerance via self-antigen expression, on the other hand they can impair T cell responses to foreign antigens by expressing suppressive factors, either directly inhibiting T cell expansion or lowering the immunogenicity of DC (**Figures 1B,C**) (Fletcher et al., 2010; Turley et al., 2010; Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011).

Similar to thymic epithelial cells (TEC), FRC from LN were shown to constitutively express multiple peripheral tissue restricted antigens (PTA), including known autoimmune targets (**Figures 1C, 2A**) (Fletcher et al., 2010; Turley et al., 2010). They share this feature with other LN stromal cells, such as lymphatic and blood endothelial cells (LEC and BEC) (Cohen et al., 2010; Fletcher et al., 2010), although the PTA are only partially overlapping. However, the best characterized regulator of promiscuous gene expression, Autoimmune regulator (Aire), was found only in a rare EpCAM-expressing cell type in the outer T zone of LN (Gardner et al., 2008), while Aire is poorly expressed in FRC, LEC, and BEC (Fletcher et al., 2010) (our unpublished observation). Another potential positive regulator of promiscuous gene expression, deformed epidermal autoregulatory factor 1 (Deaf1), was observed to be strongly expressed in FRC as well as other LN stromal cell types (Fletcher et al., 2010; Turley et al., 2010). What is the functional consequence of PTA expression by FRC? Turley and colleagues reported that in transgenic mice expressing a truncated ovalbumin (OVA), transferred OVA-specific CD8+ T cells in peripheral LN (pLN) proliferated followed by their partial deletion, even when MHC I was absent on hematopoietic cells. Surprisingly, the intestinal fatty acid binding promoter used to drive transgene expression led to OVA-peptide expression

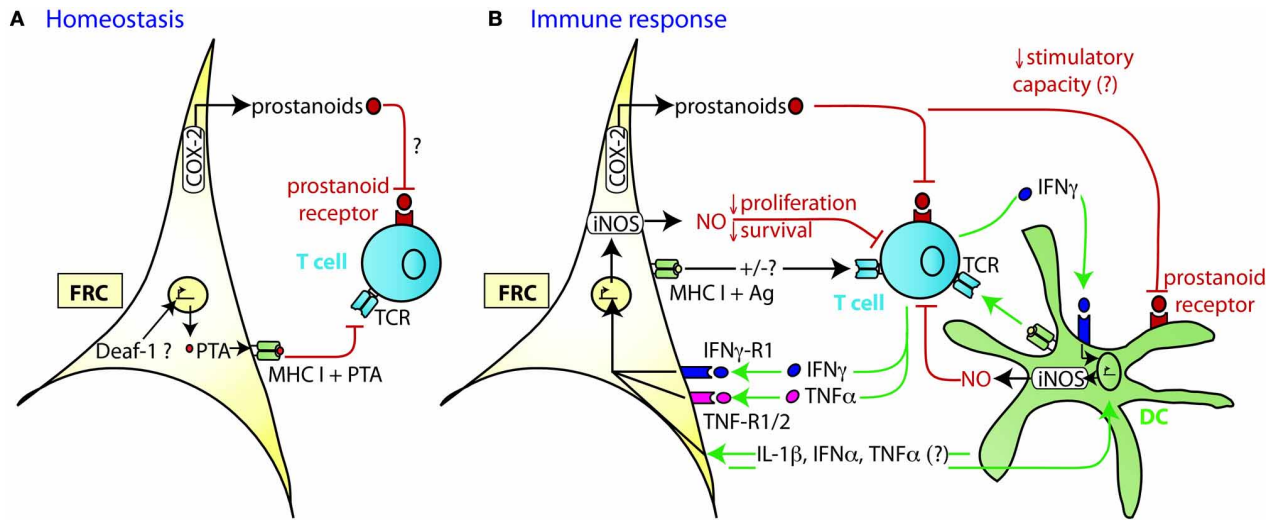

FIGURE 2 | Mechanism of TRC-mediated negative regulation of T cell expansion. (A) During homeostasis LN FRC express self-antigens (PTA), possibly in a Deaf-1 dependent fashion. PTA presented in the context of MHC I may lead to peripheral tolerance induction via clonal deletion of self-reactive T cells. *Cox-2* is constitutively expressed in FRC, presumably leading to the production of PGE_2 or other prostanoids that may attenuate T cell priming or proliferation. **(B)** During immune response DC capture antigen (Ag) in the periphery, home to the T zone of SLO and present peptides of foreign antigens to recirculating T cells. Within the first 20 h upon TCR triggering, T cells produce a first wave of IFNγ and TNFα cytokines, before entering cell cycle. In case of a very strong immune response, the high level of pro-inflammatory cytokines induces transient iNOS expression in the neighboring FRC and DC. The resulting NO release then acts as a negative feedback loop slowing down T cell proliferation over the following days presumably to ensure that the strong T cell response does not endanger tissue integrity. High concentrations of NO may also induce T cell apoptosis. The attenuating effect by FRC can occur in trans, without cognate interaction with T cells. Constitutive expression of COX-2 dependent factors, such as PGE_2, may contribute to this attenuation of T cell expansion, by acting directly or indirectly (via DC) on the proliferating T cells. Not only is there crosstalk between FRC and T cells, but also between FRC and DC which leads to a much stronger release of NO by these two cell types if IFNγ is present. While the factors exchanged between FRC and DC have not yet been identified, the may include IL-1β, IFNα, and TNFα, all of which can trigger iNOS expression.

on MHC I selectively in gp38$^+$ CD31$^-$ FRC of pLN along with some expression of CD40 and CD80 (Lee et al., 2007; Fletcher et al., 2010). Based on these findings it was proposed that FRC can induce peripheral T cell tolerance but more direct evidence is needed to strengthen this notion. When these transgenic mice were bred to mice deficient in inducible nitric oxide synthase (iNOS, NOS2), OVA-specific T cell expansion was enhanced (Lukacs-Kornek et al., 2011). As FRC and LEC but not DC or macrophages expressed iNOS in this system it suggests stromal cells use nitric oxide (NO) to limit T cell expansion and possibly tolerance induction. While FRC can express MHC class II under certain conditions, e.g., upon IFNγ stimulation (Lee et al., 2007; Ng et al., 2012), its functional significance for tolerance and immunity has remained unexplored. Also unclear is why the typically inducible cyclooxygenase-2 (COX-2) enzyme is constitutively expressed by FRC in naive LN (Siegert et al., 2011). COX-2 is the rate-limiting enzyme for the generation of prostanoids, including prostaglandin E_2 (PGE_2), that can both enhance or suppress T cell immunity [reviewed in (Gualde and Harizi, 2004; Kalinski, 2012)]. It remains to be tested whether COX-2 participates in peripheral T cell tolerance induction. In conclusion, the existing evidence suggests that FRC and LEC assist DC in peripheral tolerance induction of self-reactive CD8+ T cells by expressing self-antigens and NO.

Besides their tolerance-promoting role as unconventional antigen presenting cells (APC), we and others recently provided evidence for the surprising capacity of FRC to attenuate the proliferation of CD4+ and CD8+ T cells in acute inflammation, independently of antigen presentation by FRC (Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011) (**Figures 2A,B**). FRC did not interfere with initial TCR signaling in primed T cells but with entry or progression in cell cycle and possibly with survival. The inhibitory effect by FRC was mainly mediated by iNOS dependent NO production, with iNOS being strongly induced in FRC by the synergistic action of IFNγ and TNFα. These two cytokines are produced by CD8$^+$ T cells less than 24 h after priming and appear to be sensed by neighboring FRC (**Figure 2B**) (Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011), consistent with IFNγ having been detected over a wide area inside activated LN T zones (Beuneu et al., 2010; Perona-Wright et al., 2010). This early burst of cytokines before blastogenesis has previously been documented (Mempel et al., 2004; Beuneu et al., 2010) and induces crosstalk between these two cell types, leading to a negative feedback loop limiting T cell expansion in case of acute inflammation. NO is a highly reactive gas which is thought to be rapidly consumed by neighboring cells. NO or NO-derived reactive nitrogen species may negatively regulate T cells via nitrosylation of diverse amino acid residues, leading to down regulation of the TCR complex (Kasic et al., 2011). Furthermore, depletion of the iNOS substrate L-arginine may inhibit T cells, through down-regulation of CD3ζ (Rodriguez et al., 2002). Lastly, NO may directly block STAT5 phosphorylation thereby interfering with IL-2 signaling (Bingisser et al., 1998) [reviewed in Bogdan (2001), (2011)]. Notably, NO expression by FRC attenuated T cell proliferation without abrogating it (Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). We therefore propose that FRC form micro-niches within the T zone where T cell proliferation is either enhanced or diminished, depending on the context or phase of immune response. Although IFNγ-stimulated FRC express other known suppressive molecules, such as programmed cell death ligand 1 (PD-L1) or Indolamin-2,3-dioxygenase (IDO) (**Figure 1B**), they do not seem to be implicated in the inhibition of T cell proliferation in the *in vitro* activation systems used (Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). The function of PD-L1 on FRC may be protection from CD8+ T cell-mediated cytotoxicity, as in chronic LCMV infection (Mueller et al., 2007). A role for IDO in FRC remains to be established. As both PD-L1 and IDO have roles for regulatory T cells and suppressive myeloid cells, it would be interesting to address the function of FRC in the generation, maintenance or activation of these regulatory cells (Francisco et al., 2010; Le Blanc and Mougiakakos, 2012).

T cell expansion was also strongly attenuated *in vitro* when antigen-pulsed DC were used instead of T cell mitogens. In this setting, both iNOS and COX-2 were responsible for the strongly reduced T cell expansion. In comparison to proliferation, T cell effector function (killing, IFNγ expression) was less strongly affected (Siegert et al., 2011). Part of the attenuating effect on T cell expansion may be due to diminished DC immunogenicity. Overnight incubation of bone-marrow derived DC (BMDC) with FRC led to a 50% reduction of CD8+ T cell stimulation by these "FRC-conditioned" DC (Siegert et al., 2011). No differences in the expression of MHC I or CD80/86 by DC were observed suggesting alterations in the cytokine milieu as the most likely cause. PGE_2 may be the culprit: While it stimulates immature DC in the periphery, it seems to be suppressive for mature DC in SLO and decreases their APC function (Gualde and Harizi, 2004). Interestingly, in presence of activated T cells crosstalk between DC and FRC strongly increases iNOS expression in both cell types. Similar to a previous report on rat lung fibroblasts (Lavnikova and Laskin, 1995), it is always only a fraction of FRC and DC which express iNOS, even when FRC clones or abundant IFNγ are used (Serbina et al., 2003; Siegert et al., 2011). It remains to be shown whether this is due to the cell cycle stage or a potential negative feedback loop preventing excessive iNOS activity. Currently, the signals exchanged between FRC and DC leading to iNOS expression is unclear. It seems likely that pro-inflammatory cytokines, like IL-1β and TNFα produced by activated DC are sensed by FRC leading to iNOS induction. Many cytokines were recently described to be produced by FRC (Fletcher et al., 2011; Malhotra et al., 2012), but the FRC-derived signals stimulating iNOS expression in DC remain to be identified. In contrast to these fairly rapid attenuating effects of FRC on DC, earlier studies have suggested that stromal cells can also have long-term effects on DC by promoting the development of regulatory DC which inhibit T cell responses. Adult splenic stromal cells co-cultured for 1 week with splenic c-kit$^+$ precursor cells induced the development of CD11c^{low} CD45RB$^+$ regulatory DC expressing IL-10 (Svensson et al., 2004). In another study, neonatal splenic stromal cells converted LPS-activated BMDC into NO-expressing regulatory DC, in a process dependent on cell-cell contact and TGFβ (Zhang et al., 2004). In both studies, the splenic stromal cells were only partially characterized and it is unclear whether these included FRC-like cells. These latter

observations may be relevant for tissue development and chronic immune responses.

That FRC exert a suppressive effect on acute T cell expansion was best described in co-culture experiments, but *in vivo* experiments also support this notion. When wildtype (WT) mice that had previously received OVA-specific $CD8^+$ T cells were infected with OVA expressing vesicular stomatitis virus (VSV-OVA), iNOS expression was observed only in a subset of FRC and, to a lesser extent, DC within draining LN (Siegert et al., 2011). This expression was detectable 24 h after infection, but not at later time points. However, the negative effects of iNOS on T cell expansion were clearly visible on day 4 and 8 suggesting NO either affects T cells only during the early activation phase or it permanently retards T cell proliferation due to longer lasting modifications in the target cells. Consistent with a negative role of iNOS *in vivo*, VSV-OVA infected $iNOS^{-/-}$ mice showed an exaggerated expansion of antigen-specific T cells compared to WT mice (Siegert et al., 2011). When $iNOS^{-/-}$ mice were immunized with OVA-loaded BMDC (Khan et al., 2011) a similar role for NO in restraining the T cell response was found. However, when the antigen was directly targeted to LN resident non-inflammatory DC using OVA-coupled anti-DEC205 antibodies, a decrease rather than an increase in antigen-specific T cell expansion was observed in iNOS-deficient mice (Khan et al., 2011). These findings support a model in which iNOS-mediated inhibition of T cell expansion is observed only in strong T_{H1}-type immune responses characterized by high levels of IFNγ and TNFα. Consistent with this notion, T_{H2} cytokines did not induce iNOS in FRC and *in vitro* expansion of T_{H2} cells was not inhibited by FRC or iNOS (Niedbala et al., 1999; Khan et al., 2011). Currently it cannot be ruled out that other known iNOS sources, such as subsets of DC and macrophages, localize to LN T zones during T_{H1}-type responses and contribute to these attenuating effects (Bogdan, 2001; Serbina et al., 2003; Siegert et al., 2011). Besides using NO within their lysosomes to kill phagocytosed microbes, both DC and macrophages (or myeloid-derived suppressor cells) can inhibit T cell proliferation *in vitro* by producing extracellular NO (Albina et al., 1991; Serbina et al., 2003; Zhang et al., 2004; Gabrilovich and Nagaraj, 2009; Siegert et al., 2011). In an attempt to better define the effect of iNOS expression in FRC versus DC, immune responses were investigated in irradiated $iNOS^{-/-}$ mice receiving WT BM, but results were not conclusive as these mice showed unexpected defects in LN swelling, probably due to iNOS-dependent vascular defects induced by irradiation (Khan et al., 2011; Siegert et al., 2011). In the future, mice with cell type-specific deletion of iNOS are needed to firmly establish the relative roles of the various iNOS sources in the regulation of T cell expansion.

IFNγ stands out as a major regulator of FRC function: It strongly induces iNOS, IDO-1, PD-L1 as well as several molecules involved in antigen presentation (**Figures 1B,C**) (Mueller et al., 2007; Fletcher et al., 2010; Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011; Ng et al., 2012). Typically, IFNγ is regarded mostly as a pro-inflammatory cytokine because it enhances various aspects of the immune response, including the activation of macrophages, clearance of intracellular pathogens, up-regulation of MHC molecules or the promotion of T_{H1} responses. However, IFNγ also has immune-regulatory functions: It negatively modulates expression of tissue-destructive enzymes, diminishes the recruitment of inflammatory cells such as neutrophils, suppresses T_{H17} differentiation and positively regulates T_{REG} differentiation under certain circumstances [reviewed in (Kelchtermans et al., 2008; Hu and Ivashkiv, 2009)]. Consistent with this dual role, IFNγ can augment or suppress autoimmunity, depending on the context and timing. A prominent example is experimental autoimmune encephalomyelitis (EAE) (Kelchtermans et al., 2008; Hu and Ivashkiv, 2009). In one EAE mouse model autoimmune disease is enhanced in IFNγ-deficient mice as indicated by the increase of proliferating T cells in the spleen and the central nervous system (Chu et al., 2000). This finding may be related to the observation that T_{H1} cells limit their own expansion in the later phase of the immune response by expressing IFNγ (Feuerer et al., 2006; Haring et al., 2006). In autoimmune disease some of the protective effects mediated by IFNγ seem to be due to its capacity to induce iNOS expression. Support for this notion comes from the observation that self-reactivity and the associated pathology observed in experimental models for EAE or myasthenia gravis are enhanced in $iNOS^{-/-}$ mice (Bogdan, 2001, 2011; Shi et al., 2001; Xiao et al., 2008). The protective effect of iNOS was also observed with mycobacteria that are a critical part of complete Freund's adjuvant (CFA). When rodents are immunized with CFA they often do not develop subsequent autoimmunity, a phenomenon termed "adjuvant immunotherapy." Interestingly, Kahn and colleagues showed that this protective CFA effect depends on iNOS, as well as on IFNγ and TNFα which are the likely inducers of iNOS within lymphoid tissues (Kahn et al., 2001). Possibly, IFNγ-induced NO production in FRC or other host cells participates in the active down-regulation of T cell responses to limit tissue damage associated with acute inflammation or autoimmunity. In the future it will be of interest to test whether iNOS expression in myeloid cells, FRC or other cell types is critical for this protection.

IS ATTENUATION OF T CELL PROLIFERATION A FEATURE OF ALL MESENCHYMAL CELLS?

The suppressive activity recently observed with FRC is reminiscent of the increasing number of reports showing that mesenchymal stem/stromal cells (MSC), as well as adult fibroblasts from non-lymphoid tissues, potently inhibit T cell proliferation, both *in vitro* and *in vivo*. Some reports also state that MSC reduce the cytotoxicity and IFNγ expression in effector T cells (Haniffa et al., 2007; Jones et al., 2007; Uccelli et al., 2008; Haniffa et al., 2009; Khan et al., 2011; Siegert et al., 2011). Various mechanisms of suppression have been described for MSC and non-lymphoid fibroblasts, as summarized in **Table 1** (Nauta and Fibbe, 2007; Uccelli et al., 2008). It suggests that these suppressive pathways are either redundant, activated in a context-dependent way or simply due to differences in the assays used. Similar to FRC, IFNγ-dependant induction of iNOS in MSC appears to be a major inhibitory pathway (Nauta and Fibbe, 2007; Ren et al., 2008; Uccelli et al., 2008), but the production of suppressive COX-1/2-dependent prostanoids has also been reported for MSC (Nauta and Fibbe, 2007; Ren et al., 2008; Uccelli et al., 2008). The expression of several other inhibitory molecules observed in MSC may

Table 1 | Suppressive molecules known to be expressed in MSC versus their characterization in FRC.

Suppressive molecules expressed by MSC	Constitutive expression				References	
	MSC (human and murine)		FRC (murine)		MSC	FRC
	Effect on T cell activation		Expressed?	Effect on T cell activation		
COX-2 (PGE_2 ?)	Attenuated T cell proliferation, Impaired maturation of monocytes into DC		Yes	Attenuated CD8 T cell proliferation	4–7	1–3
HGF	Attenuated T cell proliferation		Yes	ND	4–7	2, 3
TGFβ	Attenuated T cell proliferation		Yes	No effect	4–7	1, 2, 3
IL-6	Impaired maturation of monocytes into DC		Yes	ND	4–7	2, 3
HO1	Attenuated T cell proliferation		Yes	ND	4–7	2, 3
Soluble HLA-G5 / Qa-2 or H2-Q8	Attenuated T cell proliferation and cytotoxicity		Yes	ND	4–7	2, 3
FasL	Induction of T cell apoptosis		No	ND	4–8	2, 3
	Induced expression					
	Induced by	Effect on T cell activation	Induced by	Effect on T cell activation		
iNOS	IFNγ	Attenuated T cell proliferation	IFNγ, ILß, TNFα, IFNα	Attenuated T cell proliferation (CD4 and CD8)	4–7	1, 9, 10
IDO-1/2	IFNγ	Attenuated T cell proliferation	IFNγ	No effect	4–7	1, 9
IL-10	T cell contact	Release of sHLA-G5	ND	No effect	4–7	1

MSC have been shown to interfere with the maturation and antigen presentation of DC and with T cell proliferation, both by cell-cell contact and various soluble factors. Similar observations have been obtained for FRC. FRC attenuate T cell proliferation mostly by NO production, but also lower the immunogenicity of DC in unknown ways. COX-1/2 dependent prostanoids produced by FRC may me responsible for this effect on DC. Interestingly, several candidates previously described in MSC appear to be expressed by FRC as well, as suggested by recent gene array results. Abbreviations: HGF, Hepatocyte Growth Factor; TGFβ Transforming Growth Factor beta; HO1, Heme Oxygenase-1; ND, not determined. References: (1) Siegert et al. (2011) (2) gene array data from Fletcher et al. (2011) (3) gene array data from Malhotra et al., 2012 for 2 and 3: NCBI GEO accession number GSE15907, immgen.org, (4) reviewed in Uccelli et al., 2008, (5) reviewed in Haniffa et al. (2009), (6) Sato et al. (2010) (7) reviewed in Nauta and Fibbe (2007), (8) Akiyama et al. (2012) (9) Lukacs-Kornek et al. (2011) (10) Khan et al. (2011).

be conserved in FRC and should be the focus of future studies (**Table 1**). Again, reminiscent of FRC, MSC are able to present antigens in the context of MHC II, without it being a requirement for their inhibitory effect (Chan et al., 2006; Stagg et al., 2006; Uccelli et al., 2008). For antigen presentation by MSC the IFNγ level seems to be crucial: while low levels induce MHC II expression and APC function, both are lost with higher IFNγ concentrations (Chan et al., 2006). It remains to be tested whether APC function of FRC is regulated in a similar fashion. If yes, then this regulation would be opposite to that for iNOS expression. Importantly, MSC have proven to successfully suppress autoimmune disease and graft versus host disease (Gvhd) in mice and humans (Haniffa et al., 2007; Jones et al., 2007; Uccelli et al., 2008; Sato et al., 2010; Le Blanc and Mougiakakos, 2012). This year Canada approved MSC transfers as a preventive treatment for Gvhd during bone marrow transplantations (Prochymal®, www.osiris.com). While MSC can home both to SLO and inflammatory sites it is currently not clear where and when T cell inhibition takes place. In conclusion, most mesenchymal cell types share with FRC T cell suppressive characteristics, independent of their differentiation state. Interestingly, non-mesenchymal cells such as blood and lymphatic endothelial cells from LN also suppress T cell activation via an IFNγ/NO-dependant pathway (Khan et al., 2011; Lukacs-Kornek et al., 2011). In addition, epithelial cells from healthy kidney and skin or gut tumors also potently suppress T cell expansion, indicating that this characteristic is shared with many other cell types (Siegert et al., 2011).

WHY DO NON-HEMATOPOIETIC CELLS CONTRIBUTE TO IMMUNE REGULATION?

The emerging concept is that hematopoietic cells are not the sole regulators of T cell tolerance and T cell responses but that many tissue cells can contribute, including FRC within LN. At present we can only speculate on the reasons for this complex regulation. One possible explanation is that the immune system is a powerful but also dangerous weapon that needs to be tightly controlled at many levels and anatomical sites to allow efficient immune-mediated protection while avoiding immune-mediated pathology. During homeostasis the body may keep most tissues in a slightly immune-suppressed state to avoid the activation of low affinity T cells that recognize self-antigens or harmless antigens. During inflammation there may be an upper limit to the extent of expansion allowed for a given T cell population and a given organ. In this context, the structural cells may act as mechanical and chemical sensors of the ongoing T cell response in an inflamed organ and adjust population dynamics to ensure ordered tissue growth and maintenance of functional structures. If excessive inflammatory signals are present many tissue-specific resident cells, including fibroblasts inside SLO or non-lymphoid tissues, may be activated to put a brake on the immune response.

A key function of fibroblasts is to maintain tissue integrity. In case of damage, they have repair functions with the aim of re-establishing homeostasis and full functionality. Both self-reactive and beneficial, but hyperactive, T cell responses are a serious threat for SLO and the body as a whole. Disruption of SLO structure is frequently associated with immunodeficiency and therefore to be avoided [reviewed in (Junt et al., 2008; Mueller and Germain, 2009)]. So, giving structural tissue cells, like FRC, some control over a strong ongoing T cell response appears to be a reasonable solution to proactively protect the body. Similar to the multiple cell types expressing self-antigens, and thereby shaping the peripheral T cell repertoire, several cell types—hematopoietic and non-hematopoietic—are likely to participate in the regulation of responses to foreign antigens and danger signals. It will be an important challenge to identify individual contributions, as well as coordination, between these various regulatory cells. Interestingly, many cell types of the immune system have a subset which suppresses or regulates adaptive immunity, including regulatory T and B cells, regulatory DC and alternatively activated M2 macrophages (Steinman et al., 2003; Lund and Randall, 2010; Murray and Wynn, 2011; Rudensky, 2011). Therefore, it may not be surprising that LN FRC turn out to be both positive and negative regulators of adaptive immunity. Similar to the other cell types, it remains to be established whether there is a dedicated FRC subset for immune suppression, or whether they are plastic and adopt an inhibitory activity, depending on the localization (e.g., suppressive micro-niches within the T zone), the activation status (e.g., cytokine milieu and cell-cell interactions modulating FRC) and the time (e.g., phase of the immune response). In fibroblasts and FRC some characteristics appear to stay imprinted and they correlate with the anatomical localization and the environment during cellular development (Chang et al., 2002; Hammerschmidt et al., 2008; Buettner et al., 2011; Molenaar et al., 2011). It will be a challenge for future investigations to assess in detail the complex effects FRC have on T cells, either in the naive, activated, or memory stage. These effects are likely to be strongly dependent on FRC density, functional specialization, environmental factors (positive and negative), and strength and type of T cell response. Many of these studies will rely on *in vitro* assays where physiological settings need to be mimicked as closely as possible. These findings will need confirmation from *in vivo* experiments. Mouse lines expressing Cre recombinase in an FRC-specific manner are now available (Kraman et al., 2010; Chai et al., 2011; Onder et al., 2011) and should help to selectively manipulate molecules in FRC.

While FRC-like networks have been described for other SLO, such as Peyer's patches and spleen (Katakai et al., 2008; Mueller and Germain, 2009), it remains to be established whether they are functionally similar to LN FRC. In the placenta, mucosal surfaces, skin and tumors, tissue fibroblasts could also contribute to immune regulation, in a way comparable to LN FRC. Tertiary lymphoid tissues (TLT), which often develop at ectopic sites in response to chronic inflammation, like diabetes or atherosclerosis, also contain various stromal cell types found in LN, including FRC-like cells expressing CCL19/21 and IL-7, both in mice and humans (Manzo et al., 2005; Gräbner et al., 2009; Peduto et al., 2009; Link et al., 2011; Perros et al., 2012). While these FRC-like cells seem to have features positively regulating adaptive immunity within TLT, it is currently unclear whether they also regulate it negatively. Given the prominent IFNγ and iNOS expression in many chronic inflammatory diseases, a contribution by fibroblastic cells appears likely. In case of TLT developing during autoimmune disease it would be of interest to test whether these stromal cells could be trained to suppress self-reactive T cell responses over prolonged periods of time. Alternatively, depleting these ectopic FRC or reducing their positive regulatory factors could be another promising avenue assuming TLT are critical for pathogenesis.

As LN FRC have both positive and negative effects on immune responses, they do not seem ideal candidates for a cellular therapy in autoimmunity. This is in contrast to MSC and skin fibroblasts, which are predominantly immunosuppressive. We postulate that during evolution, when LN developed in mammals, normal tissue fibroblasts were included to structure the LN (Boehm et al., 2012). In that process they may have kept their tissue-protective, immune-suppressive features, but have specialized further to include functions helping lymphocytes to survive (IL-7), to localize and migrate correctly (CCL19/21), to be silenced (PTA) or to be activated (conduits, IL-7, CCL19/21). The identification of the transcription factors, siRNA or epigenetic elements regulating the polarization of tissue fibroblasts should be an exciting field for future studies. Most importantly, future research may give clinicians the ability to modulate fibroblast function, either to promote or suppress immunity, thereby leading to therapies for treating infections, cancer and autoimmune disease.

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Follicular dendritic cells, conduits, lymphatic vessels, and high endothelial venules in tertiary lymphoid organs: parallels with lymph node stroma

Sharon Stranford [1] ***and Nancy H. Ruddle*** [2,3] ******

[1] *Department of Biological Sciences, Mount Holyoke College, South Hadley, MA, USA*
[2] *Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT, USA*
[3] *Department of Immunobiology, Yale University School of Medicine, New Haven, CT, USA*

Edited by:
Mark Christopher Coles, University of York, UK

Reviewed by:
Mark Christopher Coles, University of York, UK
Natalio Garbi, University of Bonn, Germany
Theresa T. Lu, Hospital for Special Surgery/Weill Cornell Medical Center, USA

****Correspondence:***
Nancy H. Ruddle, Department of Epidemiology of Microbial Diseases, Yale School of Public Health, 60 College Street, New Haven, CT 06520-8034, USA.
e-mail: nancy.ruddle@yale.edu

In this communication, the contribution of stromal, or non-hematopoietic, cells to the structure and function of lymph nodes (LNs), as canonical secondary lymphoid organs (SLOs), is compared to that of tertiary lymphoid tissue or organs (TLOs), also known as ectopic lymphoid tissues. TLOs can arise in non-lymphoid organs during chronic inflammation, as a result of autoimmune responses, graft rejection, atherosclerosis, microbial infection, and cancer. The stromal components found in SLOs including follicular dendritic cells, fibroblast reticular cells, lymphatic vessels, and high endothelial venules and possibly conduits are present in TLOs; their molecular regulation mimics that of LNs. Advances in visualization techniques and the development of transgenic mice that permit *in vivo* real time imaging of these structures will facilitate elucidation of their precise functions in the context of chronic inflammation. A clearer understanding of the inflammatory signals that drive non-lymphoid stromal cells to reorganize into TLO should allow the design of therapeutic interventions to impede the progression of autoimmune activity, or alternatively, to enhance anti-tumor responses.

Keywords: autoimmunity, chronic inflammation, cancer, secondary lymphoid organ, tertiary lymphoid tissue

INTRODUCTION

The non-hematopoietic or stromal cells present in secondary lymphoid organs (SLOs) – lymph nodes (LNs), spleen, Peyer's patches (PP)- provide the structural and functional underpinnings that allow the most efficient encounter of lymphocytes with their cognate antigens. In this communication, we compare and analyze the contribution of stromal cells in SLOs to a particular type of cellular accumulation, the tertiary lymphoid tissue/organ (TLO).

The immune system generates a repertoire of antigen specific lymphocytes during development of these cells in primary lymphoid organs (the thymus, fetal liver, and bone marrow). Mature lymphocytes regularly transit through SLOs where encounter with cognate antigen is facilitated, leading to an effective immune response. Strategically positioned throughout the body, LNs are encapsulated, highly organized structures; T cells and dendritic cells (DCs) are concentrated in the paracortical region, with B cells and follicular DCs (FDCs) in the cortical region. In the mature LN, resident stromal cells play several critical roles, providing: (1) a meshwork supporting lymphocyte attachment, (2) chemokine signals directing cell subset positioning, (3) a gateway for entrance for lymphocytes (high endothelial venules; HEVs), (4) connections between nodes (lymphatic vessels; LVs), (5) entry points for antigen and antigen presenting cells (APCs; afferent LVs), (6) microvessels, allowing rapid import of small antigens and chemical signals (conduits formed by fibroblastic reticular cells; FRCs), and (7) sites of self-antigen presentation (FRCs, eTACs, LVs).

While SLOs arise during development at key locations in the body, chronic immune activity in the adult can give rise to similar organized accumulations of lymphoid cells in almost any non-lymphoid tissue. These TLOs closely resemble SLOs (particularly LNs) with regard to cellular composition, organization, chemokines, and vasculature. TLOs have been noted during chronic inflammatory processes, including autoimmunity, chronic graft rejection, persistent infection (summarized in Drayton et al., 2006), atherosclerosis (Grabner et al., 2009), and cancer (Martinet et al., 2011; Bergomas et al., 2012). TLOs can also be induced experimentally by tissue-specific expression of certain inflammatory mediators (summarized in Drayton et al., 2006), including members of the lymphotoxin (LT) family (Kratz et al., 1996; Drayton et al., 2003), cytokines crucial for lymphoid organ development and maintenance (Ruddle and Akirav, 2009). Like SLOs, TLOs display organized leukocyte subtype compartmentalization driven by lymphoid chemokines (CCL19, CCL21, and CXCL13), the formation of germinal centers, and a highly organized vascular system, including HEVs, LVs, and perhaps conduits (**Figure 1**). Data suggesting that TLOs function as local sites of antigen presentation and lymphocyte activation, including somatic hypermutation and class switching in B cells (Schroder et al., 1996), suggest that they facilitate local antimicrobial responses, as well as epitope spreading (McMahon et al., 2005) and autoimmune exacerbation.

Not all inflammatory infiltrates organize into TLOs. Furthermore, in some instance, TLOs can progress from a relatively benign to a destructive phase. In type 1 diabetes in the NOD mouse, initial

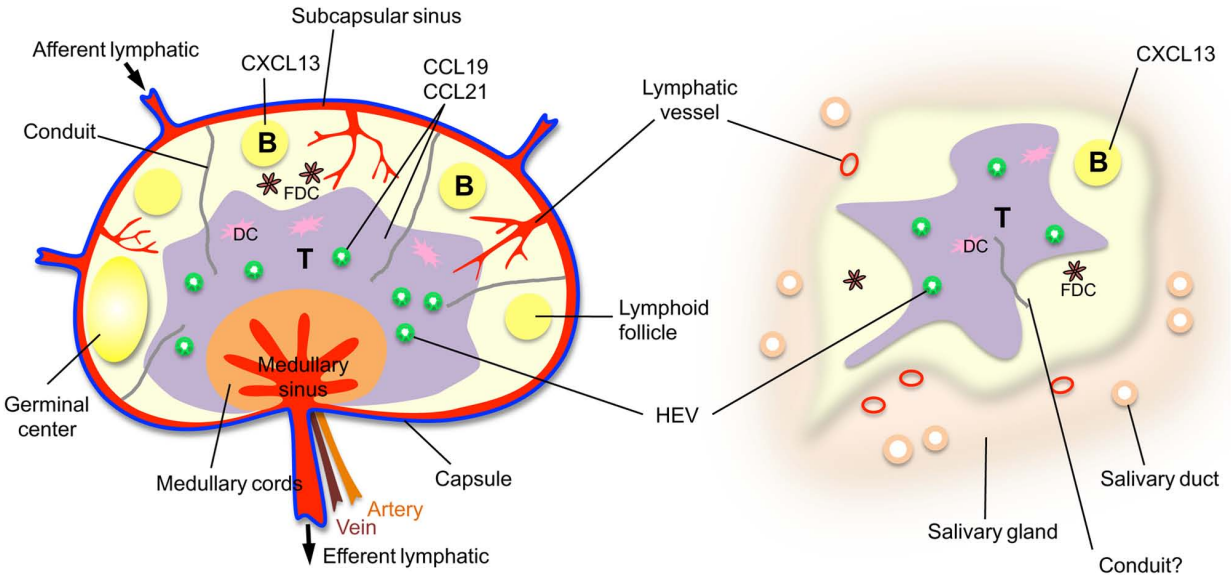

FIGURE 1 | A comparison of a lymph node with a TLO, a salivary gland from an individual with Sjögren's syndrome. Note that the HEVs and lymphatic vessels are colored to indicate the existence of mice with green fluorescent HEVs (Bentley et al., 2011) and red fluorescent lymphatic vessels (Truman et al., 2012) that have been developed to facilitate *in vivo* imaging.

pancreatic infiltrates are characterized by HEV development and minimal islet destruction, whereas later stages demonstrate frank islet destruction and diabetes (Andre et al., 1996). In this model, pancreatic TLOs disappear after removal of the β cell antigen, being replaced by tissue fibroblasts.

In the following sections, we present a detailed comparison of the stromal characteristics of SLOs and TLOs.

LYMPHOID TISSUE ORGANIZER CELLS

In the early stages of SLO development, various initiating events can activate stromal cells, called lymphoid tissue organizer (LTo) cells ($CD4^-CD3^-IL7^+VCAM\text{-}1^+CXCL13^+LT\beta R^+$), which recent data suggest may be derived from adipocyte precursors (Bénézech et al., 2012). LTo cells then activate lymphoid tissue inducer (LTi) cells ($CD4^+CD3^-LT\alpha\beta^+$, $IL7R^+$ $CXCR5^+$) and they in turn, by their cytokine production, activate LTo cells. Although, LTo cells have not yet been isolated from TLOs, LTβR has been shown to induce aortic smooth muscle cells, which are implicated in TLOs in atherosclerosis (Grabner et al., 2009), to express some genes characteristic of LTos, including CXCL13 and VCAM-1. Both CXCL13 (Hjelmstrom et al., 2000) and VCAM-1 (Kratz et al., 1996) have been observed in TLOs. However, it is possible that signals from other cells could take over the function of LTos in adult TLOs and LNs. Cells with LTi characteristics have been noted in NOD (Evans and Kim, 2009) and RIPCXCL13 (Link et al., 2011) pancreatic TLOs suggesting that they may play a role in the development of these ectopic tissues.

FOLLICULAR DENDRITIC CELLS

Follicular DCs form a network supporting B cell follicles in LNs. They are characterized by expression of complement receptors (CR1 and CR2), FcγRIIb, and markers defined by the antibodies FDCM1, FDCM2, and C4. Their roles include capture of antigen–antibody complexes for presentation to B cells and expression of the chemokine, CXCL13, which draws B cells and T follicular helper cells to the follicles via CXCR5.

Follicular DCs ($FDCM1^+CR1^+$) have been noted in TLOs. Their reticular network and association with B cells (Drayton et al., 2003) in TLOs suggest functional similarities with SLOs. As noted above, CXCL13 is found in TLOs, including those associated with *Helicobacter pylori* (Mazzucchelli et al., 1999), rheumatoid arthritis (Loetscher and Moser, 2002), Sjögren's syndrome (Barone et al., 2005), inflammatory transgenes (Kratz et al., 1996; Hjelmstrom et al., 2000; Drayton et al., 2003), and cancer (Bergomas et al., 2012). However, although FDCs are a major source of CXCL13 in LNs, monocytes and macrophages have also been shown to secrete this chemokine in the TLOs associated with rheumatoid arthritis and ulcerative colitis (Carlsen et al., 2004), suggesting additional chemokine sources in chronic inflammation, including LTo cells as noted above. Antigen presentation by FDCs has not been investigated in TLOs.

FIBROBLASTIC RETICULAR CELLS AND CONDUITS

The T cell zone of SLOs contains a dense, three-dimensional network of stromal cells called FRCs. This subset of LN stromal cells expresses podoplanin (gp38) but not the lymphatic and blood endothelial cell marker, PECAM (CD31; Turley et al., 2010). FRCs produce the extracellular matrix scaffolding that forms a series of very fine microvessels called conduits, detected using an antibody (ER-TR7) that recognizes an undefined antigen (Katakai et al., 2004). Conduits extend from the subcapsular sinus through the LN cortex, with greatest density in the paracortical T cell zone, and terminate at HEVs. These microchannels are approximately

4–5 nm in diameter and contain a dense network of collagen fibers that collectively limit access to molecules over 70 kDa in size (Sixt et al., 2005).

In addition to their role in forming conduits, FRCs also participate in the establishment and organization of lymphoid organ microenvironments (reviewed in Turley et al., 2010). They are the primary source of the T cell zone-restricted chemokines CCL19 and CCL21, both ligands for CCR7, present on T cells and DCs. These chemokines establish the T zone boundary, facilitate T cell and DC recruitment, and enhance DC maturation and function. FRCs also produce IL-7, critical for naïve T cell survival. The FRC network forms the three-dimensional scaffold along which T cells and DCs migrate; via their shared association with FRCs, interactions between naïve T cells and DCs expressing cognate antigen are facilitated. FRCs express transcripts for some self-antigens and it is has been suggested that they may be involved in self-tolerance (Lee et al., 2007). Additional LN stromal cells, termed eTACs, which are $gp38^{-}$ERTR-7^{-}, have also been reported to present self-antigens (Gardner et al., 2008).

Conduits (Anderson and Anderson, 1975) are believed to serve three key roles in SLOs (reviewed in Mueller and Ahmed, 2008). Given their narrow diameter and positioning, conduits can funnel small lymph-borne antigens from afferent lymphatics to the T cell zone, where FRC-associated resident DCs can receive them. Movement through these microvessels is much more rapid than that which could be achieved by simple filtration through the cell-dense tissue of the LN. Although this reticular network is less dense in the B cell zone, conduits may also serve as a pathway for low molecular weight antigens to reach follicles and facilitate B cell responses (Roozendaal et al., 2009). Finally, conduits serve as channels for transmitting small molecule chemical signals, such as chemoattractants from the surrounding tissue, to the paracortical region of the LN, ultimately reaching the endothelial cells lining HEVs.

Fibroblastic reticular cells and conduit-like structures have been noted in several studies of TLOs. Link et al. (2011) visualized these stromal elements using two-photon microscopy to detect injected low molecular weight fluorescent dyes, as well as via confocal microscopy and staining with ER-TR7 antibody. In TLOs from NOD or rat insulin promoter (RIP) LTα and RIPCXCL13 transgenic mice FRC-like stromal cells were observed, with the greatest density of staining in the T cell enriched areas of the TLO. In another study of NOD mice, ER-TR7 reactivity was found surrounding pancreatic islets and throughout the acinar tissue, consistent with the expression of this marker on fibroblasts. However, it was also found within the infiltrate (Penaranda et al., 2010), suggesting a supporting stromal network does exist in this case as well. Likewise, using fluorescently labeled ER-TR7 antibodies, we have observed dense staining patterns that resemble conduits in the T cell rich areas of the kidney infiltrates of RIPLTα mice (**Figure 2**). However, similar, although less dense, staining could also be seen in kidney samples from control mice, consistent with this as a fibroblast marker. Pulmonary arteries of patients with idiopathic pulmonary arterial hypertension who develop circulating autoantibodies to various vascular self-antigen include TLO-like structures and ER-TR7 antibody positivity (Perros et al., 2012). In non-lymphoid human tissues undergoing chronic inflammation (e.g., the liver of patients with primary biliary cirrhosis or the salivary glands of Sjögren's patients), conduit-like $gp38^{+}CD31^{-}$LYVE-1^{-} networks similar to those in LNs were seen in most of the T cell dense regions, but not in control tissues.

Although cells with the characteristics of FRCs have been observed in TLOs, their functions are unclear. In the absence of a defining capsule and subcapsular sinus it is more difficult to appreciate these structures as conduits transporting low molecular molecules. Whether they can secrete tissue-organizing cytokines, serve as scaffolding for the migration of T and B cells or channel low molecular weigh substances toward TLO compartments remain unanswered questions. Cells phenotypically similar to eTACs have been noted in NOD pancreatic TLOs (Gardner et al., 2008) raising the question as to whether self-antigen presentation by stromal cells occurs in TLOs and exacerbates or induces tolerance in those locations.

LYMPHATIC VESSELS

Afferent LVs deliver antigen and APCs to LNs and efferent vessels serve as routes for cell traffic to downstream LNs. Recently, an

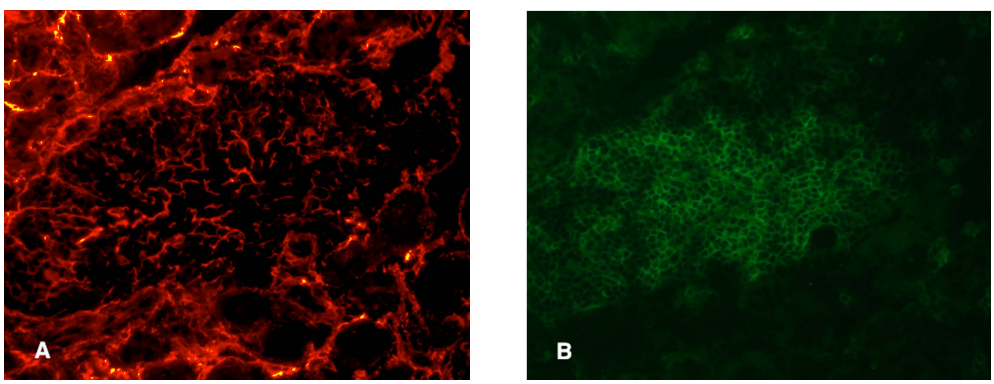

FIGURE 2 | Presumptive conduit in a RIPLTα TLO. Infiltrate of a RIPLTα mouse with a kidney infiltrate, stained with ER-TR7 (red; **A**) anti-CD3 (green; **B**). Note the presence of the fine network of ER-$TR7^{+}$ cells underlying the infiltrate.

additional LV function was described: expression of self-antigen under the control of AIRE for presentation to T cells (Cohen et al., 2010). In LNs, DCs accumulate in the subcapsular sinus and transmigrate through the floor of that sinus into the T cell zone, while T cells access the parenchyma of the LN through the peripheral medullary sinuses (Braun et al., 2011). Egress from the LN is regulated in part by the high concentration of S_1P in the lymph and the re-expression of the receptor, S_1P_1, on T cells as they leave the node and enter into the efferent lymph (Cyster and Schwab, 2012).

The presence of vessels with typical lymphatic markers, including LYVE-1 and Prox1, has been documented in the TLOs arising in both clinical settings (autoimmune disease and chronic graft rejection) and experimental models (summarized in Kerjaschki et al., 2004; Drayton et al., 2006; Furtado et al., 2007; Liao et al., 2007; Grabner et al., 2009; Mounzer et al., 2010). Although some macrophages also express LYVE-1, additional characteristics including gp38 expression, thin walls, and absence of red blood cells convincingly demonstrate their identity as LVs.

It has been suggested that TLOs differ from SLOs by the absence of a capsule. Thus, the trafficking patterns analogous to those in LNs (Braun et al., 2011), with the DCs percolating and T cells migrating through the peripheral medullary sinus to the parenchyma, might not occur in TLOs. However, TLOs in a variety of chronic kidney diseases (Mandache and Penescu, 2011) are in contact with a fibrous capsule, suggesting that, similar modes of migration in TLOs may be possible.

During ontogeny, LVs develop after the embryonic blood vessels have formed, sprouting off from the cardinal vein (Srinivasan et al., 2007), orchestrated by the homeobox genes (Sox18 and Prox1) and growth factors and their receptors (VEGFC and VEGFR3), and requiring platelets (Abtahian et al., 2003). LTα (Mounzer et al., 2010), DCs and T and B cells have been implicated in the regulation of LVs in inflammation (Angeli et al., 2006; Liao and Ruddle, 2006; Chyou et al., 2011). Regulation of lymphangiogenesis in TLOs is less well understood, although DCs have been implicated in a transgenic model of thyroiditis (Muniz et al., 2011). LTα regulates LVs in RIPLTαTLOs with less dependence on the LTαβ complex (Mounzer et al., 2010). On the other hand in the CXCL13 induced model of thyroiditis, LVs are inhibited by treatment with a LTβR-Ig (Furtado et al., 2007).

In some TLOs LVs are packed with lymphocytes (Liao et al., 2007) suggesting that they may act to transport activated lymphocytes to downstream LNs, similar to their function in SLOs. Continual administration of FTY720, an S_1P receptor agonist that prevents egress of lymphocytes from LNs (Mandala et al., 2002), prevents diabetes in NOD mice (Penaranda et al., 2010). This is only effective if the mice have already developed pancreatic TLOs. Treatment results in additional accumulation of lymphocytes in the pancreatic TLOs, which is reversed upon cessation, resulting in rapid islet destruction and diabetes. These data suggest that lymphocyte trafficking through LVs in TLOs in NOD mice is under regulation of the lymph S_1P gradient and expression of its receptor by T cells as in a canonical LN. The recent advances in *in vivo* imaging of LNs with the development of mice with red fluorescent LVs (Truman et al., 2012) may allow further analysis of the function of LVs in TLOs.

HIGH ENDOTHELIAL VENULES

High endothelial venules, which are post-capillary venules with cuboidal endothelium, are the sites of entry of naïve lymphocytes from the blood stream into LNs. This is accomplished by the interaction of molecules on the surface of lymphocytes with ligands expressed by HEVs. Peripheral node addressin (PNAd), defined by the MECA-79 antibody, is composed of any of a variety of chemically modified core glycoproteins, including GlyCAM-1, CD34, Sgp200, and podocalyxin, to become functional L-selectin ligands (Rosen, 2004). The several enzymes that mediate these post-translational modifications include FucT-IV, FucT-VII, and GlcNAc6ST2 (also called HEC-6ST, LSST, GST-3, HEC-GlcNAc6ST, gene name *Chst4*; Hiraoka et al., 1999, 2004; Hemmerich et al., 2001a,b; Homeister et al., 2001), GlcNAc6ST2 is uniquely expressed in high endothelial cells, with the exception of a population of cells in the intestine (Liao et al., 2007; Kawashima et al., 2009). Binding to PNAd slows down (tethers) L-selectin^{hi} lymphocytes in their progress through the blood vessels, allowing interaction with chemokines and integrins, eventually facilitating migration of lymphocytes toward chemokines located in the paracortical region (T cells, DCs) or cortex (B cells). PNAd rapidly replaces MAdCAM-1 after birth in mouse peripheral LNs (Mebius et al., 1997), but is expressed in mucosal LNs together with MAdCAM-1, the ligand for the integrin $\alpha_4\beta_7$.

High endothelial venules are prominent features of TLOs and their presence can be considered the defining characteristic that distinguishes these organized structures from other forms of inflammatory infiltrate, as they are so crucial for the entrance of naïve cells. HEVs in TLOs (summarized in Drayton et al., 2006) are characterized by expression of MAdCAM-1, PNAd, and chemokines, particularly CCL21 (Hjelmstrom et al., 2000), as well as the crucial sulfating enzyme GlcNAc6ST2 (Bistrup et al., 2004). HEVs have also been noted in the inflammatory infiltrates associated with atherosclerosis (Grabner et al., 2009) and several tumors (Martinet et al., 2011; Bergomas et al., 2012). In fact, the presence of high numbers of these vessels is associated with improved clinical outcomes in breast cancer (longer disease free survival and reduced metastases; Martinet et al., 2011), suggesting that the HEVs in tumor TLOs could enhance the entrance and priming of naïve lymphocytes into effector cells at the tumor site and lead to improved outcome.

Regulation of HEVs in TLOs appears to be similar to that in SLOs. In LNs, $LT\alpha_3$ regulates MAdCAM-1 (Cuff et al., 1998) and the $LT\alpha_1\beta_2$ complex regulates PNAd (Drayton et al., 2003, 2004; Browning et al., 2005) through the alternative NFκB pathway (Drayton et al., 2004). An LTβ antagonist, LTβR-Ig, inhibits HEVs in LNs (Browning et al., 2005; Liao and Ruddle, 2006; Liao et al., 2007) and also in the TLOs that arise in salivary and lacrimal glands of NOD mice (Fava et al., 2011a,b).

High endothelial venules function in LNs as entry sites for naïve cells. A similar portal function is assumed in TLOs because of the presence of naïve cells at those sites (Kratz et al., 1996) and evidence of epitope spreading (Miller et al., 2007). However, the actual migration of naïve lymphocytes from the blood stream via

HEVs into the parenchyma has not yet been visualized in real time in TLOs. Our development of mice with green fluorescent HEVs (Bentley et al., 2011) and their *in vivo* imaging in LNs (Truman et al., 2012) will enable similar analysis in TLOs and resolve whether HEVs function as entry points for naïve cells.

IMPLICATIONS

A better understanding of the stromal structure in TLOs could lead the way to therapeutics directed specifically at such structures. Likewise, a clearer appreciation of the inflammatory signals that drive the organization of stromal cells in non-lymphoid tissues into TLO-like structures might allow the design of therapeutic interventions to impede the progression of chronic inflammation, including autoimmune activity. Local interference with the chemokine signals that recruit and organize T and B cells into functional pro-inflammatory structures in non-lymphoid tissues might dampen autoimmune responses and allow for more effective conventional therapy for autoimmune diseases. Since regulatory T cells have been observed in some TLOs, a better understanding of and ability to enhance the tolerance inducing capabilities of APCs might help restrain self-reactivity, limiting exacerbation of autoimmune disease. Treatment with agents that enhance the alternative NF-κB pathway could encourage the development of HEVs in tumors, enhancing the entrance of naïve cells and providing useful new strategies in cancer. Although this concept is actually in contradistinction to treatments currently used to prevent angiogenesis in the tumor setting, understanding the complexity of the stroma in TLOs could provide creative new approaches to disease control.

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The role of non-hematopoietic stromal cells in the persistence of inflammation

Francesca Barone*, Saba Nayar and Chris D. Buckley*

Centre for Translational Inflammation Research, Arthritis Research UK, Rheumatology Research Group, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham Research Laboratories, Queen Elizabeth Hospital, Birmingham, UK

Edited by:
Mark C. Coles, University of York, UK

Reviewed by:
Masaaki Murakami, Osaka University, Japan
Nancy Ruddle, Yale University School of Medicine, USA

****Correspondence:***
Francesca Barone and Chris D. Buckley, Centre for Translational Inflammation Research, Arthritis Research UK, Rheumatology Research Group, School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham Research Laboratories, Queen Elizabeth Hospital, Birmingham B15 2WD, UK.
e-mail: f.barone@bham.ac.uk; c.d.buckley@bham.ac.uk

Inflammation results from the complex interaction between hematopoietic and stromal cells and growing evidence supports a key role for the stroma in driving the switch from acute resolving to persistence in chronic inflammatory diseases. Stromal cells have also been shown to play a critical role in cancer biology, being involved in cancer growth, dissemination, and inhibition of the autologous immune response, ultimately favoring persistence and metastatic spread. Similarly, blood and lymphatic endothelial cells contribute to tissue homeostasis during physiological inflammation but also lead to discorded leukocyte and tumor cell accumulation in pathological inflammation and cancer. This review aims to summarize the role that pathogenic stroma plays in the pathogenesis of diseases such as cancer and chronic inflammation.

Keywords: endothelium, lymphatics, rheumatoid arthritis, ectopic lymphoneogenesis, fibroblasts

INTRODUCTION

Inflammation involves the complex interaction between infiltrating cells, belonging to the immune system and tissue-resident stromal cells. The stroma, formerly considered the theatrical stage of the inflammatory process, has acquired, in recent years the role of director of the immune response, regulating the process of leukocyte recruitment, organization of leukocytes within the tissue and exit via the escape route of the lymphatic endothelium.

In physiological conditions, stromal cells provide an important structural component for tissues. Stroma consists of extracellular matrix (ECM), mesenchymal cells and a scaffold of nerves, epithelium, blood, and lymphatic vessels. Tissue-resident macrophages, although considered by some as part of the tissue stroma, are largely bone marrow-derived and bear the hallmark of the hematopoietic tissue.

Under pathogen threat or in the target tissue of chronic inflammation and cancer, stromal cells acquire novel features, critical for the development of the pathological process and functional for its persistence. This review will provide the reader with a better understanding of the role of pathogenic stroma in inflammation. As both epithelium and nerves appear to be more implicated in disease initiation, rather than persistence, this review will focus on the role of fibroblasts, lymphatic and blood vessels.

FIBROBLASTS

The most abundant cell type within tissue stroma is the fibroblast (Filer et al., 2006). Fibroblasts are traditionally defined by their spindle shaped morphology and their ability to adhere to plastic culture vessels *in vitro* (Tarin and Croft, 1969). They are believed to arise from three distinct cellular origins: primary mesenchyme, local epithelial-mesenchymal transition (EMT), or bone marrow-derived precursors (circulating fibrocytes; Abe et al., 2001; Kalluri and Neilson, 2003). It is widely accepted that the majority of fibroblasts originate from primary mesenchymal cells and that, upon appropriate stimulation, fibroblasts proliferate to generate new progeny (Iwano et al., 2002; Parsonage et al., 2005). In physiological conditions fibroblasts provide mechanical strength to tissues by producing ECM components (type I, III, and V collagen and fibronectin), factors that regulate ECM turnover, such as metalloproteinases (MMPs) and proteins involved in the formation of basement membrane (type IV collagen and laminin; Marinkovich et al., 1993; Sabatelli et al., 2001; Tomita et al., 2002). The intimate relationship between fibroblasts and mesenchymal stromal cells (MSC) and the clinical challenge to use MSC for tissue repair has driven renewed interest in fibroblasts as therapeutic target. Our group has largely contributed to this characterization utilizing antibodies raised against different components of this heterogeneous population. This screening exercise has provided us with some key markers that, together with others present in the literature (**Table 1**), can now be used to better understand distribution, function, and plasticity of the complex fibroblast family (Buckley et al., 2005; Halder et al., 2005).

Fibroblast behavior has mainly been explored in three pathological conditions: chronic inflammation, tissue fibrosis, and cancer. Interestingly, while these three conditions dramatically differ in etiology and genetic predispositions, they converge in that there are profound modifications both in terms of phenotype and function occurring to the stromal component. Whether these newly acquired properties are intrinsic to changes occurring in the fibroblasts or derive from the conditioning of the pathogenic infiltrating cells is still under investigation and seems to differ in the diverse conditions (Buckley et al., 2001).

Table 1 | Fibroblast markers.

Marker	Function	Fibroblast subtype	Other expressing cells
Vimentin	Intermediate filament associated protein	Miscellaneous	Endothelial cells, myoepothelial cells, neurons
α-SMA	Intermediate filament associated protein	Myofibroblasts	Vascular smooth muscle cells, pericytes, myoepithelial cells
Desmin	Intermediate filament associated protein	Skin fibroblasts	Muscle cells, vascular smooth muscle cells
FSP1	Intermediate filament associated protein	Miscellaneous	Invasive carcinoma cells
Discoidin-domain receptor 2	Collagen receptor	Cardiac fibroblasts	Endothelial cells
FAP	Serine protease	Activated fibroblasts	Activated melanocytes
α1β1 integrin	Collagen receptor	Miscellaneous	Monocytes, endothelial cells
Prolyl 4-hydroxylase	Collagen biosynthesis	Miscellaneous	Endothelial cells, cancer cells, epithelial cells
Pro-collagen 1α2	Collagen-1 biosysnthesis	Miscellaneous	Osteoblasts, chondroblasts
CD248	Unknown	Miscellaneous	Pericytes
VCAM-1	Cell adhesion	Miscellaneous	Activated endothelial cells

Adapted from Kalluri and Zeisberg (2006).

Fibroblasts play a crucial role in determining the site at which inflammation occurs, and influence the persistence of the inflammatory process (Takemura et al., 2001). Different events have been shown to take place in order to elicit the modifications required for fibroblast activation. Signals derived from the surrounding infiltrating cells, such as proinflammatory cytokines have been shown to play a key role in the activation of rheumatoid arthritis (RA) synovial fibroblasts (Ohata et al., 2005). Similarly, leukocyte-derived signals such as IL-4 (Th2), interferon gamma (Th1), and TNF have been shown to modify the fibroblast transcriptional profile (Parsonage et al., 2003). Nonetheless, a growing body of evidence suggests that intrinsic events such as the occurrence of epigenetic modifications in the fibroblast genome might contribute to the persistence of the activated phenotype (Ospelt et al., 2011).

Once activated, synovial fibroblasts have been shown to produce TNFα, IL-1, and IL-6, cyclooxygenase-2, the polysaccharide hyaluronan, as well as inflammatory chemokines (e.g., IL-8, CCL5, CXCL1; Szekanecz et al., 2003; Iwamoto et al., 2008), thus sustaining leukocyte recruitment in to the inflamed synovium. Fibroblasts play not only a key role in immune cell recruitment but also in leukocyte aggregation within tissue. Pathogenic fibroblasts have been implicated in the formation of tertiary lymphoid organs (TLOs), lymph node like structures, resulting from the organized aggregation of leukocytes inside tissue target of inflammatory processes (Buckley et al., 2000). Recent work of Peduto et al. (2009) has described early modifications occurring to normal stromal fibroblasts following inflammatory stimuli, like the re-expression of the fibroblast embryonic marker gp38, that result in the acquisition of a lymphoid like phenotype able to sustain TLO formation. Gp38 is a glycoprotein characterized by Farr et al. (1992), later named podoplanin due to its low level constitutive expression in kidney podocytes (Breiteneder-Geleff et al., 1997). Gp38 (or podoplanin in humans) is expressed by lymphoid stromal cells within the T cell areas of peripheral lymphoid tissue (Farr et al., 1992), in the medulla and paracortex of lymph nodes, within the peri-arteriolar region of the splenic white pulp (PALS), on the lymphatic endothelial cells (Schacht et al., 2003) and on thymic epithelial cells (Farr et al., 1992). The role of gp38 + fibroblasts in the production of lymphoid cytokines and chemokines in secondary lymphoid organs has been reviewed elsewhere and will not be discussed further in this review (Astarita et al., 2012).

In physiological conditions, within non-lymphoid tissue, fibroblasts do not express gp38. Interestingly, the phenomenon of up-regulation of this marker coincides with the capacity of tissue-resident fibroblasts to "convert to a lymphoid-like" functional phenotype. Lymphoid-like fibroblasts express CD157 (BP-3) and produce IL-7 and lymphoid chemokines CXCL13 and CCL19 which are able to drive accumulation and segregation of the leukocytes in distinct compartments within the inflamed joints (Buckley et al., 2000, 2001; Bradfield et al., 2003; Peduto et al., 2009). The histological finding of TLOs in RA synovium has been associated with severe disease progression and erosions (van de Sande et al., 2011). TLOs are not specific to RA and other chronic diseases, such as Sjogren's syndrome, Hashimoto thyroiditis, and Crohn's disease share a similar pattern of fibroblast activation and production of lymphoid cytokines/chemokines (Aloisi and Pujol-Borrell, 2006).

Rheumatoid arthritis synovial fibroblasts produce survival factors (e.g., type I interferon, IL-15, BAFF) that inhibit leukocyte apoptosis (Pilling et al., 1999; Burger et al., 2001). Gp38 expression is associated with the acquisition of a motile, contractile phenotype and it has been detected in cells derived from various types of cancers (i.e., vascular tumors, tumors of the central nervous system, malignant mesothelioma, squamous cell carcinomas, and germ cell tumors). Gp38 expression seems to identify more aggressive forms of tumors, with higher invasive and metastatic potential (Schacht et al., 2005; Raica et al., 2008). Gp38 is expressed both by tumor cells and by the cancer-associated fibroblasts (CAF), a population of fibroblasts that surrounds and mingle with the malignancy favoring its organization and metastasis in to the surrounding tissue. The expression of gp38 in the context of tumor-associated lymphangiogenesis will be later discussed. CAF as well as fibroblasts from the inflamed synovium are also characterized by FAP (fibroblast activation protein) expression (Ospelt et al., 2010).

Fibroblast activation protein, also known as "seprase," is a cell-surface 170 kDa type II transmembrane serine protease (Rettig et al., 1986; Aoyama and Chen, 1990), belonging to the family of post-prolyl aminopeptidases (Niedermeyer et al., 1998). Dipeptidyl peptidase IV (DPPIV or CD26) is the most studied closest member to FAP, with 61% nucleotide sequence and 48% amino acid sequence identity (Scanlan et al., 1994). FAP was identified as an inducible antigen by F19 monoclonal antibody and expressed on developing (Rettig et al., 1988; Garin-Chesa et al., 1990; Niedermeyer et al., 2001) and reactive mesenchyme of various tumors, transformed cell lines, and granulation tissue of wound healing (Rettig et al., 1986, 1988; Aoyama and Chen, 1990; Garin-Chesa et al., 1990; Kelly et al., 1994; Monsky et al., 1994). When over-expressed in epithelial and fibroblastic cell lines, FAP has been proven to affect cell adhesion, migration, proliferation, and apoptosis (Wang et al., 2005).

Recently a novel immunosuppressive role for FAP-positive fibroblasts has been shown in the tumor environment. By using a FAP-DTR mice, in which deletion of FAP + fibroblasts is induced upon diphteria toxin administration, Kraman et al. (2010) have shown that depletion of FAP-expressing cells in Lewis lung carcinoma and pancreatic ductal adenocarcinoma causes rapid hypoxic necrosis of both tumor and stromal cells by a process involving IFNγ and TNFα. These studies support the hypothesis that FAP activity and FAP-expressing fibroblasts facilitate tumor growth both directly as well as acting on the immune cells recruited against the malignancy. This suggests that cancerous cells, early in the disease establishment are able to modify the local environment and induce the formation of a stroma able to protect the same malignancy against the self-immune-surveillance, thus establishing a novel immunological role for stromal cells in cancer persistence and spreading.

VASCULAR STRUCTURES

LYMPHATIC VESSELS

Striking changes in the lymphatic vasculature are associated with inflammation, which include acute and chronic infections, autoimmune diseases such as RA, Crohn's disease, wound healing, cancer, and transplant rejection (Tammela and Alitalo, 2010; Alitalo, 2011). Neo-lymphangiogenesis is a critical mechanism regulating changes in interstitial fluid. Deregulated activation of its cascade results in defective leukocyte drainage and persistence of the inflammatory process. Recent studies show that induction of the NF-κB pathway activates Prox1 and this in turn activates the expression of the VEGFR-3 promoter, leading to increased receptor expression on lymphatic endothelial cells. This phenomenon enhances the responsiveness of pre-existing lymphatic endothelium to VEGFR-3 ligands, VEGF-C and VEGF-D, which stimulates lymphangiogenesis (Alitalo et al., 2005; Zhang et al., 2007; Watari et al., 2008; Kang et al., 2009; Flister et al., 2010). Other proinflammatory cytokines, e.g., IL-1 and TNFα are known to induce VEGF-C/D expression in infiltrating and tissue-resident cells such as macrophages, dendritic cells (DCs), mast cells, and fibroblasts (Ristimaki et al., 1998; Hamrah et al., 2003; Cursiefen et al., 2004; Alitalo et al., 2005; Baluk et al., 2005; Kataru et al., 2009; Kunder et al., 2009, 2011; Yao et al., 2010; Zumsteg and Christofori, 2012). Similarly, LTα secreted by lymphocytes at the site of inflammation has been documented to support inflammatory lymphangiogenesis (Mounzer et al., 2010). Data from models of inflamed cornea in mice and renal transplant induced inflammation in humans have shown that inflammation-mediated lymphangiogenesis does not occur solely by proliferation or continuous sprouting of existing lymphatic vessels but also includes incorporation of BM-derived lymphangiogenic progenitors (such as CD11b + macrophages) into the existing or growing lymphatic vessels. These CD11b + progenitors have the capability to transdifferentiate into LYVE + vessels under pathological conditions, contributing to the increased lymphatic vessel density observed at inflammatory sites (Maruyama et al., 2005; Kerjaschki et al., 2006; Maby-El Hajjami and Petrova, 2008; Lee et al., 2010). Lymphangiogenesis is thought to facilitate resolution of inflammation, providing drainage of tissue edema, clearance of inflammatory cells and also favoring adaptive immune cell function by promoting macrophage and DCs mobilization to the draining lymph nodes. Inhibiting lymphangiogenesis by blocking VEGFR activity exacerbates pulmonary edema and favors the establishment of chronic mycoplasma infection (Baluk et al., 2005; Polzer et al., 2008; Kataru et al., 2009; Huggenberger et al., 2011; Kim et al., 2012). Taken together, these data suggest that the signals that activate the induction of the inflammatory process also program its resolution. It is believed that such a coordinated series of events is altered in chronic inflammation, where the number of the infiltrating leukocytes overcome the drainage capacity of the newly developing lymphatics. It is debatable whether newly formed lymphatic vessels are able to establish functional connections and deliver antigen, fluid, and cells to the draining lymph nodes. The inability of lymphatic vessels to deliver antigens to the draining lymph node could favor the persistence of TLOs, where an excess of antigen is presented in structures whose stroma component is potentially unable to exert the tollerogenic activity attributed to lymph node stroma (Kline and Thomas, 1976; Eliska et al., 1986; Joos et al., 1993; Ruggiero et al., 1994; Angeli et al., 2006; Angeli and Randolph, 2006; Thaunat et al., 2006; Li et al., 2011). In this context the ectopic expression of CCL21 on newly formed (but yet non-functional lymphatic vessels) might compete with the chemokine gradient established by pre-existent functional lymphatic vessels, thus contributing to the entrapment of leukocytes and DCs within the TLOs (Kerjaschki et al., 2004; Burman et al., 2005).

Lymphatic vessels have also been shown to play a role in immune regulation. For example the decoy chemokine receptor D6, which acts as a scavenger of inflammatory CC chemokines, is known to be expressed on lymphatic endothelium (Nibbs et al., 2001; Jamieson et al., 2005; Martinez de la Torre et al., 2005). In addition, inflamed lymphatic endothelium mediates suppression of DC maturation via CD11b interaction with ICAM-1 receptor (expressed on lymphatic endothelial cells; Podgrabinska et al., 2009).

However, lymphangiogenesis is a double-edged sword, as tumor cells can use this process as a mechanism to drive metastasis. Tumor cells enter lymphatic vasculature by invading pre-existing lymphatic vessels or by eliciting neo-lymphangiogenesis on the periphery of the tumor, stimulating the secretion of growth factor (VEGF) on tumor-associated fibroblasts and macrophages (Jeltsch

et al., 1997; Karpanen et al., 2001; Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001; Schoppmann et al., 2002). Interestingly, high density of lymphatic vessels correlates with high incidence of lymph node metastasis and poor prognosis in some cancers (Beasley et al., 2002; Alitalo et al., 2005; Cao, 2005; Kyzas et al., 2005). As mentioned above, gp38 is highly expressed on the lymphatic endothelium and its expression correlates with poor prognosis and increased risk of lymph node metastasis (Schacht et al., 2005; Kitano et al., 2010). Lymphangiogenesis has also been found to play a pathogenic role in transplantation biology where it sustains delivery of donor antigens to the recipient lymph node, ultimately favoring the generation of an immune response against the transplanted tissue (Tammela and Alitalo, 2010; Alitalo, 2011; Seeger et al., 2012). These data suggest a key role for lymphatic vessel function and homeostasis in the regulation of the balance between immunity and tolerance as well the persistence of inflammation compared to its resolution.

BLOOD VESSELS

Similar to the fibroblasts and lymphatic vessels, blood vessels undergo remodeling during inflammation. Blood endothelium changes its structure and phenotype and participates in the inflammatory response mainly regulating leukocyte recruitment into the tissue. This phenomenon is characterized by loss of vascular integrity, which results in exposure of the sub-endothelium matrix and efflux of plasma-protein rich from the intravascular space (Pober and Sessa, 2007). The newly formed extravascular matrix supports leukocyte extravasations and it is associated with the expression of leukocyte adhesion molecules such as E-selectin, VCAM-1, and ICAM-1 (Adams and Shaw, 1994; Clark et al., 2006; Ley and Reutershan, 2006). Inflammatory stimuli such TNFα, IL-1, certain bacteria and viruses, physical and oxidative stress (Pober and Sessa, 2007), and anti-endothelial cell antibodies (found in systemic inflammatory disease such as vasculitides; Meroni et al., 1995) all elicit NF-κB translocation and binding to promoter regions of genes commonly up-regulated during blood endothelial cell activation (Bierhaus et al., 1997; Hunt and Jurd, 1998; Pober and Sessa, 2007). Failure to restore homeostasis of the blood endothelium contributes to chronic inflammatory disease and edema. Activated blood endothelial cells synthesize cytokine such as IL-6, which regulates the acute phase response, and chemoattractants, such as IL-8 and MCP-1 that help establishing the chemotactic gradient necessary for the influx of various inflammatory cells into the site of inflammation (Pober and Cotran, 1990; Mantovani et al., 1997; Middleton et al., 1997). Blood endothelial cells are also able to act as an antigen-presenting cell, expressing class II HLA molecules, in a phenomenon that has been shown to contribute to transplant rejection (Pober et al., 1996). Expression of co-stimulatory molecules such as OX40, ICOS, and CD2, known to be important in the formation and activation of T cell memory, has been documented in activated human endothelial vessels (Shiao et al., 2005), suggesting a role for the endothelium not only in leukocyte recruitment but also in their education.

Additional endothelial changes are observed in various chronic inflammatory diseases, such as Sjogren's syndrome, thyroiditis, and RA. As mentioned above, chronically inflamed organs often acquire TLOs that are accompanied by conversion of flat venular endothelial cells into tall and plump endothelial cells that very closely resemble high endothelial venules (HEVs) found in the T cell rich area of the lymph node. These ectopic HEVs are characterized by expression of the lymph node trafficking code, Peripheral node addressin (PNAd) that binds L-selectin expressed on naïve/central memory T lymphocytes and mature DCs. This homing machinery, supported by the ectopic expression of CCL21, allows HEVs in peripheral tissue to misguide influx of CCR7 + memory T cells into the inflamed tissue leading to amplification and maintenance of chronic inflammation (Girard and Springer, 1995; Barone et al., 2005; Manzo et al., 2005; Aloisi and Pujol-Borrell, 2006). Our group has recently demonstrated that endothelium cultured with fibroblasts derived from inflamed synovium behaves differently in terms of leukocyte recruitment compared to endothelium cultured with healthy dermal fibroblasts (McGettrick et al., 2009). While reinforcing the concept that fibroblasts convey site specificity to the immune response, these data highlight the active role of the stroma in the shaping of the immune response from its earliest phases.

Growth of new blood vessels from existing ones is a very important component of many diseases including cancer and chronic inflammation (Pandya et al., 2006; Costa et al., 2007). Angiogenesis in these conditions ensures continuous oxygen and nutrient supply to pathogenic cells, thus sustaining their growth and survival. Several cells types including malignant cells, synovial fibroblasts, keratinocytes, and monocytes/macrophages are capable of producing classic angiogenic factors (such as VEGFα, angiopoetin, and PDGF) when the environment becomes hypoxic. Moreover, inflammatory cytokines such as IL-1, TNFα (low dose), and IL-8 have been reported to be pro-angiogenetic, thus supporting this process while exerting other proinflammatory activity. Blood vessels formed during pathological angiogenesis are structurally and functionally abnormal. Tumor vasculature is highly disorganized, vessels are tortuous and dilated, with uneven diameter, excessive branching and shunts that lead to chaotic and variable blood flow, often resulting in the establishment of hypoxic and acidic areas in the malignant tissue. The chaotic architecture and disturbed blood flow contributes to lower therapeutic effectiveness for several drugs (Jain, 1988; Giaccia, 1996; Helmlinger et al., 1997; Baish and Jain, 2000; Eberhard et al., 2000). Malignancy-associated neo-vascularization shows a non-uniform pattern of adhesion molecules that coupled with distorted blood supply (Huang et al., 1997; Eliceiri and Cheresh, 1999) might explain why leukocyte-endothelial interaction is low in cancer. Similarly, the new vessels formed at the site of inflammation also exhibit structural and functional abnormalities. In RA, the vascular network is reported to be dysfunctional as it is unable to restore tissue oxygen homeostasis and the rheumatoid joints are believed to remains markedly hypoxic during disease (Taylor and Sivakumar, 2005). In both tumor and RA impaired angiogenesis ultimately favors the selection of cells that are metabolically resistant to lack of oxygen, thus reducing the effectiveness of therapy aimed to disturb the neo-angiogenetic process.

CONCLUSION

Current treatments targeting leukocytes have led to a dramatic change in the management of inflammatory disease. However, this approach does not result in a definitive cure and even in patients that achieve clinical remission, relapse occurs once treatment is withdrawn. This suggests that other non-leukocyte targets need to be addressed if persistent inflation is to be resolved. In this review we have explored the role of stromal cells in disease pathogenesis and suggest that stromal cells provide an attractive, site-specific target for therapy. Combined therapies, aimed at silencing the stroma, provide an alternative approach to curing persistent inflammatory disease.

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Mesenchymal stem cells are mobilized from the bone marrow during inflammation

Jasper J. Koning[1], Gijs Kooij[1], Helga E. de Vries[1], Martijn A. Nolte[2] and Reina E. Mebius[1]*

[1] *Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, Netherlands*
[2] *Department of Hematopoiesis, Adaptive Immunity Lab, Sanquin Research and Landsteiner Laboratory AMC/UvA, Amsterdam, Netherlands*

Edited by:
Mark C. Coles, University of York, UK

Reviewed by:
Masaaki Murakami, Osaka University, Japan
Oliver Pabst, Hannover Medical School, Germany

****Correspondence:***
Reina E. Mebius, Department of Molecular Cell Biology and Immunology, VU University Medical Center, PO Box 7057, 1007 MB, Amsterdam, Netherlands.
e-mail: r.mebius@vumc.nl

Mesenchymal stem cells (MSCs) show great therapeutic potential for the treatment of various immune mediated diseases, including Multiple Sclerosis (MS). Systemic administration of MSCs during experimental allergic encephalomyelitis (EAE), an animal model for MS, was shown to reduce the infiltration of T cells, B cells, and macrophages into the CNS. Whether endogenous MSCs are mobilized and potentially modulate the severity of disease is not known. Here we show that during the acute phase of EAE, MSCs numbers in the bone marrow were severely reduced, which restored to control levels during the progressive phase of the disease. The number of bone marrow MSCs inversely correlated with the number of both CD4 and CD8 T cells present in the bone marrow indicating a link between activated T cells and MSC mobilization. Analysis of CD70-transgenic mice, which have a constitutively activated immune system and elevated number of activated T cells in the bone marrow, showed severely reduced number of bone marrow MSCs. Transfer of T cells that were activated through their CD27 receptor reduced the number of bone marrow MSCs dependent on IFN-γ. These data provide a mechanism by which MSCs can be mobilized from the bone marrow in order to contribute to tissue repair at a distant location.

Keywords: MSC mobilization, experimental allergic encephalomyelitis, inflammation and MSC, bone marrow and MSC, MSC and IFNg, CD70

INTRODUCTION

Mesenchymal stem cells (MSCs) are described as multipotent stromal cells, mainly residing in the bone marrow, although they can also be found in low numbers in almost all adult tissues (Kuznetsov et al., 2001; da Silva et al., 2006). MSCs can differentiate into various cells of the mesodermal lineage which include osteoblasts, adipocytes, and chondrocytes as well as myelosupportive stroma which, together with endothelial cells, are all constituents of the heterogeneous bone marrow stroma (Friedenstein et al., 1966; Kuznetsov et al., 1997; Prockop, 1997; Pittenger et al., 1999; Tokoyoda et al., 2010). Stromal cells within the bone marrow support the maintenance of hematopoietic stem cells (HSCs) (Mendez-Ferrer et al., 2010).

In the bone marrow, MSCs were identified as a population of nestin-expressing cells which exclusively contained colony-forming-unit fibroblast (CFU-F) activity and depletion of nestin^{pos} cells reduced the HSC-activity in bone marrow indicating that these cells are HSC-niche components (Mendez-Ferrer et al., 2010). However, whereas HSCs crucially depend on stem cell factor (SCF) produced by niche cells, deletion of SCF from nestin^{pos} cells did not affect HSC frequency in the bone marrow (Ding et al., 2012), suggesting that other cell types also contribute to HSC maintenance through SCF production. Indeed, both endothelial as well as leptin receptor-expressing perivascular cells were identified as the source of SCF and deletion of either population clearly reduced HSCs numbers in bone marrow (Ding et al., 2012). Therefore, multiple cell types within the heterogeneous bone marrow stroma support HSC-niche maintenance under homeostatic conditions.

In analogy to the supportive function of lymph node stromal cells in immune cell homeostasis (Koning and Mebius, 2011), bone marrow stromal cells can also support maintenance of various lymphocytes (Tokoyoda et al., 2010). The lack of distinctive structures such as lymphatic endothelial cells as well as specialized high endothelial venules in the bone marrow however, may reflect differences in the functional role of these organs in lymphocyte homeostasis. Verily, whereas lymph node stromal cells are involved in the initial phase of immune responses, bone marrow stromal cells participate in maintaining memory cells, both plasma cells and CD4^{pos} T cells (Tokoyoda et al., 2010). Memory T cells reside in contact with VCAM-1^{pos} stromal cells that express IL-7, a T cell survival cytokine (Fry and Mackall, 2005; Tokoyoda et al., 2009). Bone marrow plasma cells locate near CXCL12 abundant reticular (CAR) cells, while the preponderance of HSCs also locates near CAR cells. Therefore, whereas in lymph nodes specialized micro-environmental stromal subsets, which support specific types of immune cells, have been described (Koning and Mebius, 2011), such subdivisions for bone marrow stromal cells are less well defined. Nevertheless, the overlapping supportive and suppressive roles of stromal cell microenvironments in lymphocyte homeostasis and function, respectively, are shared by all stromal cells despite distinct anatomical locations (Jones et al., 2007). MSCs are precursors for stromal cells in various organs, and they share immunosuppressive effects with stromal

cells. Therefore, MSCs are thought to serve as good candidates to treat immune mediated diseases including Multiple Sclerosis (MS) (Uccelli et al., 2008).

MS is a chronic inflammatory disease resulting in demyelination and axonal loss throughout the central nervous system (CNS), with unknown cause and only limited treatment options (Ewing and Bernard, 1998; Noseworthy et al., 2000; Lassmann et al., 2001). Spontaneous remyelination that contributes to functional recovery is limited resulting in a relentless increase in disability during disease progression (Scolding and Franklin, 1998; Bruck et al., 2003; Fancy et al., 2010).

The effectiveness of administration of MSCs for treatment of MS patients needs to be explored in more detail, but animal studies in rodents show promising perspectives for future treatments. The most commonly used animal model for MS research is murine experimental allergic encephalomyelitis (EAE). This model resembles both the inflammatory phase, i.e., the generation of autoreactive myelin specific T cells, as well as the neurodegenerative phase of the disease, i.e., destruction of the myelin sheath around the axons and subsequent loss of axons, as observed in human disease (Steinman, 2001). Several studies have shown that systemic administration of MSCs at disease onset ameliorated EAE and decreased infiltration of T cells, B cells and macrophages into the CNS (Zappia et al., 2005; Kassis et al., 2008). In these studies MSCs exerted immunomodulatory effects via inhibition of T cell activation and proliferation.

A recent study indicates that MSCs also harbor direct neuroprotective effects. It was demonstrated that administration of MSCs at the onset of EAE remarkably reduced the levels and activity of anti-oxidant molecules *in vivo* (Lanza et al., 2009). Subsequently, using an *in vitro* model system, the authors showed that upon induction of oxidative stress within a neuroblastoma celline, MSC-conditioned medium suppressed the upregulation of anti-oxidant molecules indicating a direct neuroprotective effect of MSCs (Lanza et al., 2009).

While it was shown that MSCs migrate to the brain upon *i.v.* administration after the experimental induction of stroke, only a small percentage of these cells enters the CNS parenchyma (Eglitis et al., 1999). And although MSCs were reported to transdifferentiate *in vitro* into neural cells (Kopen et al., 1999) most studies so far indicate that MSCs do not transdifferentiate *in vivo* during EAE, despite their presence in spinal cord (SPC) and brain after systemic administration (Zappia et al., 2005; Gerdoni et al., 2007). Therefore, the positive effect of MSC administration on the disease course of EAE is mostly through modulation of immune cells although direct neuroprotective effects may also play a role.

All studies which addressed a potential therapeutic effect of MSCs on EAE disease outcome focused on administration of exogenous MSCs (Zappia et al., 2005; Gerdoni et al., 2007; Kassis et al., 2008; Lanza et al., 2009). However, so far there is no data concerning the behavior of endogenous MSCs during the course of EAE. Since the bone marrow is the major source of MSCs, we investigated the presence of bone marrow MSCs during the course of MOG induced EAE. We found severely reduced numbers of bone marrow MSCs at the peak of disease, which restored to control levels upon progression into the chronic phase. Activated CD4 T cells in the CNS, which produce pro-inflammatory molecules such as IFN-γ, TNF-α, IL-17, lymphotoxin, and GM-CSF, are considered to play a central role in the pathogenesis of MS and EAE (Zamvil and Steinman, 1990; Sospedra and Martin, 2005; Segal, 2010; Codarri et al., 2011). Analysis of the immune cells within the bone marrow revealed a significant negative correlation between $CD4^{pos}$ and $CD8^{pos}$ T cells and MSC, such that high numbers of either T cell subset coincided with low numbers of bone marrow MSCs, suggesting a T cell mediated effect on MSC mobilization. Analysis of MSC numbers in the bone marrow of mice with constitutively activated T cells showed a strong reduction of MSCs in the bone marrow. Indeed, transfer of T cells, which were subsequently activated through their CD27 receptor, demonstrates a role for T cells in reducing the number of MSCs. While prolonged production of IFN-γ in the bone marrow seemed to reduce MSC numbers, short term mobilization by T cells was independent of T cell derived IFN-γ.

RESULTS

REDUCED NUMBER OF MESENCHYMAL STEM CELLS IS PRESENT IN THE BONE MARROW DURING EAE

Over the past years there has been increasing evidence that administration of MSCs decreases the severity of EAE (Zappia et al., 2005; Kassis et al., 2008; Lanza et al., 2009). However, so far no data has been presented concerning the behavior of endogenous bone marrow MSCs during the course of EAE. Therefore, we induced EAE with recombinant myelin oligodendrocyte glycoprotein (rMOG) and analyzed total numbers of MSCs in the bone marrow, the major reservoir for MSCs, at various timepoints after disease induction (day 8, 15, and 29). At day 8 after disease induction, mice are still in the inductive phase and exhibit no clinical signs yet. However, at day 15 after disease induction, mice suffered from severe clinical signs varying from hind leg bending (score 2) to complete hind leg paralysis (score 4) which is accompanied by infiltration of immune cells, such as macrophages as well as T cells, in white matter lesions of the brain (Kooij et al., 2009). During the progressive phase of the disease (day 29), clinical symptoms were slightly improved (**Figure 1A**).

Total numbers of MSC, present in the bone marrow, were determined using the colony-forming units-fibroblast (CFU-F) assay, which exploits specialized medium to allow selective outgrowth of MSCs. At day 8, shortly before clinical onset of the disease, no change in the amount of bone marrow MSCs could be observed when compared to CFA induced control animals (**Figure 1B**). Strikingly, at the peak of the disease (day 15), we observed a dramatic reduction in the amount of MSCs present in the bone marrow (**Figure 1B**). This decrease in bone marrow MSCs appeared to be transient, since numbers were comparable to control mice at the chronic phase (day 29), when clinical symptoms started to improve (**Figures 1A,B**). In mice that exhibit no clinical symptoms despite disease induction, we could not observe changes in MSC numbers (data not shown).

The decrease in absolute numbers of MSC at day 15 was not the result of a decrease in bone marrow cellularity, since total bone marrow cell counts were comparable to CFA control mice (**Figure 1C**). Bone marrow MSCs are precursors for bone marrow mesenchymal stromal cells and although it appeared that

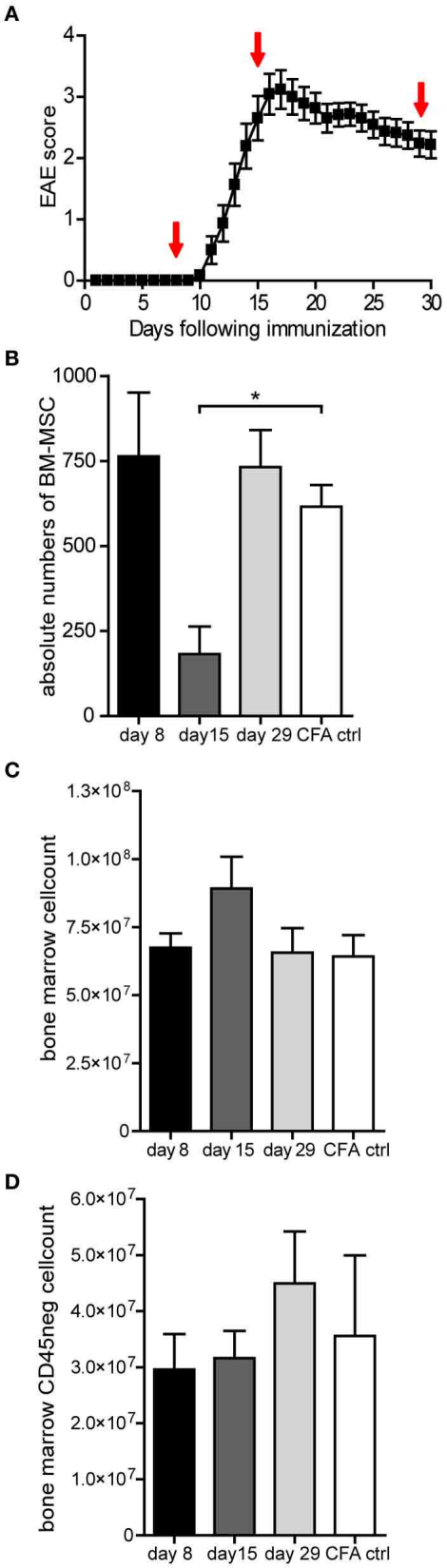

FIGURE 1 | Continued

FIGURE 1 | The number of MSCs decreases transiently in the bone marrow during EAE. (A) Clinical signs of rMOG (1–125) induced EAE showing mean clinical scores (± SEM). Mice were examined daily for clinical signs of EAE and were scored as followed: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, complete hind limb paralysis; 4, hind limb paralysis plus forelimb paralysis; and 5, moribund or dead. Bone marrow was isolated at day 8, 15, and 29 after immunization as indicated by arrows ($n = 3$ per group). **(B)** Numbers of MSCs present in the bone marrow during the course of EAE analyzed at the indicated timepoints after EAE induction. Total numbers of MSCs were determined by colony forming unit (CFU) assay and numbers of CFUs are expressed as mean ± SEM ($n = 3$ or more per group, one way ANOVA, $^*p < 0.05$). **(C,D)** Bone marrow cell counts and total number of $CD45^{neg}$ cells of EAE mice during the course of EAE analyzed at the indicated timepoints. Data represent mean ± SEM of EAE induced mice ($n = 3$ or more per group).

MSCs were mobilized from the bone marrow, it is conceivable that MSCs have differentiated locally into bone marrow mesenchymal stromal cells, thus remaining within the bone marrow. To address whether the number of bone marrow stromal cells had increased, the total number of $CD45^{neg}$ cells in the bone marrow was determined by FACS analysis at day 8, 15, and 29 after disease induction. These analyses showed no significant changes, suggesting that the stromal compartment did not expand substantially (**Figure 1D**). These data suggest that MSCs are mobilized from the bone marrow during the course of EAE.

THE NUMBER OF MSCs NEGATIVELY CORRELATES WITH BOTH CD4 AND CD8 T CELLS DURING EAE

Mobilization of MSCs is a multistep process, in which the initial release from their niche is followed by active migration of MSCs across bone marrow endothelium to eventually reach the bloodstream (Pitchford et al., 2009). The factors involved in mobilization of MSCs are not completely elucidated but it has been shown that treatment with VEGF followed by CXCR4-antagonist infusion leads to mobilization of MSCs out of the bone marrow (Pitchford et al., 2009). Recombinant-MOG-induced EAE is primarily a T cell mediated disease (Zamvil and Steinman, 1990), although the disease additionally involves other immune cells (Hemmer et al., 2002). These immune cells may in fact migrate to the bone marrow, where they could mediate the mobilization of MSCs. We therefore analyzed the composition of the immune cells in bone marrow with FACS analysis during the course of the disease and correlated the number of immune cells with the number of MSCs present in the bone marrow. Interestingly we observed a significant negative correlation between the number of both $CD4^{pos}$ and $CD8^{pos}$ T cells and MSCs, such that high number of T cells correlated with low numbers of MSCs (**Figures 2A,B**), independent of time after induction of the disease. We did not observe such a correlation with B cells, dendritic cells, granulocytes, or macrophages (**Figures 2C–F**).

Therefore, we hypothesized that activated T cells that migrate to the bone marrow during the course of EAE may in fact be involved in the mobilization of MSCs. Alternatively, high numbers of activated T cells in bone marrow samples could influence the outcome of the CFU assays through the production of growth factors, which may suppress MSC proliferation or induce MSC apoptosis. To test whether activated $CD4^{pos}$ or $CD8^{pos}$ T cells

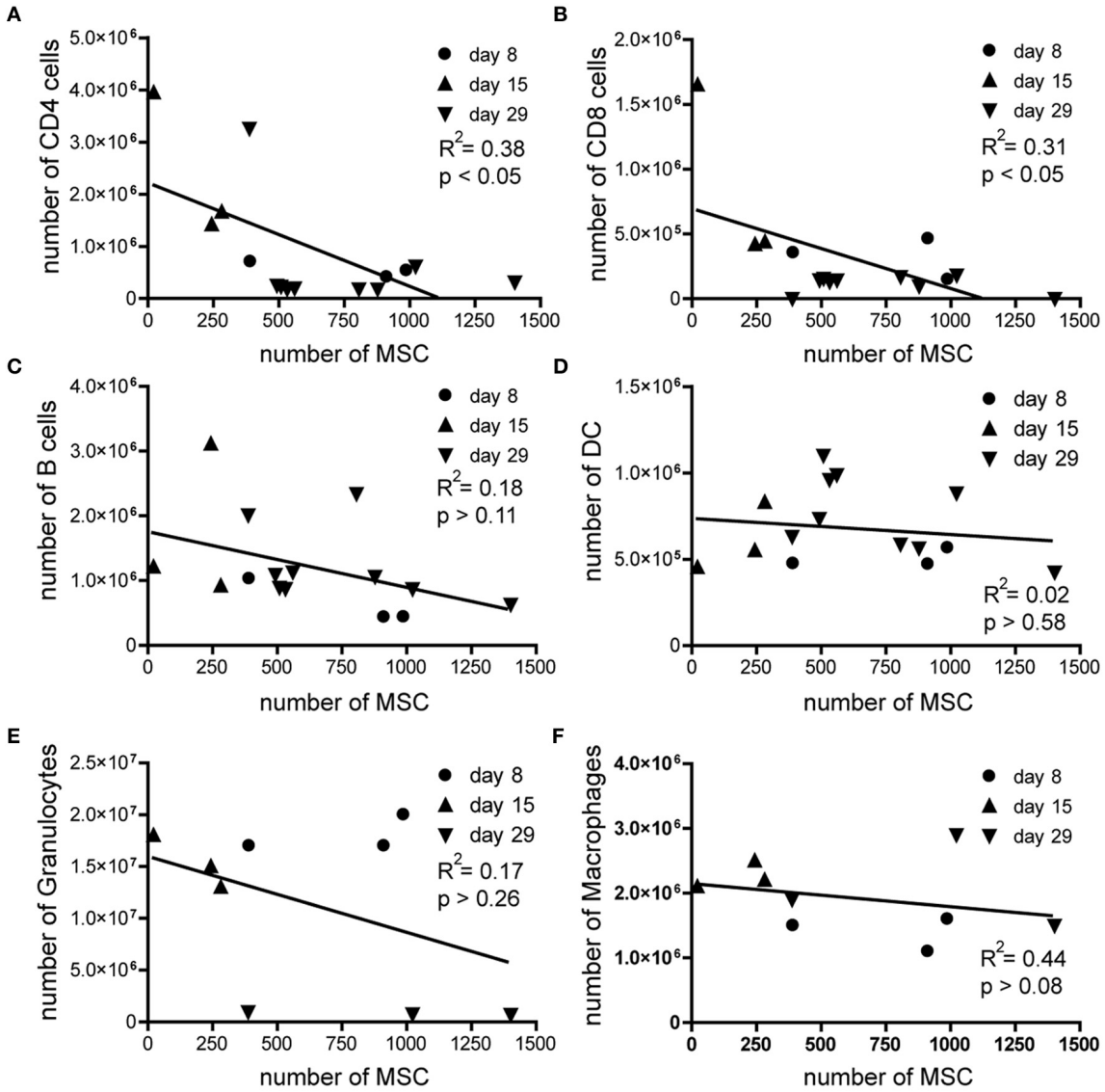

FIGURE 2 | The number of MSCs negatively correlates with both CD4 and CD8 T cells during EAE. Absolute numbers of MSCs plotted against the absolute numbers of **(A)** CD4 T cells, **(B)** CD8 T cells, **(C)** B cells, **(D)** Dendritic cells, **(E)** Granulocytes, and **(F)** macrophages during the coarse of EAE. Correlation was analyzed by using linear regression analysis.

in general could produce factors which could inhibit the proliferation of MSCs *in vitro*, thereby affecting the outcome of the CFU-F assay, we cultured bone marrow MSCs of healthy mice with supernatants of *in vitro* CD3-CD28 beads activated $CD4^{pos}$ or $CD8^{pos}$ T cells. We labeled MSCs with CFSE and cultured them for 3 days, allowed them to proliferate under normal culture conditions, in the presence or absence of different concentrations of supernatants from activated $CD4^{pos}$ or $CD8^{pos}$ T cells. Upon cell division, CFSE is equally divided over the daughter cells and thus reduced cell division will result in higher MFI levels of the measured CFSE. Although the supernatants contained IFN-γ and TNF-α (data not shown), indicating that T cells were indeed activated, we observed no difference in the capacity of MSCs to proliferate upon addition of various concentrations of supernatant derived from activated $CD4^{pos}$ T cells when compared to MSCs cultured under normal conditions (**Figure 3A**). The same results were obtained with supernatants from activated $CD8^{pos}$ T cells (**Figure 3A**). Furthermore, culturing CFSE labeled MSCs with different concentrations of various recombinant cytokines, inflammatory molecules, growth factors and potential mobilization factors, revealed that none of these factors substantially inhibited the proliferation of MSCs *in vitro* (**Figure 3B**). Together, these data indicate that factors produced by activated T cells did not directly affect the proliferation of MSCs and therefore did not influence our CFU assays. Consequently, our data suggest that other mechanisms are

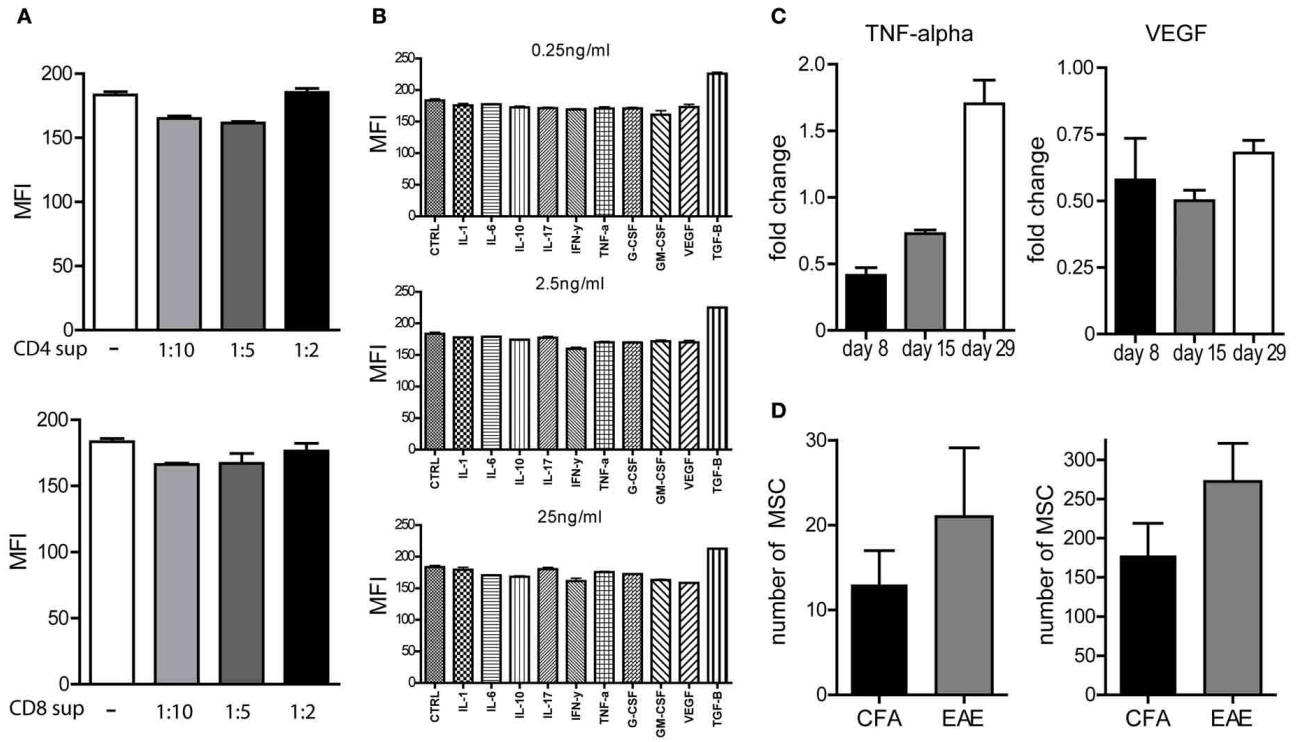

FIGURE 3 | Proliferation of MSCs not affected upon cytokine treatment. **(A,B)** *In vitro* proliferation of CFSE labeled bone marrow derived MSCs from healthy mice in the presence of different concentrations of supernatants from CD3/CD28 beads activated CD4 or CD8 T cells **(A)** or various growth factors or cytokines **(B)** during 72 h measured by MFI of CFSE. Experiments were performed three times in duplo and a representative example is shown. **(C)** TNF-alpha and VEGF-A levels determined by Luminex assay in supernatants of bone marrow samples which were re-stimulated *in vitro* with rMOG for 72 h. Experiments performed in duplo and data represent fold change compared to CFA induced control mice ($n = 3$ per group). **(D)** Absolute numbers of MSCs in cervical lymph nodes and spinal cord in EAE animals at day 29 after immunization ($n = 3$, mean ± SEM).

involved in the observed decrease in numbers of MSC in the bone marrow during the course of EAE.

To test whether T cells present in the bone marrow produce inflammatory mediators that could instigate the mobilization of MSCs, we determined whether TNF-α, IFN-γ, and IL-17 could be detected in supernatants of complete bone marrow samples upon *in vitro* restimulation with rMOG for 72 h. Surprisingly, restimulation did not result in detectable levels of IFN-γ or IL-17 (data not shown) and only low levels of TNF-α, which was elevated at day 29 when compared to control bone marrow samples, could be detected (**Figure 3C**). Since VEGF-A has been shown to be directly involved in mobilization of MSCs from the bone marrow (Pitchford et al., 2009) and $CD4^{pos}$ T cells are able to produce VEGF-A upon activation (Matsuyama et al., 2002), we also determined the levels of VEGF-A in these supernatants. Even though detectable levels of VEGF-A could be measured, we did not observe increased VEGF-A levels in bone marrow samples derived from EAE mice when compared to controls, which could account for the mobilization of MSCs (**Figure 3C**).

Since we showed that the number of bone marrow MSCs is transiently decreased during the course of EAE, we addressed whether these cells could be found at elevated numbers at other locations in the body. We could detect CFU-F activity only in the meninges of EAE mice, and not in control animals, at day 15 after disease induction, although the numbers of precursors were very low (data not shown). In addition, we determined the numbers of MSCs in SPC, and cervical lymph nodes (cLNs) of mice at the chronic phase of EAE (day 29), however, CFU-F analysis of cLNs as well as SPC did not reveal significant differences (**Figure 3D**).

Collectively, the observed decrease of MSCs within the bone marrow of diseased animals is paralleled by an increase of T cells within the bone marrow. Since inflammatory mediators could not be identified as the responsible effector molecules, direct cell-cell contact between $CD4^{pos}$ or $CD8^{pos}$ T cells and MSCs within the bone marrow could regulate the number of bone marrow MSCs during the course of EAE.

IFN-γ MEDIATED CHRONIC IMMUNE ACTIVATION DEPLETES MSCs IN BONE MARROW

To address whether activated T cells could indeed affect the presence of MSCs in the bone marrow during chronic immune activation, we analyzed the number of MSCs in the bone marrow of B cell specific CD70 transgenic (CD70TG) mice. These mice have a constitutively activated T cell compartment since transgenic over-expression of CD70 by B cells induces T cells to continuously produce high levels of IFN-γ, eventually leading to depletion of B cells from bone marrow, spleen, and lymph nodes (Arens et al., 2001; de Bruin et al., 2010). When these mice are

crossed with IFN-$\gamma^{-/-}$ mice, B cell numbers remain normal when compared to IFN-$\gamma^{-/-}$ mice, indicating that constitutive production of IFN-γ by T cells induces B cell depletion (Arens et al., 2001). In addition, B cell depletion is also a result of extensive triggering of CD27 expressed on hematopoietic precursors (Nolte et al., 2005).

Analysis of the amount of MSCs present in the bone marrow of CD70TG mice revealed a strong reduction in absolute numbers of MSCs when compared to WT controls. Strikingly, this effect was eliminated when CD70TG mice were crossed to IFN-$\gamma^{-/-}$ mice (**Figure 4A**). It has been reported that CD70TGIFN-$\gamma^{-/-}$ mice still showed an activated T cell compartment as indicated by increased numbers of $CD44^{hi}CD62L^{neg}$ T cells in both lymph nodes and bone marrow (Arens et al., 2001; de Bruin et al., 2010), therefore, it is unlikely that the observed decrease in bone marrow MSCs in CD70TG mice is a result of the presence of activated T cells but rather highlights the role of IFN-γ produced by T cells in reducing the number of MSCs in the bone marrow. Additionally, the observed differences were neither the result of changes in bone marrow cellularity nor differentiation of MSCs into stromal cells, as we observed no changes in total cell count or the amount of $CD45^{neg}$ cells in CD70TG mice, respectively (**Figures 4B,C**).

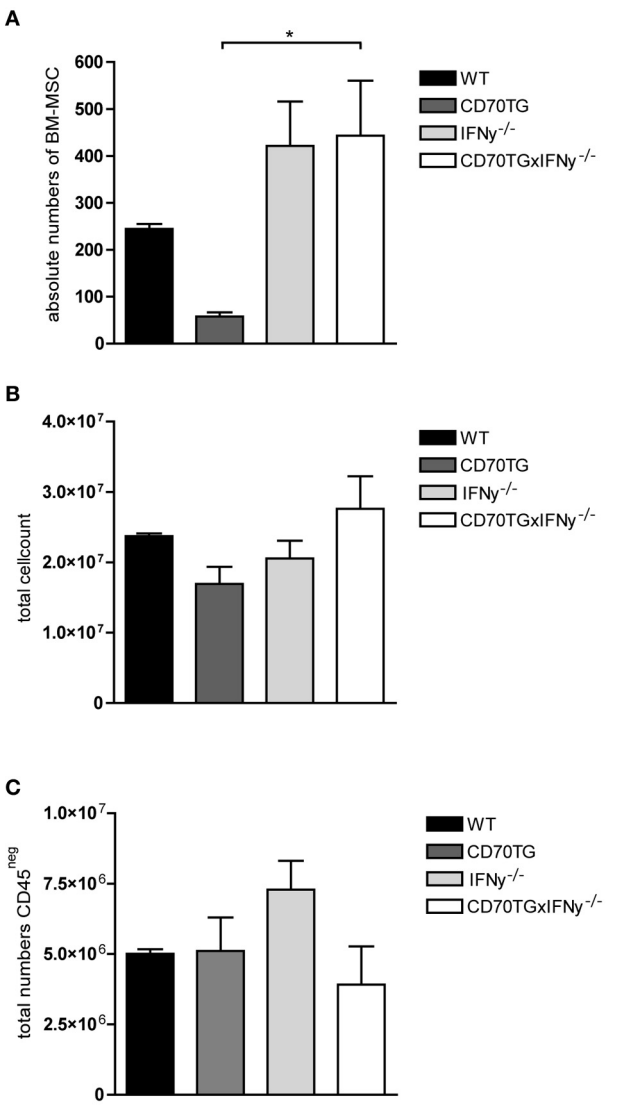

FIGURE 4 | Chronic immune activation depletes MSCs from the bone marrow. (A) Absolute numbers of MSCs in bone marrow in WT, CD70TG, and CD70TG/IFN-$\gamma^{-/-}$ mice. **(B)** Total cell count, and **(C)** absolute numbers of $CD45^{neg}$ cells in bone marrow of WT, CD70TG, and CD70TG/IFN-$\gamma^{-/-}$ mice. Data represent mean ± SEM of 3 animals per group (ANOVA; $^{*}p < 0.05$).

MOBILIZATION OF MSCs DEPENDS ON T CELL DERIVED IFN-γ

To more clearly indicate that indeed IFN-γ producing activated T cells are responsible for the reduction of MSC in the bone marrow we adoptively transferred WT T cells into CD70TGxCD27$^{-/-}$ mice. Upon transfer, only the donor T cells will become activated through CD27-CD70 interaction, while they subsequently differentiate into effector T cells (**Figures 5A,B**) and accumulate in the bone marrow. Transfer of WT T cells clearly reduces absolute number of MSCs within 3 weeks when compared to CD70TGxCD27$^{-/-}$ mice (**Figure 5D**). To assess whether this effect is IFN-γ mediated, we also adoptively transferred IFN-$\gamma^{-/-}$ T cells into CD70TGxCD27$^{-/-}$ mice. The transfer of an equal amount of IFN-$\gamma^{-/-}$ T cells was not sufficient to reduce number of MSCs within the same time period (**Figure 5D**). Transfer of WT T cells or IFN-$\gamma^{-/-}$ T cells did not affect bone marrow cellularity (**Figure 5D**). Strikingly, we observed increased numbers of CD8 T cells but not CD4 T cells in the bone marrow of CD70TGxCD27$^{-/-}$ that received WT T cells (**Figure 5C**).

Upon analysis of MSC number after 5 days of transfer of WT T cells or IFN-$\gamma^{-/-}$ T cells we did not observe differences between the two groups (data not shown) suggesting that the reduction of MSCs depends on prolonged T cell activation and IFN-γ production.

Together, these data indicate that mobilization of MSC out of the bone marrow is mediated via IFN-γ production by activated T cells.

DISCUSSION

MSCs have emerged as potential therapeutic treatment of MS, since they possess strong immunosuppressive capacity. In EAE, the most commonly used animal model for MS, it was shown that transfer of MSCs before or at the onset of clinical disease, reduced the clinical signs of the disease. By suppressing immune cell activation and thereby the infiltration of immune cells into the CNS, the administration MSCs resulted in reduced demyelination and axonal loss (Zappia et al., 2005; Gerdoni et al., 2007; Kassis et al., 2008; Lanza et al., 2009). Here we addressed whether endogenous MSCs could affect the ongoing disease. MSCs can be found in almost all organs, but they are most frequent in bone marrow (da Silva et al., 2006). We observed a strong reduction in the amount of bone marrow MSCs at the peak of the disease, which returned to control levels during the chronic phase of the disease. We hypothesize that this strong reduction in bone marrow MSCs is due to mobilization of MSCs into the periphery. Analysis of the hematopoietic compartment revealed that high levels of both $CD4^{pos}$ and $CD8^{pos}$ T cells coincided with low numbers of MSCs

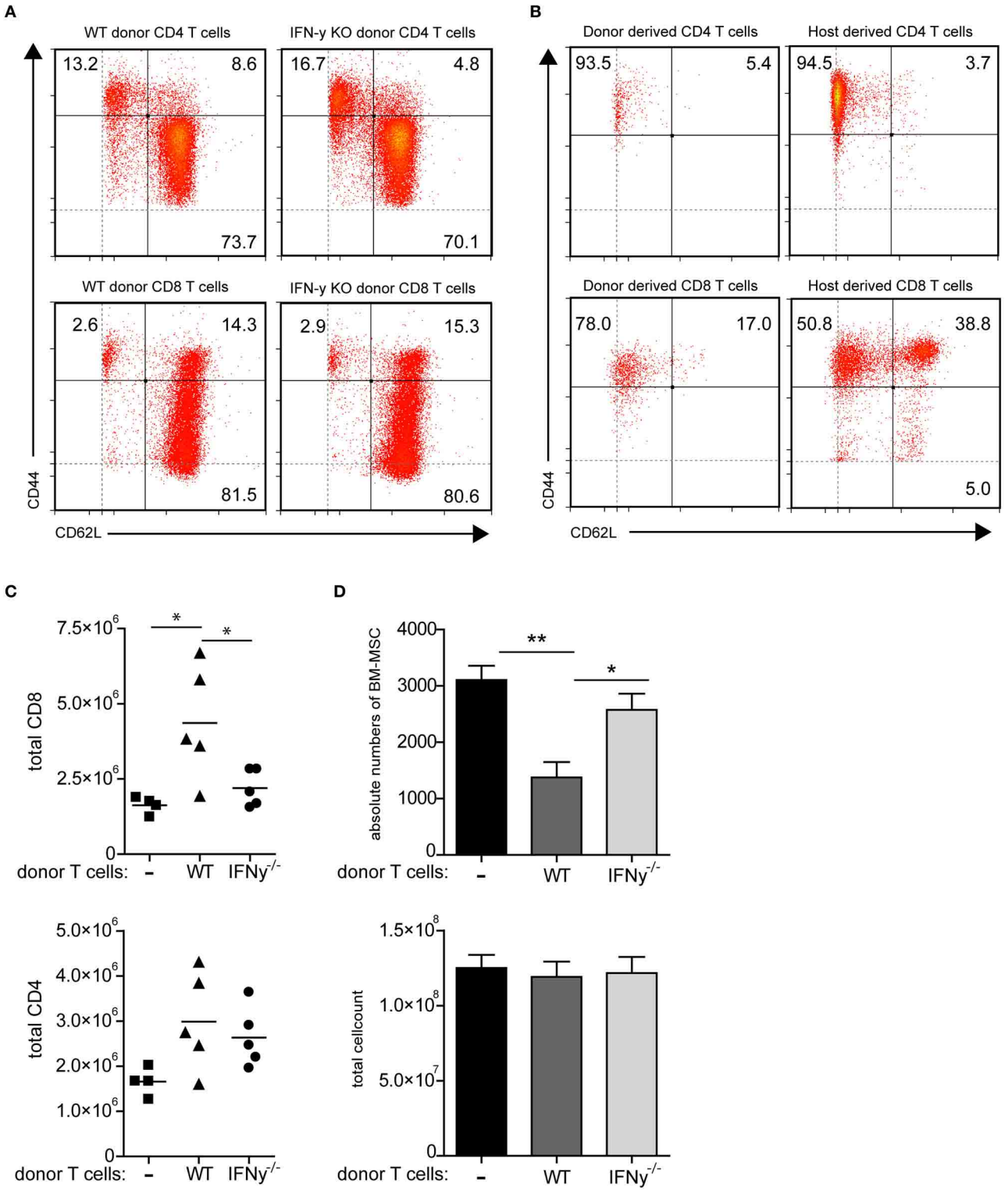

FIGURE 5 | Mobilization of MSCs depends on T cell derived IFN-γ. **(A,B)** Phenotype of WT and IFN-$\gamma^{-/-}$ donor CD4 and CD8 T cells before adoptive transfer **(A)** and phenotype of donor WT T cells and host donor T cells in the bone marrow of CD70TGxCD27$^{-/-}$ mice after 3 weeks of adoptive transfer **(B)**, effector memory; CD44^{pos}CD62L^{neg}, central memory; CD44^{pos}CD62L^{pos}, naive; CD44^{neg}CD62L^{pos}. **(C)** Absolute numbers of CD8 or CD4 T cells in bone marrow of CD70TGxCD27$^{-/-}$ recipients that received no T cells, WT T cells or IFN-$\gamma^{-/-}$ T cells. **(D)** Absolute numbers of MSCs and total cell count in bone marrow of CD70TGxCD27$^{-/-}$ recipients that received no T cells, WT T cells or IFN-$\gamma^{-/-}$ T cells. Data represent an average of 5 animals per group (±SEM, ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$).

in the bone marrow. We propose that upon inflammation, activated T cells, which migrate to the bone marrow, can mediate the mobilization of MSCs in an IFN-γ dependent way.

Several factors have been proposed to mediate the mobilization of MSCs from the bone marrow. For instance, elevated levels of MSCs are found within 48 h in peripheral blood after experimentally induced femoral vascular injury. The mobilization of MSCs from the bone marrow in this model is possibly mediated by VEGF and G-CSF, since these factors were significantly elevated within 24 h (Wang et al., 2008). Others have shown that combined treatment of mice with VEGF and G-CSF is not sufficient to mobilize MSCs, but that interrupting the SDF1-CXCR4 axis together with VEGF pretreatment is mandatory for MSC mobilization (Pitchford et al., 2009). In both studies, mobilization is not mediated by immune cells and in the latter study, mice were not exposed to tissue injury.

Under normal, non-pathogenic conditions, the bone marrow harbors both memory T cells together with T cells that continuously circulate through the body. Additionally, the bone marrow also functions as a secondary lymphoid organ, since priming of naïve T cells can take place within the bone marrow (Feuerer et al., 2003). Moreover, the bone marrow can actively recruit activated T cells which is mediated through alpha 2-integrin (Di Rosa and Pabst, 2005; Tokoyoda et al., 2009). We hypothesized that activated T cells present in the bone marrow could influence the number of bone marrow MSCs. To test this, we analyzed B cell specific CD70TG mice, which contain T cells that are constitutively activated and produce IFN-γ continuously. As a control we used CD70TGxIFN-$\gamma^{-/-}$ mice, which still have an activated T cell compartment, but lack the production of IFN-γ (Arens et al., 2001). Whereas in CD70TG mice, numbers of MSCs were severely reduced, this phenomenon was completely abrogated in CD70TG mice that were deficient for IFN-γ, demonstrating that the reduced MSC numbers were a result of continuous IFN-γ production in the bone marrow, most likely derived from activated T cells. Activated T cells express the adhesion molecule CD44, which serves as a ligand for hyaluronic acid that colocalizes with CXCL12, produced in the bone marrow by both endothelial cells and stromal cells including MSCs (Avigdor et al., 2004). Therefore, during immune activation, T cells could specifically localize close to MSCs, secrete IFN-γ locally, thereby mediating MSC mobilization. Since the CD70TGxIFN-$\gamma^{-/-}$ mice still contain an activated T cell compartment, including CD44 expressing activated T cells, it is unlikely that mobilization of MSCs is caused only through physical interaction with activated T cells, but rather suggests that paracrine secretion of IFN-γ by T cells is needed. Indeed, $CD4^{pos}$ and $CD8^{pos}$ T cells in the bone marrow of CD70TG mice were shown to produce high levels of IFN-γ upon measurement by FACS analysis (Arens et al., 2001). Remarkably, systemic increase of IFN-γ levels could not be measured in these mice suggesting that the action of IFN-γ on MSCs is mediated when T cells and MSCs are in close proximity. In addition, upon activation, T cells are able to produce VEGF-A (Matsuyama et al., 2002). Whether VEGF-A production by activated T is under the influence of IFN-γ and abrogated in CD70TG/IFN-$\gamma^{-/-}$ is unknown but could explain the lack of mobilization in these mice, despite an active T cell compartment.

The observation that adoptive transfer of WT T cells but not IFN-$\gamma^{-/-}$ T cells into CD70TGxCD27$^{-/-}$ resulted in decreased MSC numbers in the bone marrow supports the hypothesis of T cell mediated, IFN-γ dependent reduction of MSCs. Shortly after transfer, we were unable to observe differences between CD70TGxCD27$^{-/-}$ mice that received WT T cells or IFN-$\gamma^{-/-}$ T cells indicating that only upon prolonged T cell activation and IFN-γ production by donor T cells, MSC numbers are reduced from the bone marrow.

We did not observe increased cell death upon *in vitro* stimulation of MSCs with IFN-γ (data not shown), suggesting that MSCs are resistant to IFN-γ mediated apoptosis as was observed to occur with B cells. Analysis of complete EAE bone marrow samples upon *in vitro* restimulation with MOG did not reveal increased IFN-γ levels, suggesting that either antigen specific T cells are suppressed or completely absent from the bone marrow during EAE, or that IFN-γ production by MOG-specific T cells cannot be measured in the supernatants. Decreased effector functions of T cells upon *in vitro* restimulation would be in line with other publications showing that MSCs are able to inhibit T cell activation (Zappia et al., 2005; Gerdoni et al., 2007; Kassis et al., 2008). Therefore, the interaction between IFN-γ producing T cells and MSCs will critically control both cell subsets, since suppression of activated IFN-γ producing T cells by MSCs may sustain the MSC population in the bone marrow, while loss of this control will ensure their mobilization from the bone marrow.

It is unclear whether bone marrow MSCs that disappear from the bone marrow during the disease, localize to distinct organs. Based on CFU-F assays, we could observe MSCs in the meninges of EAE mice at day 15 (data not shown). Although the numbers of CFUs were very low, these colonies could never be observed in control mice. Several studies indicate that upon systemic administration, MSCs migrate to lymphoid organs as well as to the brain and SPC (Zappia et al., 2005; Kassis et al., 2008). We did not observe significant differences between MSCs present in SPCs of diseased versus non-diseased mice, although this may need further analysis at different timepoints. It could however be that subsequent differentiation of MSCs within the affected tissue prevents their CFU-F capacity in our assays, thus limiting our ability to detect them.

The observation that bone marrow MSCs are mobilized from the bone marrow at the peak of disease could have a similar effect on the course of the disease as the therapeutic administration of exogenous MSCs, which is most effective before or at the peak of disease. These data suggests the need for a temporal systemic increase of MSCs before clinical disease onset to suppress the priming and activation of autoimmune/antigen specific T cells. Future studies that induce the active mobilization and proliferation of endogenous bone marrow MSCs before onset of clinical disease would indicate whether the endogenous MSCs can dampen the effects of T cell mediated autoimmunity.

EXPERIMENTAL PROCEDURES

ANIMALS

EAE was induced in female FVB mice (NKI, Amsterdam) or C57BL/6 (Charles River, France), 8–12 weeks of age and housed at the VU University Medical Center. B cell-specific

CD70TG (Arens et al., 2001), IFN-$\gamma^{-/-}$, CD70TGxIFN-$\gamma^{-/-}$, CD70TGxCD27$^{-/-}$, and wild-type control mice were housed and used at the AMC, Amsterdam. All experimental procedures were reviewed and approved by the Ethical Committee for Animal Experiments of the VU (Vrije Universiteit) University Medical Center (Amsterdam, The Netherlands).

INDUCTION OF EAE

EAE was induced in 8–12-week-old female mice via subcutaneous immunization with 200 μg rMOG (1–125; synthesized as described Adelmann et al., 1995) mixed (volume ratio 1:1) with Complete Freund's Adjuvant (CFA; Difco Laboratories) containing 500 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (MBT; Difco). Control (CFA) animals were injected with saline mixed with CFA containing 500 μg of heat-killed MBT. All animals were additionally injected intraperitoneally (i.p.) with 200 ng pertussis toxin derived from *Bordetella pertussis* (Sigma, Zwijndrecht, The Netherlands) in 200 μL saline at the time of, and 24 h after immunization. Mice were examined daily for clinical signs of EAE and were scored as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, complete hind limb paralysis; 4, hind limb paralysis plus fore limb paralysis; and 5, moribund or dead. Mice were euthanized at day 8, 15, or 29.

SINGLE CELL SUSPENSION

Bone marrow single cell suspensions were obtained according to the following protocol. Freshly isolated femur and tibia were flushed once with 1 ml icecold DMEM (Gibco) supplemented with 2% FCS, 2% antibiotics, and glutamine *(Flush Fraction)*. To release MSCs from the bone marrow, femur, and tibia were subsequently incubated with DMEM containing Blendzyme 2 (150 μg/ml), DNAse I (200 ug/ml, both Roche Applied Sciences, The Netherlands), 2% FCS, and 2% antibiotics for 20 min at 37°C under continuous stirring. After incubation, enzymatic activity was stopped and femur and tibia were flushed once more with 1 ml cold DMEM with 2% FCS and 2% antibiotics and all cells were collected *(Blendzyme 2 fraction)*.

Lymph node, SPC and meninges single cell suspension were obtained as follows. Isolated cLNs, SPC, and meninges were cut extensively into small pieces followed by enzymatic digestion in DMEM containing Blendzyme 2 (150 μg/ml), DNAse I (200 ug/ml), 2% FCS, and 2% antibiotics for 15 min at 37°C under continuous stirring. After incubation, enzymatic activity was stopped by adding cold DMEM containing 10% FCS and 2% antibiotics.

All single cell suspensions were washed once with an excess of medium to remove any residual Blendzyme 2 and resuspended in DMEM (Gibco) supplemented with 2% FCS, 2% antibiotics, and glutamine, and cells were counted. For flow cytometry analysis, cells were passed through a 70 μm filter to remove cell clumps.

ADOPTIVE TRANSFER

T cells were obtained from lymph nodes and spleen single cell suspensions from either WT (CD45.1^{pos}) or IFN-$\gamma^{-/-}$ mice. Single cell suspensions were generated by mincing the organs through 40 μm cell strainers followed by erythrocyte lysis with ACK lysis buffer. Subsequently, cells were incubated with CD4 and CD8 microbeads (Miltenyi Biotec) and enriched by MACS positive selection using automacs with LS columns (Miltenyi Biotec). Enriched cells with a purity of >95% as determined by flow cytometry, were resuspended in PBS and 5–10 $\times 10^6$ in 200 μl PBS cells were intravenously injected into CD70TGxCD27$^{-/-}$ mice. Mice were sacrificed and analyzed 5 days or 21 days after T cell transfer.

COLONY FORMING UNIT ASSAY

Colony forming unit (CFU) assays were performed according to the following protocol: single cell suspensions were seeded at three different concentrations in 2 ml Mesencult + stimulatory supplements (Mesencult Proliferation Kit, Stem Cell Technologies, France) and 2% antibiotics/well in a 6-wells plate. CFU assays were always performed in duplo. After 2 weeks of culture, adherent cells were washed twice with PBS, fixed in methanol and stained with Giemsa. CFU's were counted using a stereo microscope. A cluster of cells containing ≥10 cells was considered a colony.

FLOW CYTOMETRY

Single cell suspensions were seeded at 2×10^6 cells/well in a 96 well plate (4°C). All cells were incubated with 10% (vol/vol) normal mouse serum (NMS) to block aspecific binding of primary antibodies. Subsequently, staining with the appropriate antibodies was carried out for 30 min at 4°C.

The following antibodies, recognizing CD45R (clone RA3-6B2), CD8 (clone 53-6.7), and CD31 (clone ERMP12) were affinity purified from the supernatants of hybridoma cell cultures by using protein G-sepharose (Pharmacia, Uppsala, Sweden). The antibodies were either biotinylated or labeled with Alexa-Fluor-488 or Alexa-Fluor-647 (Invitrogen Life Technologies). Unless stated otherwise, the following antibodies were obtained from eBioscience; CD8-FITC (clone Ly-2), CD45.1-FITC (BD Biosciences, Clone A20), GR-1-Alexa-Fluor-488 (clone RB6-8C5), CD8-PE (clone Ly-2), CD19-PE (BD Pharmingen clone ID3), CD44-PE (clone IM-7), F4/80-PE (clone BM8), SCA-1 PE-Cy5.5 (clone D7), CD45-PE-Cy7 (clone 30F-11), CD4-APC (clone GK1.5), CD62L-APC (clone MEL-14) IFN-y-APC (clone XMG1.2), CD11c-APC (clone N418), CD45 Pacific Orange (Caltag Laboratories, clone 30-F11), and CD11b-biotinylated (Beckman Coulter, clone M1/70). Biotinylated antibodies were visualized with Streptavidin-APC-Cy7 (eBioscience). Sytox Blue dead cell stain (Molecular Probes) was used to discriminate between live and dead cells. Data were acquired on a Cyan ADP High Performance Research Flow Cytometer (Beckman Coulter) and were analyzed with Summit Software v4.3. Single stained cells were used to compensate for spectral overlap. Fluorescence Minus One (FMO) stained cells were used to set boundaries between positively and negatively stained cells.

In vitro RESTIMULATION AND LUMINEX ASSAY

Bone marrow single cell suspensions were seeded at 1×10^6 cells/well in a flat-bottomed 96 well plate in media [IMDM supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2% penicillin, streptomycin, and L-glutamine (PSG)]. Cells were stimulated with either various concentrations (3–30 μg/ml)

rMOG (1–125) or PMA (20 ng/ml; Sigma)/ionomycin (500 ng/ml; Sigma) as a positive control. Supernatants were harvested after 72 h and stored in −20°C until further analysis. IFN-γ, IL-17, TNF-α, VEGF, and GM-CSF were measured using Luminex assay (Millipore Corporation, USA, cat.nr. mpxmcyto-70k) according to the manufacturer's instructions.

For in vitro restimulation of bone marrow T cells, 1×10^6 total bone marrow cells were restimulated for 6 h with PMA (10 ng/ml) and Ionomycin (1 μM) in the presence of Brefeldin A (Golgiplug™, BD biosciences). The percentage of IFN-γ producing T cells was measured with flow cytometry.

In vitro MSC PROLIFERATION

Bone marrow single cell suspensions from healthy C57BL/6 mice were obtained as described above. To expand MSCs *in vitro*, bone marrow single cell suspensions were cultured overnight in Mesencult + stimulatory supplements and 2% antibiotics. After 24 h, non-adherent cells were removed and fresh medium was added. After two passages, cells were used for proliferation assays. Hereto, cells were harvested upon trypsinization and washed extensively with PBS. Cells were labeled with 5 μM 5,6-carboxyfluorescein succinimidyl ester (CFSE, Moleculare Probes, Invitrogen) at 1×10^7 cells/ml for 10 min at 37°C. After washing, the cells were seeded at 0.1×10^6 cells per well in a 6-wells plate supplemented with 2 ml Mesencult + stimulatory supplements. Cells were allowed to adhere for 2 h and subsequently incubated with the following stimuli at various concentrations (0.25 ng/ml, 2.5 ng/ml, and 25 ng/ml); IL-1, IL-6, IL-10, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, VEGF, and TGF-β (all peprotech, UK).

T cell conditioned medium was prepared as follows; spleens were isolated from C57BL/6 mice and minced through 70 μm gauze to obtain single cell suspensions. Red blood cells (RBCs) were removed with lysis buffer (150 mM NH_4, 1 mM $NaHCO_3$, pH 7.4). CD4 or CD8 T cells were labeled with either CD4-PE-Cy7 (clone GK1.5) or CD8-PE-Cy7 (clone 53-6.7) antibodies (both eBioscience) and isolated with the Easysep Mouse PE Positive selection kit (Stem Cell Technologies, France) according to manufacturer's instructions. Purity was checked by flowcytometry (>95% purity). Subsequently, purified T cells were stimulated with CD3/CD28 beads (BD Bioscience) and after 72 h, T cell conditioned supernatants were collected and stored at −20°C. For bone marrow MSC proliferation assay, T cell conditioned medium was diluted 1:2, 1:5, or 1:10 and added to plate adherent MSCs. For all proliferation assays, unstimulated CFSE labeled cells were used as controls. After 72 h, cells were harvested by trypsinization and CFSE dilution was analyzed on a Cyan ADP as a measurement for proliferation. Sytox Blue dead cell staining was used to discriminate between live and dead cells.

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Stromal cell induction of regulatory dendritic cells

***Benjamin M. J. Owens*† *and Paul M. Kaye**

Centre for Immunology and Infection, Hull York Medical School and Department of Biology, University of York, York, UK

Edited by:
Burkhard Ludewig, Kantonal Hospital St. Gallen, Switzerland

Reviewed by:
Scott Mueller, The University of Melbourne, Australia
Nicola Laraine Harris, École Polytechnique Fédérale de Lausanne, Switzerland

***Correspondence:**
Paul M. Kaye, Centre for Immunology and Infection, Hull York Medical School and Department of Biology, University of York, Wentworth Way, York YO10 5DD, UK.
e-mail: paul.kaye@york.ac.uk

†***Current address:***
Benjamin M. J. Owens, Translational Gastroenterology Unit, Nuffield Department of Medicine, Experimental Medicine Division, University of Oxford, Oxford, UK.

Dendritic cells (DCs) are specialized antigen presenting cells of bone marrow origin that can exist in tissues in either an immature or mature state. DCs have a myriad of roles in immunity and tolerance induction, but are perhaps best known for their role in the activation and differentiation of naïve T cells at the onset of an acquired immune response. Over the past decade, a body of literature has developed that suggests that DCs, as well as many other myeloid cell populations, are also capable of exerting "regulatory" effects on T cell responses. However, relatively little is known regarding the mechanisms by which such regulatory myeloid cells arise *in vivo*. In this mini-review, we first define the characteristics of "regulatory" DCs (rDCs) and then focus on the contribution of non-hematopoietic stromal cells to their generation within specific tissue microenvironments. We also highlight areas of research that warrant future attention, arguing for a focusing of efforts toward a better understanding of the features of stromal cell populations that enable the induction of rDCs. Finally, we discuss how an understanding of stromal cell-myeloid cell interactions may lead to new therapeutic strategies for cancer, autoimmunity, and infectious disease.

Keywords: stromal cells, dendritic cells, Immune regulation, IL-10, inflammation, infection

INTRODUCTION

Dendritic cells (DCs) lie at the interface of innate and adaptive immunity, playing a critical role in the initiation of effective T cell-mediated immune responses. Paradoxically, DCs also have the potential to exert powerful negative regulatory effects on the immune system (Steinman et al., 2003). This potential for dampening immunity has spawned great interest in the context of cell-based therapeutic intervention in a variety of autoimmune and inflammatory contexts (Kalantari et al., 2011). Alongside several populations of conventional $CD11c^{hi}$ DCs (cDCs), there are many other myeloid cell populations capable of antigen presentation and exerting both positive and negative effects on T cell responses. This has given rise to a literature that contains a significant level of confusion. That many of these myeloid cells also share phenotypic characteristics has only compounded the problem. For example, the assignment of cells as DCs based solely on CD11c expression has not always been helpful (Drutman et al., 2012). The relationship between cDCs and other myeloid cells is further complicated by the existence of convergent differentiation pathways, particularly under inflammatory conditions (Geissmann et al., 2010). Nevertheless, recent data strongly support the concept that cDCs belong to a distinct immune cell lineage (Meredith et al., 2012; Satpathy et al., 2012). The identification of key lineage-related transcription factors should allow rapid progress in determining the relationships between cDCs and other myeloid cell populations and add some clarity to studies of the regulatory properties of these cells.

Whilst the regulatory potential of cDCs *in vivo* has long been appreciated (Hawiger et al., 2001), consensus regarding the phenotypic features of cDCs and myeloid cells with regulatory properties has been hard to reach. Historically, "immature" cDCs were classed as those that had yet to receive a pathogen-derived signal and existed in the tissues in a state of readiness for antigen presentation, with high levels of endocytosis to facilitate antigen capture and large intracellular pools of MHCII. Upon pathogen recognition by TLRs or other pattern recognition receptors, cDCs "mature" and in so doing, shut down endocytosis in favor of MHCII-peptide display, heightened expression of co-stimulatory molecules, and the secretion of cytokines that direct naïve T cell differentiation. Early literature suggested that immature cDCs may also be endowed with regulatory function, although this may represent an oversimplification (Kleindienst et al., 2005). Conversely, all subsets of splenic cDC have recently been shown to be capable of producing the regulatory cytokine IL-10, even after TLR induced maturation (Maroof and Kaye, 2008; Owens et al., 2012). In the context of chronic *Leishmania donovani* infection, IL-10-producing cDCs are capable of antigen presentation and the induction of naïve T cell proliferation *in vitro* (Owens et al., 2012), making them functionally distinct from rDCs as we define below. Against this background, where pleiotropic function characterizes cDCs, it becomes pertinent to ask whether there are distinct populations of DCs (regulatory DCs; rDCs) in which regulatory function is hardwired, and how stromal cell populations can contribute to their generation (**Figure 1**). The remainder of this review will focus on addressing this question.

REGULATORY DCs: CHARACTERIZATION AND FUNCTION

Amongst cytokines, IL-10 has become synonymous with the concept of regulation, yet as discussed above cDCs under appropriate circumstances are quite capable of producing this cytokine. Hence,

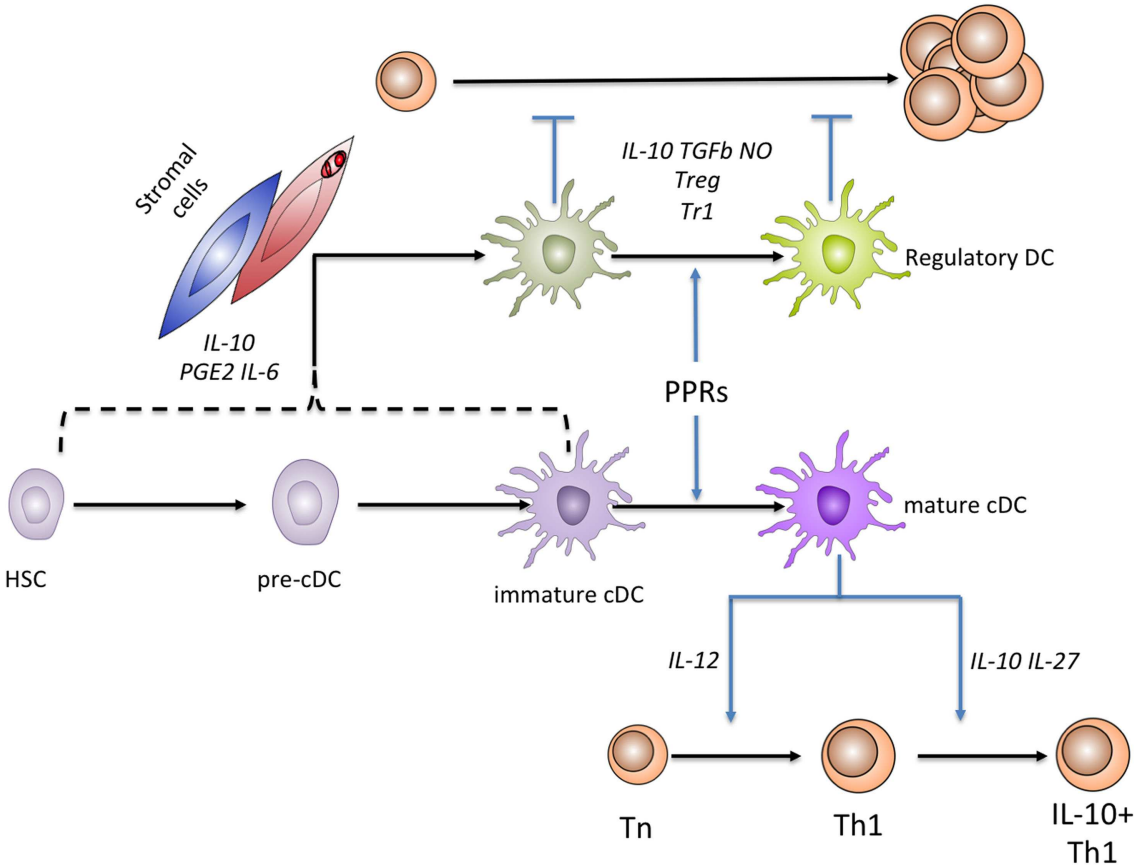

FIGURE 1 | Relationships between rDCs and cDCs. cDCs are known to originate from a hematopoietic stem cell precursor, through a sequence of events (not shown) that culminates in production of tissue precursor cells (pre-cDCs). In tissue, cDCs exist as immature cells, but functionally mature in response to microbial sensing mediated through various pattern recognition receptors (PRRs). Mature cDCs have an enhanced capacity to prime naïve T cells and induce their differentiation (shown here for Th1 cells). Mature cDCs may also help terminate excessive T cell responses by inducing a "self regulatory" phenotype, e.g., through the induction of IL-10 production by Th1 cells. Under steady state conditions, stromal cells (blue) are able to induce the generation of IL-10 and PGE_2-producing rDCs from c-kit$^+$ progenitors by production of PGE_2, IL-6, and IL-10, as well as direct interactions mediated by Fibronectin. During the chronic inflammation associated with *L. donovani* infection, IL-10-producing rDC generation is enhanced by the production of CCL8 and CXCL12 by splenic stromal cells that in many cases are directly parasitized (red). The stage(s) of development at which cDCs or their precursors are able to differentiate into regulatory DCs is not yet defined. Functionally, regulatory DCs are best defined by their capacity to inhibit the proliferative capacity of T cells. rDCs employ multiple mechanisms to suppress naïve T cell proliferation, including the direct production of IL-10 and the induction of FoxP3$^+$ nTreg and IL-10$^+$ Tr1 Treg populations.

IL-10 alone could not be a sufficient criterion by which to distinguish rDCs. Although there is evidence of a rDC population with functions that are distinct from cDCs, there is currently nothing known as to the extent of plasticity within this group of myeloid cells. In particular it is not yet clear whether rDCs represent a terminally differentiated DC phenotype, or a transient functional state reflecting phenotypic changes of myeloid cells in distinct tissue microenvironments. Despite such ambiguity in the nature of rDCs, some of the strongest evidence in support of the existence of this population has come from the study of how fibroblasts and endothelial cells impact on DC development from hematopoietic stem cells or committed myeloid progenitors.

Stromal cell induction of rDC differentiation can occur in multiple tissues even in the absence of pathogen recognition and inflammation, suggesting that this is a normal homeostatic process. To date, stromal cell-induced rDCs have been reported in murine spleen (Svensson et al., 2004; Zhang et al., 2004; Tang et al., 2006; Nguyen Hoang et al., 2010; Xu et al., 2012), liver (Xia et al., 2008), kidney (Huang et al., 2009), lung (Li et al., 2008), and tumor tissue (Liu et al., 2009). Despite their divergent tissue localization, the majority of studies reporting stromal cell-induced rDCs have characterized them as populations of CD11c^{lo} MHCII$^{lo/int}$ CD11b$^+$cells, based on surface protein expression assessed by flow cytometry. Splenic rDCs have also been reported to express CD45RB (Wakkach et al., 2003; Svensson et al., 2004), although the functional significance of this is not known. Expression of co-stimulatory molecules such as CD40, CD80, and CD86 is generally lower on rDCs than cDCs, suggesting an impaired capacity to deliver activatory signals to naïve T cell populations, although high co-stimulatory molecule expression (particularly CD80) by rDCs has been reported in some contexts (reviewed by Svensson and Kaye, 2006).

Several other characteristics of stromal cell-induced rDCs allow for their more rigorous identification. Multiple studies have reported that rDCs are major producers of the anti-inflammatory cytokine IL-10, including those from spleen (Svensson et al., 2004; Zhang et al., 2004; Tang et al., 2006), liver (Xia et al., 2008), kidney (Huang et al., 2009), and lung (Li et al., 2008), suggesting that IL-10 production is a conserved feature of rDCs, irrespective of tissue localization, or origin. This preferential IL-10 production can be driven by TLR triggering, and at least partially relies on ERK signaling (Qian et al., 2006). However, conventional CD11c^{hi}MHCII^{hi}cDCs are also capable of abundant IL-10 production (Saraiva and O'Garra, 2010), a process particularly pronounced during chronic parasitic infection (Maroof and Kaye, 2008; Owens et al., 2012) indicating that IL-10 production is not unique to rDCs.

Distinctive functional properties of rDCs provide perhaps the best method for their identification, as rDCs utilize multiple pathways in order to exert their immune regulatory function. Several reports suggest that rDCs are capable of modulating T cell proliferation *in vitro* and *in vivo*, with splenic stromal-induced rDCs suppressing proliferation via IL-10, TGFβ, and/or nitric oxide (NO) production (Svensson et al., 2004; Zhang et al., 2004; Tang et al., 2006), and pulmonary stromal-induced rDCs inhibiting T cell proliferation via production of Prostaglandin-E_2 (PGE_2; Li et al., 2008). Importantly this capacity for stromal-induced rDCs to suppress T cell proliferation does not appear to be dependent on the presence of stromal cells within the assays, as stromal-induced rDCs are capable of suppressing T cell proliferation during *in vitro* co-culture solely with T cells, as well as after adoptive transfer *in vivo*. This therefore indicates a regulatory process distinct from that recently reported for lymph node (LN) fibroblastic reticular cells (FRCs), whereby nitric oxide produced by LN FRCs regulates T cell proliferation via direct effects on T cells, in addition to modulating cDC function (Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). Stroma-induced rDCs within tumors are able to utilize an alternative mechanism involving the direct production of Arginase-1 (Liu et al., 2009). This results in the metabolism of L-Arginine and consequent suppression of T cell proliferation *in vitro* and *in vivo*. In contrast, splenic stroma-induced rDCs specifically recruit CXCR3$^+$ Th1 cells to more efficiently suppress their proliferation, via the IFNα/β-dependent production of IP-10 after TLR triggering (Qian et al., 2007). More recent evidence has suggested that splenic rDCs are also able to directly induce apoptosis of activated CD4$^+$ T cells by a process involving NO, Fas-Ligand, and IFNγ (Xu et al., 2012), a mechanism also reported to occur in the liver (Xia et al., 2008). This capacity for direct suppression of CD4$^+$ T cell proliferation, even after TLR induced "maturation," is a cardinal feature of rDCs that allows for them to be distinguished from cDCs.

In addition to their direct modulatory effects on activated T cells, rDCs can also employ indirect immune regulatory mechanisms, in many cases by the induction of specialized populations of regulatory T cells (Treg). Splenic stroma-induced rDCs directly induce IL-10-producing Tr1 Treg *in vitro* (Svensson et al., 2004), whereas rDCs induced by pulmonary stromal cells can induce populations of Foxp3$^+$ natural Treg (Li et al., 2008). Whether the differential induction of Treg populations from rDCs induced by stromal cells of distinct tissues reflects the polarization of rDC subsets is currently unclear. It will be important to define stroma-induced rDCs from distinct tissues in more detail to elucidate whether further functional subsets exist, in addition to the cues that drive their differentiation. However, Treg induction by rDCs does not always contribute to their regulatory potential (Tang et al., 2006) and indeed rDCs have been reported to activate NK cells in some circumstances (Qian et al., 2006). These divergent functions suggest that although rDCs are capable of suppressing the cellular components of an active immune response, in different contexts rDCs may also act in an immune stimulatory fashion.

Taking these multiple parameters into consideration, a "minimal" definition of stromal-induced rDCs can be reached as CD11c$^{lo/int}$ MHCII$^{lo/int}$ CD11b$^+$ myeloid cells, capable of IL-10 production, the direct suppression of CD4$^+$ T cell proliferation and (in some cases) the induction of Treg. Although several other functional features can also contribute to the identification or characterization of rDCs, these are either tissue-context dependent, or have not yet been assessed in all stromal-induced rDC populations.

SPECIFIC CONTEXTS LEADING TO THE INDUCTION OF rDCs BY STROMAL CELLS

It is clear that in several tissue contexts stromal cells can induce rDCs, but direct evidence as to whether this process is regulated by infection or inflammation is more limited. One exception is experimental visceral leishmaniasis (EVL), a chronic infection caused by the intracellular parasite *L. donovani* (Kaye et al., 2004). Splenic stromal cells from mice infected with *L. donovani* have an enhanced capacity to direct hematopoietic progenitors toward a rDC phenotype *in vitro* (Svensson et al., 2004), a process at least in part dependent upon infection-modulated levels of the chemokine CCL8 (Nguyen Hoang et al., 2010). The precise mechanisms by which infection itself enhances the capacity for stromal cells to support rDC induction during chronic inflammation are not known, but as stromal cells are targets of *Leishmania* infection (Bogdan et al., 2000) it is feasible that direct parasite modulation of stromal cell function may represent a strategy for manipulating host defense mechanisms in favor of the invading pathogen (Svensson and Kaye, 2006). Whether similar alterations in stromal cells occur during other parasitic infections with an abundance of CD11c^{lo}rDCs (Li et al., 2011; Smith et al., 2011), and whether rDCs are associated with chronic infection by viral or bacterial pathogens will require further investigation.

As stromal cells from organs considered mucosal (Li et al., 2008; Huang et al., 2009) or with specialized properties related to tolerance induction (Xia et al., 2008) have been reported to induce rDCs, it is possible that this is a generalized feature of stromal populations from these sites. It is not yet known whether stromal cells from other mucosal organs, such as the skin or intestine, are specialized for the induction of rDCs.

MECHANISMS OF rDC INDUCTION BY STROMAL CELLS

Unlike the relatively conserved phenotypic and functional characteristics of rDCs, there are multiple reported mechanisms by which stromal cells induce these cells. Physical contact between splenic stromal cells and mature cDCs has been shown to be required

for their polarization toward a rDC phenotype, in a process also dependent on fibronectin (Zhang et al., 2004). However, kidney stromal cells can induce rDCs by a process that does not require cell-cell contact (Huang et al., 2009), indicating heterogeneity in the mechanisms underlying stromal cell-induced rDC differentiation. This is perhaps not surprising given the heterogeneity in stromal cell populations themselves, as shown clearly by recent transcriptional analysis of stromal cell subsets within lymphoid tissue (Malhotra et al., 2012).

Diverse stromal cell-derived products such as IL-6 (Huang et al., 2009), CCL8 and CXCL12 (Nguyen Hoang et al., 2010), TGFβ (Li et al., 2008), and M-CSF (Xia et al., 2008) have been reported to impact upon the stromal cell induction of rDCs, but it is unclear as to whether redundancy exists in this system. In addition to rDCs being producers of PGE_2 and IL-10, the release of these mediators by stromal cells has also been implicated in their generation, with splenic endothelial-produced IL-10 (Tang et al., 2006) and tumor stroma-derived PGE_2 (Liu et al., 2009) playing a role in the induction of rDC populations.

A potent immunoregulatory circuit endowing human DCs with IL-27-dependent regulatory potential was recently described (Ilarregui et al., 2009), relying on the carbohydrate-binding protein Galectin-1. As Galectin-1 is expressed by stromal cells (Jung et al., 2007), this raises the intriguing possibility that stromal populations are also capable of delivering signals that skew conventional human DCs toward regulatory capacity, in addition to the differentiation of specific rDC populations.

FUTURE QUESTIONS

As evidenced by this short review, there is still much to be revealed regarding the precise mechanisms by which stromal cells induce the differentiation and/or expansion of rDCs (**Figure 1**). Indeed there are large gaps in our knowledge regarding the precise identity of rDCs, whether subsets of these cells exist *in vivo* and the lineage relationship of rDCs to conventional DC populations. Transcriptional and epigenetic profiling of rDCs from a multitude of tissue sites and disease states will allow for these questions to begin to be addressed. As many of the mediators previously reported to be important for rDC generation are produced widely within the immune system, it is important to determine how they contribute to the generation of rDCs within a defined tissue microenvironment. As it is likely that a multitude of synergistic signals underlie the induction of rDCs by stromal cells, revealing the extent of redundancy in this system will be key in finding pathways essential for rDC induction by stromal cells. In addition, extending our knowledge of the processes by which pathogens, vaccination, or chronic inflammation can modulate stromal cell function, and thus favor or suppress rDC induction *in vivo* will be crucial when considering therapeutic strategies aimed at manipulating rDC abundance or function.

Furthermore, a more detailed analysis of the stromal microenvironment that supports rDC generation *in vitro* will provide clues as to novel mechanisms responsible for their *in vivo* induction. This type of analysis has already been performed for splenic stromal cell populations capable of inducing immature DC populations *in vitro* (Despars et al., 2008), identifying gene signatures associated with this functional capacity. Extending this approach to rDC induction will likely reveal much useful data in this area.

More fundamentally, it is essential that we gain a much deeper understanding of the stromal cell populations capable of rDC induction *in vivo*. Even within the same organ it would appear that diverse stromal populations such as fibroblasts (Svensson et al., 2004) and endothelial cells (Tang et al., 2006) are capable of rDC induction. Clarity on whether distinct differences in stromal cells both within and between organs results in a differing capacity for rDC induction, or alternative mechanisms by which induction occurs, will likely reveal much about the biological processes required for stromal cells to induce rDC populations. Key to this may be identification of the tissue specific niches for rDC development (as this will define the local stromal cell population) coupled to transcriptional and/or proteomic analysis of the stromal cells at such sites. In particular the application of advanced imaging techniques such as intravital microscopy or whole mount histology could be applied to identifying stromal niches for rDC generation *in situ*, allowing for the visualization of the distinct microenvironments that facilitate the induction of these cells. However these approaches will necessitate the development of specific tools for the identification of rDCs *in vivo*. Such experiments are also likely to challenge the conclusions drawn from conventional experimental approaches.

To bring clarity to the field, the following approaches should therefore be taken to address the major outstanding research questions regarding stromal cell-induced rDCs:

- Transcriptional and epigenetic analysis of stromal-induced rDCs from distinct tissues to determine whether subsets exist and their relationship to cDCs.
- Imaging approaches to visualize the stromal cell-rDC niche *in vivo*, which will require the development of new tools for rDC identification.
- Transcriptional and epigenetic analysis of stromal cells capable of rDC induction, specifically comparing these profiles to those of stromal cells from distinct tissues and/or disease states.

Extending our knowledge of both rDCs and the stromal cells that induce them may allow for the potential therapeutic benefits of this immunoregulatory axis to be realized.

HARNESSING STROMAL CELL NICHES FOR THERAPEUTIC IMMUNE REGULATION

With patient-specific pro-inflammatory DC infusions effective in phase III trials for prostate cancer (Kantoff et al., 2010), the use of myeloid cell populations to modulate immune responses in humans is rapidly becoming a clinical reality. Applying this rationale to the design of alternative therapeutic myeloid cell infusions aimed instead at repressing immune responses is already under close scrutiny (Kalantari et al., 2011; Lutz, 2012), with progress in this area likely soon. As stromal cell-induced rDCs are potent negative regulators of inflammation and improve outcome in preclinical models of hepatic and pulmonary insult (Li et al., 2008; Xia et al., 2008), such cell populations – once characterized fully in humans – would provide a promising candidate for therapeutic infusion.

Although clearly feasible, the *ex vivo* expansion of myeloid cell populations for infusion has disadvantages. Instead, harnessing the capacity for stromal cells to regulate rDC induction by specifically targeting them for functional modulation *in vivo* could provide a method to enhance rDC induction for the amelioration of autoimmune or inflammatory disease, or conversely repress rDC induction during the immunosuppression associated with chronic infection and cancer. Stromal targeting approaches have already attracted much interest in the oncology field (Engels et al., 2012). Experimental therapeutics have included the targeting of potent cytotoxic agents to tumor stroma by conjugation to a collagen IV-specific monoclonal antibody (Yasunaga et al., 2011), specific enzymatic degradation of tumor stroma to enhance stromal remodeling (Provenzano et al., 2012), and therapeutic ablation of cancer stromal cells in murine tumor models (Kraman et al., 2010), but stromal targeting approaches are not usually considered in other therapeutic contexts.

By expanding our knowledge regarding the detailed mechanisms underlying stromal cell – regulatory dendritic cell interactions, we may advance one step closer to the ultimate goal of subtly manipulating immune function by targeting stromal cells within an inflammatory microenvironment.

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Stromal cells of the mouse spleen

Joke M. den Haan, Reina E. Mebius and Georg Kraal*

Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, Netherlands

Edited by:
Christopher G. Mueller, Centre National de la Recherche Scientifique, France

Reviewed by:
Geneviève Milon, Institut Pasteur, France
Peter Balogh, University of Pécs, Hungary

****Correspondence:***
Georg Kraal, Department of Molecular cell Biology and Immunology, VU University Medical Center, PO Box 7056, 1007MB Amsterdam, Netherlands. e-mail: g.kraal@vumc.nl

The composition and function of stromal cells in the white pulp of the spleen resemble to a large extent the situation in other secondary lymphoid organs such as lymph nodes. The stromal cells play an important role in the support and guidance of lymphocytes and myeloid cells in the T and B cell zones of the spleen. Major differences of the spleen are found in the way cells enter the white pulp and the composition of stromal cells in the red pulp. In this review, the features of stromal cells of both white and red pulp will be described in light of the function of the spleen.

Keywords: spleen, white pulp, red pulp, fibroblast reticular cells, marginal zone, marginal reticular cell

INTRODUCTION

The structure and organization of the spleen differ in many aspects from other secondary lymphoid organs such as lymph nodes, related to the complex function of the spleen as a filter of the blood as well as a lymphoid organ. The spleen is composed of compartmentalized lymphoid tissue, the white pulp, which resembles the organization of lymph nodes (Mebius and Kraal, 2005). The venous part of the spleen, the red pulp, is composed of intricate blood endothelial sinuses lined with macrophages, essential for particle clearance of the blood and removal of effete red blood cells. In addition to the immune and filter function of the spleen, the organ is a large reservoir of monocytes and can play a role in hematopoiesis during ontogeny and under pathological conditions. This variety of functions will be reflected in the local composition and function of stromal cells in the spleen, such as fibroblast reticular cells (FRC) and endothelial cells. Here, we will describe what is known about the different stromal cell types in the compartments of the spleen and their contribution to the function of the organ.

ONTOGENY

The distinct position of the spleen is reflected in its ontogeny. The molecular and cellular requirements that are essential for the development of lymph nodes and mucosa associated lymphoid organs have been described in large detail. Studies in mice deficient in various genes have made it clear that the interaction of lymphoid-tissue inducer (LTi) cells and stromal lymphoid tissue organizer (LTo) cells is crucial for the development of lymph nodes (Mebius, 2003). The hematopoietic LTi cells, expressing lymphotoxin-α1β2, seed the lymph node anlage and interact with the mesenchymal LTo cells that express the lymphotoxin-β receptor (LTβR). The interaction between the two cell types and the resulting upregulation of adhesion molecules, cytokine and chemokine production is instrumental for further local development of lymph nodes (Vondenhoff et al., 2009b). Interestingly, deficiency of either the lymphotoxin receptor or ligand leads to a complete absence of lymph node development. Similarly, deficiencies described for genes that are crucial for the differentiation or the homing and clustering of LTi cells prevent the formation of lymph nodes (Yoshida et al., 2002; Vondenhoff et al., 2009a). Yet, under all these circumstances the spleen will still be formed. In fact, products of the HOX genes, which play a more central role in embryogenesis, are necessary for spleen formation (Brendolan et al., 2007), with Pbx1 as the prime regulator of spleen organogenesis (Koss et al., 2012). Interestingly, LTi cells can be detected in the fetal spleen where they can be found at the periphery of the white pulp anlagen (Vondenhoff et al., 2008). Expression of homeostatic chemokines in stromal and endothelial cells suggests that LTi cells are attracted by these chemokines. As lymphotoxin-α1β2 can be detected on B cells but not on LTi cells in neonatal spleen, the earliest formation of the white pulp in fetal spleen occurs in an LTα1β2-independent manner (Vondenhoff et al., 2008).

Although lymphotoxin signaling is not required for the formation of the white pulp and the segregation of red and white pulp, it is important for an optimal functional development of the lymphoid part of the spleen (Futterer et al., 1998). In its absence, T and B cell compartments do form but B cell follicles lack functional follicular dendritic cells (FDCs) and there is a conspicuous absence of macrophages in the marginal zone. This impaired development can be the result of altered induction of chemokines that are necessary for the homing and retentions of lymphocytes and dendritic cells (DCs). That B cells play an important role in this process was demonstrated in mice that lack B cells (Ngo et al., 2001; Nolte et al., 2004). A profound effect was seen on the organization of the splenic T cell zone (periarteriolar lymphoid sheath, PALS) and on the number of DCs in the white pulp. This was dependent on the production by stromal cells of CCL21, a T cell zone chemokine, and the induction of gp38 (podoplanin) expression, a stromal cell marker. This induction required the interaction of B cell and stromal cells and depended on LTα1β2 expression by B cells (Ngo et al., 2001).

In other studies, it was demonstrated that the chemokines CCL21, and to a lesser extent CCL19, are involved in the localization of the marginal zone macrophages in the marginal zone (Ato et al., 2004).

STROMAL CELLS IN THE SPLEEN: THE WHITE PULP

Even though it is obvious that stromal cells are important for the organization of the white pulp and marginal zone, the question is whether there are differences between stromal cells from spleen and other lymphoid organs. The organization of the white pulp of the spleen in discrete T and B cell areas closely resembles that of the lymph nodes, but there are two major differences, the absence of high endothelial venules (HEVs) and the absence of a subcapsular sinus.

High endothelial venules are crucial for the entrance of naïve, recirculating lymphocytes from the blood into the lymph nodes. In the absence of the homeodomain transcription factor Nkx2-3 HEV do develop in the spleen and can mediate L-selectin-dependent homing of lymphocytes (Czompoly et al., 2011). In a normal spleen, both lymphocytes and antigen will enter the lymphoid white pulp from the surrounding marginal zone (Bajenoff et al., 2008). Here, the blood vessels partially end and blood-borne antigens will be picked up by macrophages and DCs, whereas lymphoid cells can actively migrate into the white pulp, depending on the expression of adhesion molecules and chemokine receptors. From homing studies using blocking antibodies the involvement of the adhesion molecules LFA-1 and α4β1 on migrating cells and the ligands ICAM-1 and VCAM-1 on stromal cells has been inferred (Nolte et al., 2002; Lo et al., 2003).

Data from intravital microscopy have confirmed earlier observations that lymphocytes predominantly enter the white pulp from the marginal zone via the bridging channels and from there into the T cell-dependent area surrounding the central arteriole (Bajenoff et al., 2008). These bridging channels are also involved in the exit of effector cells that have been activated in the white pulp and leave for the red pulp or further dissemination into the blood (**Figure 1**).

The migration of cells into the white pulp is an active process by which a layer of stromal cells, the marginal reticular cells (MRC) has to be passed. These cells form the boundary between marginal zone and the T and B cell areas of the white pulp and are characterized by the expression of MAdCAM-1 and the production of CXCL13 (Katakai et al., 2008). Furthermore, the production of chemokines by these cells may lead to the formation of local niches for cells in the marginal zone, such as the marginal zone B cells and the marginal metallophilic macrophages, and possibly DCs.

The position of the MRC at the border of marginal zone and white pulp emphasizes the overall structural resemblance of the white pulp with a lymph node. In a lymph node, the MRC form the bottom of the subcapsular sinus and play a similar role as in the marginal zone of the spleen by regulating cell entrance into the T cell zone (Katakai et al., 2008; Koning and Mebius, 2012).

In addition, MRC are in close contact with CD169 expressing metallophilic macrophages in the spleen and subcapsular macrophages in the lymph nodes and may be involved in their generation and maintenance. Recent studies indicate that these macrophages have important functions in both the innate and the adaptive immune system by producing type I IFN and viral antigens after infection, by transferring antigens to B cells and DCs, and by directly stimulating NKT cells (Phan et al., 2009; Vondenhoff et al., 2009b; Backer et al., 2010).

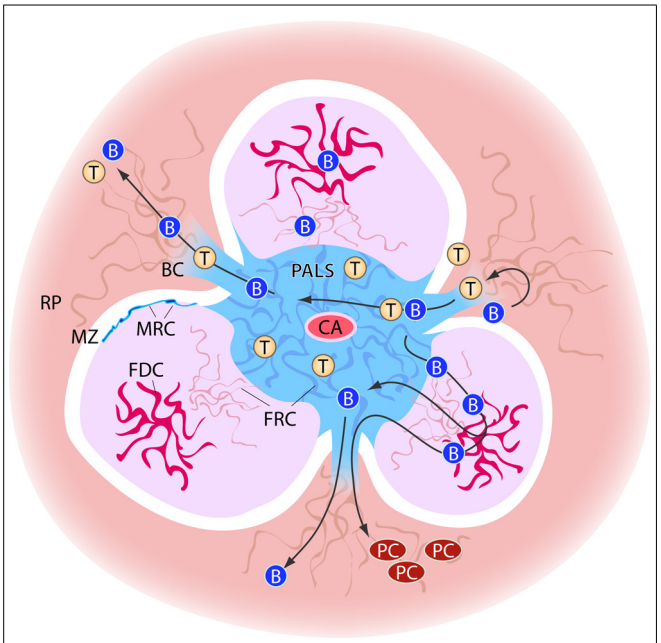

FIGURE 1 | Schematic view of stromal elements in the spleen. T and B cells enter the spleen white pulp area via from the marginal zone via bridging channels (BC). Cells first enter the T cell zone of the white pulp, the PALS (peri-arteriolar sheath) in which fibroblast reticular cells (FRC) are the predominant stromal element. In the B cell zones, the follicles, stromal cells form a less dense network and here the follicular dendritic cells (FDCs) play an important role in B cell activation. PALS and follicles are surrounded by the marginal zone (MZ). At the border of marginal zone and T and B cell zone, the marginal reticular cells (MRC) can be found. The bridging channels also form the route of exit for lymphocytes from the white pulp. B cells that have undergone differentiation and maturation in the follicles may exit the white pulp to localize as plasma cells (PC) in the red pulp (RP).

Once lymphoid and myeloid cells have entered the white pulp they are able to migrate further along a network of stromal cells, the FRC. The network extends throughout the T cell zone of the white pulp and is connected to the marginal zone (Mueller and Ahmed, 2008). Although the structure of the FRC network in the spleen has not been studied in as much detail as has been done for the comparable network in lymph nodes, based on functional studies it is very likely that the overall composition is similar to the lymph node situation.

In contrast to most connective tissues, in which the extracellular components that are produced by the fibroblasts surround the cells, in lymphoid organs a special adaptation leads to a situation where the fibroblasts surround the extracellular matrix they produce. In addition, the FRC are connected to each other forming a three-dimensional reticulum, and lymphocytes fill up the spaces of this network (Gretz et al., 1997). The extracellular matrix is highly organized in a collagen fiber network, consisting of 20–200 parallel bundles, up to 1 micron in diameter, of

mostly type I and type III collagen fibers. The complete ensheathment of the collagen bundles by a basement membrane forms a tube and the meshwork inside, formed by collagen and connecting fibrils and associated glycosaminoglycans, results in a molecular sieve through which small molecules and fluid can be transported (Lammermann and Sixt, 2008; Roozendaal et al., 2008). This tubular system is called the conduit, and an important issue of the conduit system is the size exclusion, which is set at approximately 70 kDa in the spleen (Nolte et al., 2003). Molecules or particles with a larger size cannot enter the conduit and be distributed.

Because all the FRC are connected, transport of molecules through these tubes over larger distances can be rapidly achieved. In the lymph node, it has been established that this conduit system acts as a messenger system to transport signal molecules like chemokines and cytokines from the draining region of the lymph node to the port of entry for lymphocytes, the HEV. Chemokines produced at a site of inflammation would reach the lymph node with the afferent lymph and quickly be transported to the HEVs via the conduit system. The conduit system ends in a perivenular region surrounding the venule and it is assumed that the chemokines can be transported to the lumen of the vessel by active transcytosis and presented there to attract blood-borne cells, such as lymphocytes, monocytes, or even granulocytes, to enter the node depending on the types of transported chemokine. In the spleen, a similar function of the conduit system can be envisaged, again with the difference that lymphocyte exit from the blood directly into the T cell zone has never been demonstrated. Nevertheless, an effect of small regulatory molecules on the regulation of blood distribution between marginal zone and red pulp may can be envisaged.

Although the majority of ECM products and fibers are inside the fibers, it has recently been shown that a substantial amount of matrix proteins such as laminin, fibronectin, collagen IV, and tenascin were also present on the outer surface (Sobocinski et al., 2010). These proteins can act as anchorage sites for lymphocyte to crawl along the fibers and also function as docking molecules for chemokines.

FRC also actively produce the homeostatic chemokines CCL19 and CCL21, which are ligands for CCR7 present on naïve T cells (Luther et al., 2000). These chemokines function to keep the T cells in an active migratory state, whereby they continuously move along the FRC and associated DCs. CCL19 is also involved in the homeostasis of T lymphocytes, together with the survival factor IL-7, which is also produced by the FRC (Link et al., 2007). Recently, CXCL12 was shown to be localized on FRC and involved in the migration of plasmacytoid DCs into the white pulp (Umemoto et al., 2012). In addition, podoplanin expressing stromal cells of the T cell zone stimulated LTi survival in an IL-7-independent manner (Hou et al., 2010). Furthermore, a recent study indicated a direct stimulatory role for fibroblastic reticular cells in the activation of cytotoxic T cells during viral infection via the production of the alarmin IL-33 (Bonilla et al., 2012).

In the B cell follicles, an important stromal cell can be identified which is central for B cell immunity, the FDCs. When B cell follicles develop during ontogeny the reticular framework of FRC is replaced by a less dense network of FDCs. This replacement is not complete and a conduit system still exists in adult B cell follicles where it provides an efficient mechanism for delivery of small antigens and chemokines such as CXCL13 to B cells that are in direct contact with the conduits (Roozendaal et al., 2009). Larger antigens and complexes are transported into the follicles by macrophages. In addition, it has been shown that marginal zone B cells can shuttle between the marginal zone and follicles using a combination of the chemokine receptor CXCR5 and the sphingosine 1-phosphate receptors S1P1 and S1P3 (Cinamon et al., 2008). This is regarded as an additional mechanism for systemic antigen capture and delivery to FDCs and may not involve migration via the bridging channels. FDCs trap and present antigen to B cells via the binding of immune complexes to complement receptors 1 and 2 and $Fc_{\gamma}RIIb$ (El Shikh et al., 2010). In addition, they provide survival and proliferation signals to both naïve B cells and germinal center B cells and thereby are necessary for the integrity of both primary B cell follicles and germinal centers (Wang et al., 2011). The factors secreted by FDCs include BAFF (Gorelik et al., 2003), IL-6, and the chemokine CXCL13 to attract B cells (Ansel et al., 2000). FDC generation is dependent on LTα1β2 and TNFα expression by B cells and there are no differences reported between lymph node and splenic FDCs.

OTHER STROMAL CELLS IN THE SPLEEN

With minor local differences the general picture of the stromal network of the lymphoid compartment as an important scaffold in the T and B cell zones is quite comparable between spleen and lymph nodes. In addition, the spleen contains the red pulp. This compartment has unique venous sinuses in which the lining endothelial cells are positioned in parallel way. The endothelial cells contain stress fibers that run along the long axis of the endothelial cells and which are attached to annular fibers that run around the sinuses (Mebius and Kraal, 2005). Contraction of the stress fibers leads to the formation of small slits between the endothelial through which red blood cells can leave the sinuses for the venous system of the spleen. Membrane stiffening as a result of aging will eventually prevent erythrocytes from leaving the sinuses and will result in elimination by macrophages present in these sinuses. For the human spleen is has been suggested that endothelium-derived littoral cells are important for this process of filtration and elimination (Ogembo et al., 2012).

In addition to the presence of these red pulp macrophages with their important function in erythrocyte turnover and iron metabolism, the spleen is also a reservoir of macrophage precursors, monocytes (Swirski et al., 2009), which are thought to reside in the red pulp. This monocyte depot can play an important role under circumstances of acute inflammation, where rapid recruitment of monocytes leads to improved clinical outcome, as has been demonstrated in a model of acute myocardial infarction (Leuschner et al., 2012). Interestingly, in a mouse tumor model it has recently been demonstrated that the spleen also harbors neutrophil precursor cells and that both the monocyte and neutrophil reservoir can lead to tumor progression by infiltrating the tumor (Cortez-Retamozo et al., 2012). The recruitment of the cells from the spleen was mediated by the chemokine receptor CCR2. Based on histology, it was deduced that the myeloid progenitor

cells were located in the red pulp of the spleen. Hematopoietic niches have been described in red pulp sinuses, closely associated with endothelial cells (Kiel et al., 2005), but how exactly the maintenance of myeloid progenitor cells and extramedullary hematopoiesis is supported by stromal cells in the red pulp needs further study.

The red pulp is also an important site for the localization of effector cells that have been generated in the white pulp or have migrated from other lymphoid organs. In particular, plasma cells are retained in large numbers in the red pulp. It is clear that this position enables a rapid transport of the secreted immunoglobulins into the blood and subsequently throughout the body.

During their differentiation plasma cells show increased chemotactic sensitivity to the CXCR4 ligand CXCL12 and exhibit reduced responsiveness to the B and T zone chemokines CXCL13, CCL19, and CCL21. The CXCR4 ligand CXCL12 is expressed within splenic red pulp, indicating that as B cells differentiate into plasma cells they undergo a change in chemokine responsiveness that promotes their retention in the red pulp (Ellyard et al., 2005; Hargreaves et al., 2001). In a study on human stromal cells, it was shown that stromal cells that expressed CXCL12 were able to produce IL-6, an important factor for antibody production, and expressed the adhesion molecule CD54, which is a ligand for CD11a, which is found on plasma cells (Ellyard et al., 2005).

CONCLUDING REMARKS

Both in the white pulp and in the red pulp of the spleen different stromal cells make up the basic framework of the respective compartments. Through differential production of chemokines and growth factors stromal cells can form local niches that are essential for the maintenance and function of a variety of cell types. In the white pulp, these niches are essential for the generation of immune responses. In the red pulp, stromal cells are involved in the localization of effector cells such as plasma cells, but they have also been suggested to be central to the support of myeloid progenitor cells. Together, the data emphasize the important role of various stromal cells in creating microenvironments with highly specific functions in lymphoid organs. Future work is needed to further phenotype the different stromal cell subsets that mediate these specific functions, further allowing the identification of the different molecules involved in these processes.

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Application of tissue engineering to the immune system: development of artificial lymph nodes

Tom Cupedo[1]*, Abraham Stroock[2] and Mark Coles[3]*

[1] *Department of Hematology, Erasmus University Medical Center, Rotterdam, Netherlands*
[2] *School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY, USA*
[3] *Centre for Immunology and Infection, Department of Biology and Hull York Medical School, University of York, York, UK*

Edited by:
Burkhard Ludewig, Cantonal Hospital St. Gallen, Switzerland

Reviewed by:
Joern Dengjel, Albert-Ludwigs-Universität Freiburg, Germany
Henrique Veiga-Fernandes, Instituto de Medicina Molecular, Portugal

****Correspondence:***
Mark Coles, Centre for Immunology and Infection, Department of Biology and Hull York Medical School, University of York, York YO10 5DD, UK.
e-mail: mark.coles@york.ac.uk;
Tom Cupedo, Erasmus University Medical Center, Department of Hematology, PO Box 2040, 3000 CA Rotterdam, Netherlands.
e-mail: t.cupedo@erasmusmc.nl

The goal of tissue engineering and regenerative medicine is to develop synthetic versions of human organs for transplantation, *in vitro* toxicology testing and to understand basic mechanisms of organ function. A variety of different approaches have been utilized to replicate the microenvironments found in lymph nodes including the use of a variety of different bio-materials, culture systems, and the application of different cell types to replicate stromal networks found *in vivo*. Although no system engineered so far can fully replicate lymph node function, progress has been made in the development of microenvironments that can promote the initiation of protective immune responses. In this review we will explore the different approaches utilized to recreate lymph node microenvironments and the technical challenges required to recreate a fully functional immune system *in vitro*.

Keywords: lymph node, tissue engineering, stroma, bio-materials, extracellular matrix

OVERVIEW OF LYMPH NODE STRUCTURE

Immune responses in mammals are coordinated from secondary lymphoid organs (SLOs) that are positioned at strategic locations throughout the body. Naïve T and B cells continuously pass through SLO while navigating the bloodstream. Within the SLO, T and B cells scan for the presence of their cognate antigen. In the absence of antigen recognition, cells receive homeostatic survival signals allowing them to continue their journey to the next SLO. On the other hand, when T or B cells do recognize antigen, the SLOs provide an optimal environment for cellular activation, proliferation, and selection for high affinity antibodies.

To facilitate optimal lymphocyte activation, SLOs are compartmentalized into distinct cellular micro-domains that are colonized almost exclusively by either T or B cells (Crivellato et al., 2004). The generation and maintenance of B cell follicle and the T cell zone is critically dependent on cytokines, adhesion molecules, and extracellular matrix proteins made by non-hematopoietic stromal cells (Cyster, 2005; van de Pavert and Mebius, 2010). A thorough understanding of the non-hematopoietic microenvironment of SLO is therefore indispensable for lymphoid organ tissue-engineering (Mueller and Germain, 2009).

MESENCHYMAL STROMAL CELLS

Three major subsets of functionally distinct stromal cells of mesenchymal origin are currently recognized in human and mouse lymph nodes.

T ZONE RETICULAR CELLS

T zone reticular cells [TRC, also known as fibroblastic reticular cells (FRCs)] are the major mesenchymal stromal population in the T cell areas of the lymph nodes, located at the medullary side of the B cells follicles (Kaldjian et al., 2001; Katakai et al., 2004a; Link et al., 2007). Phenotypic, TRC are characterized by expression of podoplanin (gp38, D240) alpha-smooth muscle actin (αSMA) and the production of extracellular matrix components recognized by the antibody ERTR7 (Van Vliet et al., 1984). On a functional level, TRC secrete the homeostatic chemokines CCL21 and CCL19 which act on naïve T cells and dendritic cells (DCs), respectively (Link et al., 2007). Structurally, TRC provide a scaffold to which DC that came in through the lymph are anchored and which is used by naïve T cells to navigate the lymph node in search for DC (Bajénoff et al., 2006).

FOLLICULAR DENDRITIC CELLS

Follicular dendritic cells (FDCs) are located within primary B cell follicles in resting lymph nodes and in the light zone of germinal centers during immune responses (Chen et al., 1978). In spite of their name, FDCs are not DCs and do not possess antigen processing machinery but rather are radio-resistant stromal cells of mesenchymal origin (Endres et al., 1999; Krautler et al., 2012). FDCs are decorated by complement receptors and Fc receptors and they use these to present unprocessed immune complexes to B cells during germinal center reactions, when B cells that have undergone somatic hypermutation test newly generated receptors for improved affinity (Kosco-Vilbois and Scheidegger, 1995). In addition, FDCs secrete the B cell chemoattractant CXCL13 that is essential to maintain the structural integrity of the B cell follicle under homeostatic conditions (Cyster et al., 2000; Allen and Cyster, 2008; Suzuki et al., 2010; Wang et al., 2011).

MARGINAL RETICULAR CELLS

The third major mesenchymal cell type in lymph nodes are marginal reticular cells (MRCs), which are found in the outer cortex, directly underneath the subcapsular sinus (Katakai et al., 2008). MRCs have been identified in both mouse (Katakai et al., 2008) and human (T. Cupedo and M. Coles, unpublished data) lymph nodes. Phenotypically MRCs resemble so-called lymphoid tissue organizer (LTo) cells that are responsible for lymph node development in the embryo (Cupedo et al., 2004; Katakai et al., 2008; Katakai, 2012). Adult lymph node MRCs express RANKL, VCAM-1, MAdCAM-1, and podoplanin and they secrete homeostatic chemokines (Katakai et al., 2008). The physiological importance of MRCs in lymph node functioning remains in large parts to be elucidated, but these cells were shown to be involved in the shuttling of antigens from the marginal sinus to the B cell follicles (Roozendaal et al., 2009).

ENDOTHELIAL CELLS

There are two major types of vascular network that provide nutrients, oxygen, and cellular input and output from the lymph node.

BLOOD ENDOTHELIAL CELLS

Blood vessels enter and exit the lymph node at the medullary hilus area. In the paracortical T cell zone, the smaller venules take on a characteristic cubical appearance and are known as high endothelial venules (HEV; Miyasaka and Tanaka, 2004). These HEV are the main points of entry for naïve T and B lymphocytes. They express adhesion molecules and addressins and are decorated with stromal cell-derived chemokines. In the absence of functional HEV, recirculation of naïve lymphocytes, and thus immune surveillance, are severely impaired (von Andrian and Mempel, 2003).

LYMPHATIC ENDOTHELIAL CELLS

Lymphatic endothelium is recognized by the characteristic expression of the receptor Lyve-1 (Banerji et al., 1999). Afferent lymphatic vessels originating in peripheral tissues carry lymph to draining lymph nodes (Oliver and Alitalo, 2005). This lymph also contains free as well as cell-bound antigens. The lymph vessels end in the subcapsular sinus, a lymphatic sinus that is located directly underneath the lymph node capsule (McMaster and Hudack, 1935; Oliver and Alitalo, 2005). From the sinus, larger antigens (>70 kDa) can be captured by lymph node-resident subcapsular macrophages or are carried by migratory antigen-presenting cells including DCs and neutrophils into the organ (Junt et al., 2007), smaller antigens can enter by diffusion through the conduit network and be captured by antigen presenting cells in the lymph node (Carroll and Isenman, 2012). Efferent lymphatic vessels originate in the lymph node hillus and allow cells to leave the organ and re-enter the blood stream or travel through the lymph to a subsequent lymph node (Forster et al., 2012).

CAPTURING THE STRUCTURE AND COMPLEXITY OF A LYMPH NODE

Despite their relative modest size, bio-engineering a lymph node presents a formidable challenge due to the high level of complexity resulting from the large variety of cell types, the highly organized stromal and lymphoid structure, the rapid cellular motility of lymphocytes and DCs and the staggering cell density (key features summarized in **Figure 1**). The multitude of cell types and the scale of the engineering, with complex changes in microenvironments occurring within a few microns, make engineering a lymph node more complex than the engineering of larger organs like the heart or kidneys. From immunohistological and

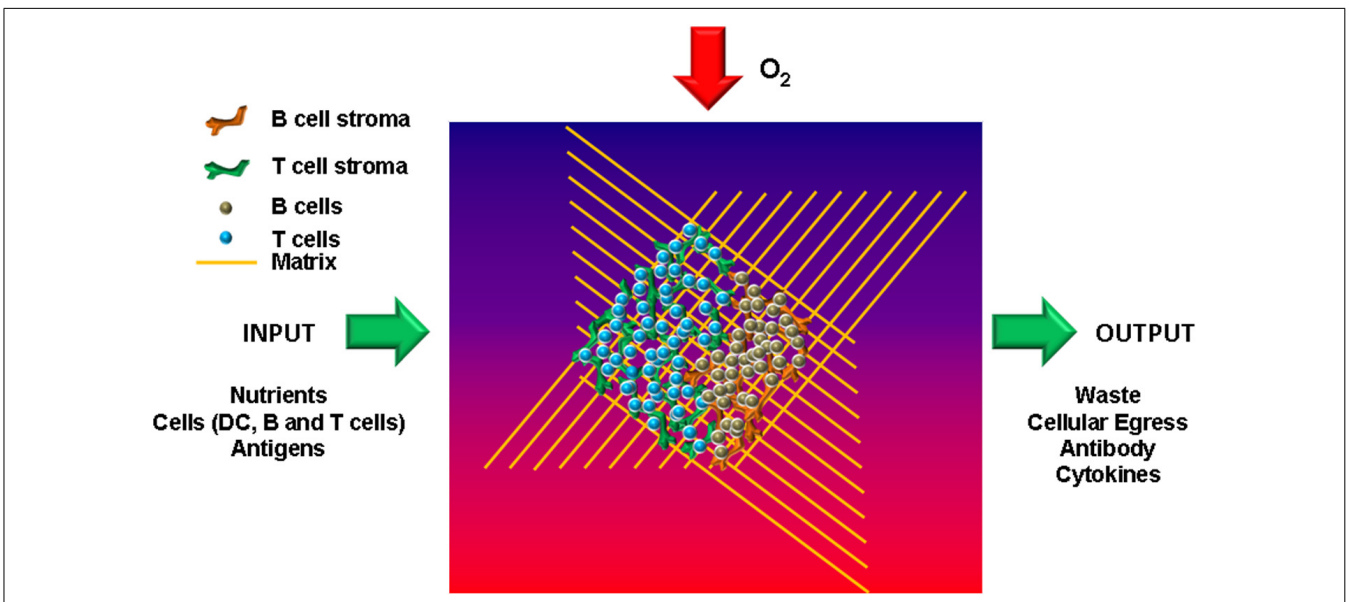

FIGURE 1 | Key features of an artificial lymph node. To recreate functional lymph nodes *in vitro* several key constituents need to be incorporated. These include specific stromal cells for T and B areas in order to generate the typical microarchitecture. In addition, nutrients, oxygen, and antigens need to be able to enter the organoid, while functional end products such as antibodies and cytokines should be able to be extracted from the organ.

flow cytometric measurements it can be estimated that within 1 mm^3 of lymph node tissue approximately 1 to 2 $\times$ 10^6 cells reside, accounting for a cell density of about one billion cells/ml (our unpublished data). Within this cell mass highly organized lymphatics, marginal reticular zone stroma, T cell zone stroma, multiple B cell follicles, vascular networks and medullary regions containing plasma cells and macrophages are present which are all highly different localized microenvironments (Crivellato et al., 2004). Even in extremely high dense tissue culture, maximum cell densities are in the range of 5 to 10 $\times$ 10^6 cells/ml, which is two orders of magnitude less dense, indicating that there is hardly any liquid phase within the lymph node parenchyma just a compact cellular environment where cellular interactions and cell–extracellular matrix interactions dictate rapid cell migration. The lack of a liquid interface indicates that free diffusion of cytokines, chemokines, and growth factors is unlikely to occur by a simple gradient as observed *in vitro* assay systems and rather is controlled through direct contact with the stromal network that produce homeostatic chemokines and cytokines, sequestration of these factors by extracellular matrix and by the rapid motility of cytokine-secreting activated lymphocytes in the lymph node. All of these effects indicate that vast differences exist between to the biology measured in a culture dish and the biology operational *in vivo* that needs to be recapitulated in the bio-engineered lymph node.

SOURCE OF MESENCHYME

Stromal cells provide the structural basis for both lymph node structure and function and their faithful incorporation into an *in vitro* system is thus a necessity for creation of a functional artificial lymph node environment. A prerequisite for lymph node modeling is therefore the establishment of mesenchymal cell cultures that can take on the function of lymph node stromal cells found *in vivo*. Unlike most other organs that involve layers of epithelium and fibroblasts or endothelium and muscle cells, the lymph node stroma contains an open lattice network of different types of mesenchymal stromal cells that permits rapid motility of immune cells.

There are three main practical approaches that can be utilized to generate mesenchymal cells for use in tissue engineering: (1) the application of immortal fibroblastic cell lines from a variety of different tissues (Suematsu and Watanabe, 2004; Tomei et al., 2009), (2) the use of primary stromal cells isolated from lymph nodes (Tomei et al., 2009), and (3) the differentiation of lymph node stroma from mesenchymal progenitor cells (Benezech et al., 2012; Zheng et al., 2012). Although fibroblastic cell lines from non-lymphoid tissues are easy to culture they are unlikely to mirror the gene expression pattern found in FRCs or FDCs. The culture of primary stromal cells from lymph nodes has been challenging, and this has only been successfully accomplished by a few groups (Katakai et al., 2004b; Onder et al., 2012). Prolonged culture of these cells has led to a loss of phenotype requiring supplement of the cells with exogenous cytokines and chemokines (Suto et al., 2009). Differentiation of lymphoid stromal subsets from progenitor cells would be the most ideal method to acquire lymphoid stromal cells, but reproducible protocols have not yet been developed.

THE OXYGEN CHALLENGE

Vascular networks are both the site of cellular input and egress from lymph nodes but more importantly they provide the high levels of oxygen and nutrients required by highly motile immune cells. Any structure larger than 80–100 μm will become highly hypoxic in the absence of oxygen supply and this problem is amplified in lymph nodes, which have highly motile cells and have a cell density of one billion cells/ml (our unpublished data). One of the key engineering challenges in developing an artificial lymph node is providing an environment that can support the oxygen need of the lymph node environment. Possible scenarios include limiting the organ size, lowering the cell density, or providing artificial blood vascular networks or hollow fibers for gas diffusion and nutrient supply (Zheng et al., 2012). Artificial vascular beds can be generated through the formation of microfluidic networks in bio-compatible gels. These networks can be seeded with endothelial cells and vascular smooth muscle cells leading to the development of vascular networks that can support vascular sprouting and would lead to the incorporation of a highly structured vasculature *in vitro* (Zheng et al., 2012). Even though including functional vascular networks into engineered organs will be technically challenging, incorporation of the efficient transport of oxygen and nutrients into the organoid is essential for capturing normal lymph node function.

TOWARD 3D CULTURE

A variety of different cellular approaches have been used by bioengineers to recreate tissues and organs *in vitro*. These include three-dimensional (3D) culture of cells in extracellular matrix or on artificial materials (Tomei et al., 2009), the formation of spheroids (Lin and Chang, 2008), use of de-cellularized tissues (Mirsadraee et al., 2006), and cell printing (Guillotin and Guillemot, 2011). Culturing of cells in a 3D environment for tissue engineering and regenerative medicine has been performed on a number of different types of materials all with their inherent advantages and disadvantages.

Non-biological materials include sponges, synthetic polymers and peptide hydrogels, electrospun polycarbonate fibers, and synthetic multilayer surfaces.

Electrospun fibers and multilayer surfaces provide a 3D surface that permits attachment and growth of stromal cells in 3D (Holmes et al., 2012). The disadvantage of this technology is that in essence it is a 3D environment where cells grow in 2D, attached to an inert material, leading to very different interactions between stromal cells as they are not forming a 3D self-supporting network. Moreover, interactions with lymphocytes and DCs will also be fundamentally different from those observed *in vivo* as they will interact only with non-adhered surface of stromal cells. Alternative synthetic matrices include sponges and polymer hydrogels (Tibbitt and Anseth, 2009). Depending on the pore size, sponges are essentially like electrospun polycarbonate, providing a mere artificial substrate for cell growth. Polymer hydrogels form into solid gels through the exclusion of water and condensation of polymer, leading to the encapsulation of cells into the hydrogel rather than the formation of 3D networks. These types of gels work extremely well for cell types such as chondrocytes but are

likely to be of limited application for engineering lymph node environments.

Biological materials including agarose, laminin, Matrigel, fibrin, and collagen gels are all commonly used in tissue engineering. Agarose is an inexpensive material that is commonly used to culture hematopoietic colonies. Its advantages are the ability to be formed into a variety of different tensile strengths and agarose gels can be used to induce a certain level of cellular organization. A major disadvantage of agarose is the fact that it cannot be biologically restructured during culture, this restricts the mobility of cells and it is technically challenging to extract cells for analysis, limiting its application. Matrigel is a mix of laminin, fibronectin, and collagen of an unknown constituency and is commonly used in tissue engineering and tumor metastasis assays (Hughes et al., 2010). Matrigel is secreted by a mouse tumor cell line and probably represents a type of extracellular matrix normally deposited in tumor microenvironments and surrounding blood vasculature. Despite its popularity, Matrigel cannot be easily remodeled by cells, and migration through Matrigel usually involves digestion of the matrix by matrix metalloproteinase proteins (MMPs), rather then active squeezing and crawling of cells through the matrix (Lammermann et al., 2008). It is also very different in constituency to the type of extracellular matrix found in lymph nodes. Collagen gels are commonly used in a variety of tissue engineering applications and different types of collagens are important components of extracellular matrix in a number of organs including lymph nodes. Collagen I bundles form the flexible outer capsule of the lymph node and the conduits that connect lymphatics to the HEVs providing structural support for the stromal cell network and channels for movement of factors and low molecular weight antigens into the parenchyma of the lymph node (Lammermann et al., 2008). Collagen IV is secreted by TRC and forms a layer of extracellular matrix around the these cells (Sixt et al., 2005). To date, there is no evidence that this matrix deposition is important to the structural integrity of the lymph node. Collagen is a highly bio-compatible material and has been applied to a number of different tissue engineering situations but is easily contracted by cells when they apply force to the collagen gel and is more challenging to work with due to its relative low tensile strength in comparison to other biomaterials (Cross et al., 2010).

Alternative tissue engineering approaches used to form 3D organ-like cultures include spheroids which have been successfully utilized to study bone biology, endothelial cell outgrowths, and the formation of breast and prostate tissues (Villadsen et al., 2007). Spheroids are formed through the culture of cells in methyl cellulose in hanging droplets, inducing the formation of a compact tissue that can take on a higher degree of organization reminiscent of the *in vivo* organ. Although this approach has worked well for a variety of different tissues, the technology has to date not been utilized for the formation of artificial lymph node microenvironments. The main reason being that spheroids form into a very compact cell aggregate which is fundamentally different from the open lattice stromal cell network found in LNs.

Cell printing and encapsulation technology have been used to form vascular networks and 3D bone structures (Fedorovich et al., 2011). However, this technology lacks the resolution to be of application when generating a complex structure like a lymph node.

Capturing the complex structure of lymph nodes with separate B and T cells zones is challenging. Most work to date has not tried to impose structure upon the bio-engineered tissues rather it has relied upon self-organization. In developing lymph node B cells form into B cell follicles guided by a complex system of stromal-derived chemokines (Mebius, 2003). Recapitulating the appropriate signal *in vitro* will be essential for achieving organized artificial lymphoid tissues.

CURRENT *IN VITRO* MODELS OF LYMPH NODE MICROENVIRONMENTS

To recreate the lymph node environment a number of different approaches have been utilized, and we will highlight several of those here. For a more comprehensive overview of the existing models see Kobayashi et al. (2011).

COLLAGEN/POLYURETHANE MATRIX

By combining type I collagen with polyurethane a composite matrix was generated that provided sufficient tensile strength to counteract the contraction induced by the stromal cells seeded in the matrix. Using immortalized TRC lines, it was convincingly shown that these cells not only behave totally different in 3D compared to 2D, but most importantly that culturing the matrix under flow induced structural remodeling and production of chemokines (Tomei et al., 2009).

BIOREACTOR

In order to recreate immunological interactions rather then to faithfully rebuild the lymph node structure, progress has been made using membrane-based perfusion systems that can be seeded with several types of matrices. After loading the bioreactor with DCs and lymphocytes the production of inflammatory cytokines as well as tissue architecture could be assessed (Giese et al., 2006, 2010).

COLLAGEN SPONGE

The best results in terms of immune responses have been achieved using collagen sponges, seeded with stromal cells and DC and subsequently transplanted in mice. Even though these sponges attract and organize B cells, form germinal centers and lead to plasma cell differentiation in a very efficient manner, they fall beyond the scope of this review as most of the these features are achieved *in vivo* (Suematsu and Watanabe, 2004; Okamoto et al., 2007).

FUTURE DIRECTIONS AND CHALLENGES

A number of factors have limited the capacity to generate artificial lymph nodes these include the size, the development of hypoxia in larger tissues, and the engineering complexity of the lymph node. One of the important engineering challenges will be to recreate a compact environment *in vitro* complete with stromal networks and to maintain cell motility, behavior, and function. This will require developing tissue engineering on an immensely small scale, the development of techniques to form stromal networks from stem cell precursors, and methods to culture cells in very high densities. So far a number of groups have shown that this can be

done *in vivo* and some of the complexity has been modeled *in vitro* using custom-designed bioreactors. Advancement in bioreactor development and the culture of stromal cells provides a platform for advancements in this bioengineering challenge.

CONCLUSION

Over the last 15 years bio-engineers have developed novel artificial organs that have started to appear in the clinic, one of the great challenges left is to successfully develop an artificial immune system that can reproduce complex immune responses *in vitro*. Through designing and engineering artificial lymph nodes immunologists will develop a tool that could be used to dissect the molecular and biophysical mechanisms controlling human immune responses in health and disease.

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