Dendritic cells in remodeling of lymph nodes during immune responses

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Summary

A critical hallmark of adaptive immune responses is the rapid and extensive expansion of lymph nodes. During this process, the complex internal structure of the organs is maintained revealing the existence of mechanisms able to balance lymph node integrity with structural flexibility. This article reviews the extensive architectural remodeling that occurs within lymph nodes during adaptive immune responses and how it is regulated by dendritic cells (DCs). In particular we focus on previously unappreciated functions of DCs in coordinating remodeling of lymph node vasculature, expansion of the fibroblastic reticular network and maintenance of lymphoid stromal phenotypes. Our increased understanding of these processes indicates that DCs need to be viewed not only as key antigen-presenting cells for lymphocytes but also as broad-acting immune sentinels that convey signals to lymphoid organ stroma and thereby facilitate immune response initiation at multiple levels.

Key words: lymph node, inflammation, stroma, dendritic cells, fibroblastic reticular cells, follicular dendritic cells, high endothelial venule,

1. Introduction

Lymph nodes act as pathogen filters, sites of encounter between antigen-presenting cells and lymphocytes and as environments for clonal expansion of antigen-specific T and B cells (1, 2). Key to all these processes is the architecture of the lymph node, which is supported by a complex interconnecting scaffold of non-haematopoetic stromal cells, including specialised fibroblasts, lymphatic endothelial cells and blood endothelial cells (1, 3). In addition to providing the physical infrastructure, stromal subsets help define different territories within lymph nodes. T cell areas contain fibroblastic reticular cells (FRCs) that account for 30-50% of total lymph node stroma and stretch throughout the paracortex forming a complex interconnected cellular network (1-3). FRCs produce the CCR7 ligands, CCL19 and CCL21, that attract T cells and promote their migration, as well as IL-7, which acts as a T cell trophic signal (4, 5). B cell follicles are supported by follicular dendritic cells (FDCs), a rarer stromal cell subset that produces CXCL13 along with growth factors such as BAFF necessary for the proliferation and maintenance of B cells (6). Additionally present throughout the node are specialized blood vessels, termed high endothelial venules (HEVs), that support the immigration of T and B cells from the bloodstream and respond to inflammatory stimuli to increase such traffic (7). After entry into the lymph node via HEVs, lymphocytes utilise the stromal cell networks to migrate to their correct anatomical location and to meet antigen-presenting cells (2).

Dendritic cells (DCs) are the classical antigen-presenting cells in lymphoid organs (8). Some DCs are constitutively resident in those organs and

differentiate locally from a blood-borne precursor (9, 10). Other DCs are termed migratory and represent lymph-borne immigrants from peripheral tissues (11). DC migration happens constitutively but is markedly accelerated after infection or inflammation (8, 11). Within the lymph node, immigrant DCs bearing antigens acquired in the periphery, or resident DCs presenting antigens acquired from afferent lymph, become strategically positioned on the stromal network for scanning by naïve T cells (12). T cells that encounter foreign antigen, together with appropriate co-signals, proliferate, differentiate and home to peripheral tissues in order to eliminate the challenge (8).

One of the hallmarks of the immune reaction is massive lymph node expansion, with organ cellularity and physical size increasing 5-10 fold following immunisation or infection (1) It is becoming increasingly clear that DCs play a key role in this process by conveying key signals to stromal components that are required to initiate the remodeling of lymph node architecture and to support organ expansion. Thus, DCs not only deliver foreign antigens and promote activation and clonal expansion of T cells but also modify the lymph node environment to support that expansion. Here we specifically discuss the role of DCs in lymph node architecture and in its remodelling at the outset of immune responses.

2. DCs in antigen presentation to T cells

An adaptive immune response requires T cells to become stimulated in response to foreign antigens processed and presented by DCs. Sessile DCs present in tissues throughout the body continuously monitor their microenvironment for signs of tissue damage or infection. In response to

these signs, DCs become motile and traffic to lymph nodes (8, 11, 13). A key molecule upregulated during this transition is the chemokine receptor CCR7. CCR7 has long been accepted as the critical receptor for entry of DCs into lymph nodes (14) but is now also known to be important for the mobilization of DCs within the tissues and for supporting their haptotactic migration towards afferent lymphatics at the start of their journey (15). Whilst CCR7 undoubtedly controls the directional migration of DCs, our understanding of mechanisms controlling DC entry into lymph nodes has been recently expanded to include additional layers of complexity. The C-type lectin CLEC-2, whose expression on DCs is greatly increased following exposure to inflammatory stimuli (16-18), has been shown to help promote the migratory behavior of the cells (16). CLEC-2 interacts with the glycoprotein ligand podoplanin (PDPN), also known as gp38, which is constitutively expressed on lymphatic endothelium and fibroblastic reticular cells (3, 19), on which DCs crawl en route to and within the lymph node paracortex (20). Triggering of CLEC-2 by PDPN promotes formation of actin-rich protrusions in DCs, via the activation of Vav and Rac1 (16). These protrusions allow DCs to spread along stromal scaffolds and support DC motility (16).

Another mechanism controlling lymph node entry of DCs operates at the level of the subcapsular sinus and involves the atypical chemokine receptor CCRL1 (21). CCRL1 is expressed by lymphatic endothelial cells that line the celling but not the floor of the subcapsular sinus (21). CCRL1 binds, sequesters and induces the degradation of CCL21 and CCL19, creating a gradient (21). This allowing incoming antigen-laden DCs to keep moving towards the inner areas of the node an provides an elegant explanation as to how they reach the

paracortex to find and prime naïve T cells.

Lymph arriving at lymph nodes via afferent lymphatic vessels flows beneath the capsule in the subcapsular sinus. Its ability to carry microbial debris and live organisms means that it can serve a rich source of antigens emanating from sites of infection. It had been suggested that DCs resident in the lymph node can acquire small soluble antigens arriving via the conduit network (see below) (22, 23). More recently, it has been found that a subset of lymph node-resident DCs can also directly sample the contents of the subcapsular sinus and thereby acquire even large size pathogen material such as bacteria or viruses that drain from sites of infection via the afferent lymph (24). Antigen acquisition by lymph node-resident DCs allows for rapid initiation of T cell responses to pathogens and complements antigen presentation by migratory DCs.

3. DCs in the filtering function of lymph nodes

In addition to acting as sites of antigen presentation, lymph nodes also act as filters for lymph draining from tissue. The inner sinus lining acts as a physical barrier between the lymph and the rest of the node. Migratory DCs and activated lymphocytes actively migrate through this barrier in order to reach the inner areas of the node. In contrast, soluble factors such as chemokines and cytokines that are sufficiently small (<70kDa) are permitted to flow through the lymph node confined within a network of conduits (25). Conduits are essentially small interconnecting tubes constructed out of collagen and other basement membrane components (23, 25, 26) excreted by FRCs. These extracellular matrix components are then bundled and held together by

the FRCs wrapping their cell bodies around them (19, 23). Conduit size exclusion is controlled by a specialised lymphatic endothelial cell population that lines the celling and floor of the sinus and that expresses the glycoprotein PLVAP (27). PLVAP allows these endothelial cells to forms diaphragms, effectively closing off the underlying conduit network to large molecules (27). There is also evidence that $PLVAP^+$ channels serve as a route for transmigrating leukocytes arriving via the afferent lymph as lymph nodes from mice lacking PLVAP become severely distorted and display an accumulation of activated B cells in enlarged follicles and a reduction in T cells (27).

If afferent lymph can carry live microbes after infection, how are they prevented from disseminating systemically? The $PLVAP^+$ cells and the conduit system act as one barrier. Another key barrier operates at the level of the subcapsular sinus where specialised $CD169^+$ macrophages take up bacteria and viruses and destroy them (28, 29) In addition $CD169^+$ macrophages orchestrate an innate immune response that reinforces the subcapsular sinus “firewall” and contributes to preventing pathogen dissemination (30). Finally, subcapsular sinus macrophages also serve as antigen-presenting cells for B cells and transfer antigens captured from the incoming afferent lymph to B cells that residing in follicles just beneath the sinus (28, 31, 32). Notably, it has recently been found that the transfer of antigen to B cells is aided by a process of macrophage migration into the follicles, which is triggered by the arrival of immigrant DCs (33). However, large scale macrophage re-localisation causes temporary loss of the protective macrophage layer, which, in mice, can take 28 days to be reinstated (33). During this period, subcapsular sinus firewall integrity and

further B cell responses are compromised, resulting in vulnerability to secondary infection (33).

4. DCs in maintenance and growth of the lymph node vascular network

Lymphocytes continuously recirculate between the blood and the lymph nodes {Lammermann:2008bk, Young:1999ww}. Key to this process are HEVs, specialized parts of the vasculature that favour lymphocyte egress from the circulation and serve as the ports of lymphocyte entry into nodes (7). It is now clear that DCs are important regulators of the conversion of blood endothelial cells into HEV cells. Close contact between lymph node resident DCs and HEVs and signaling via LTβR maintains the expression of specialized HEV markers such as GLYCAM1 even though others, such as CD31 and VE-cadherin, are DC-independent (35). Notably, in the absence of DCs, the HEV phenotype is lost and the endothelium reverts to an immature phenotype unable to support T and B cell trafficking (35).

During lymph node expansion, growth of the HEV network is a required and important step to provide additional entry points for circulating lymphocytes. In the absence of vascular growth, lymph nodes expansion is significantly attenuated (36). Like maintenance, endothelial proliferation is dependent on DCs and other myeloid cell populations. The stimulatory effect of DCs on vascular growth is so pronounced that transfer of DCs is sufficient to promote HEV proliferation (36). The signal for vascular proliferation is received via VEGFR engagement by VEGF. Dendritic cells are however not the source of this growth factor. Through the use of VEGF LacZ reporter mice and mRNA analysis of lymph node stromal cell populations, it was shown that it is in fact

FRCs that express VEGF to provide the proliferative cue (37). Some FRCs ensheath HEVs and behave as a specialised pericyte population that remains connected to the FRC network. DCs closely associate with the FRC network and provide ligands for lymphotoxin-β receptor, the signaling of which leads to the production of VEGF by FRCs (38, 39). This DC-FRC communication appears to be direct as DCs are able to elicit the same effect on fibroblast cell lines in coculture experiments *in vitro* (38). There are further reports that support the notion that the presence of DCs controls lymph node size. A recent study of found that expression of diphtheria toxin receptor in DCs can lead to an unexpected dearth of these cells, in particular of the migratory subtype, in lymph nodes (40). While the reason for this observation remains unclear, it is correlated in all cases with lymph node hypocellularity (40). One potential explanation is the lack of lymphotoxin-β receptor ligands and a failure of DCs to regulate levels of VEGF produced by FRCs, thereby reducing the vascular network and the number of entry sites for lymphocytes.

Permitting large numbers of lymphocytes to enter lymph nodes following inflammation appears at odds with the ability of HEVs to maintain an effective barrier between the blood and the lymphoid tissue. One solution to these seemingly contradictory functions is again found in the crosstalk between FRCs and the endothelium (*Fig. 1*). In inflamed lymph nodes, at the same time as numerous lymphocytes traffic through HEVs, platelets also leak from the blood into the perivascular space. Here, they immediately encounter FRCs that ensheath the endothelium. The FRCs in this space, similar to FRCs through the rest of the network, express high levels of PDPN that triggers CLEC-2, which is constitutively expressed at high levels on platelets

(41) CLEC-2 signalling via the kinase Syk leads to the release of sphingosine-1-phosphate (S1P) from platelets, which acts on the endothelium to induce upregulation of VE-cadherin, a critical junctional protein (42). The upregulation of VE-cadherin is sufficient to reinforce the endothelial cell-cell junctions and maintain the barrier function of the HEV (42), preventing further leakage of blood components into the tissue. In genetic models of ablation of either PDPN from FRCs or CLEC-2 from platelets, severe bleeding within the lymph nodes occurred, a defect which could be rescued by the transfusion of CLEC-2-sufficient platelets (42).

5. DCs in stretching of the FRC network.

In response to inflammation, HEV function is increased to facilitate ingress of lymphocytes from the blood while signals that permit lymphocyte egress from lymph nodes are downregulated, a process termed lymph node shutdown (43). The net result is an increase in numbers of naïve lymphocytes accumulating in the reacting lymph node and this is what initially increases total lymph node cellularity before antigen-specific activation results in clonal proliferation of both B and T cells. As a result of both lymphocyte trapping and proliferation, lymph node cellularity rapidly increases, placing pressure and strain on the stromal architecture and forcing its remodeling (44, 45, 46).

It had been assumed that the enlargement of lymph nodes occurs through the synchronised and coordinated proliferation of stromal and lymphocytic components (3, 44). This does seem to be true for the proliferation of blood endothelial cells, which begins very early in reactive lymph nodes (36). However, it is not the case for fibroblastic stroma. In fact, when stromal cell

subsets and lymphocytes are enumerated over time following immunisation, a lag in the proliferation of FRCs can be observed (44, 45). The length of this lag depends on the immune stimulus but can be as much as two days, during which time a marked increase in lymph node size has already taken place (44, 45). How, then, does the lymph node accommodate increased cellularity in this early phase of an immune response without increasing numbers of FRCs? The FRC network is a highly contractile structure and in fact the size of the lymph node can be controlled via the tuning of contractility of FRCs, notably, by DCs (*Fig. 2*).

Contractility in FRCs is driven by the activity of PDPN. We, and others have found that PDPN constitutively signals to drive actomyosin contractility without the requirement for engagement by an external ligand (45, 46). In this state, the cytoplasmic tail of PDPN is serine phosphorylated (45, 47) and binds to ezrin/radixin/moesin proteins (45, 48), connecting the plasma membrane to the underlying actin cytoskeleton. PDPN signaling also promotes RhoA/C activity, and activation of the RhoA/C effector Rho kinase (ROCK), which leads to phosphorylation of myosin light chain (MLC2) and the generation of contractile actin filaments (49) It is not yet clear which kinase is responsible for the phosphorylation and activation of PDPN and, furthermore, the direct connection between PDPN and RhoA activity remains to be fully characterised. Indeed, in one study, the ability of PDPN to drive contractility appeared independent of the cytoplasmic tail, raising the question of whether additional mechanisms exist to permit RhoA signaling (46). Either way, this signaling and the associated contractile activity of PDPN is tempered by sequestration within cholesterol-rich lipid rafts, which uncouples PDPN from

ezrin. This likely occurs through PDPN binding to inhibitory partners present in the membrane microdomain, including the hyaluronic acid receptor CD44, which itself can also drive contractility. Evidence suggests that when podoplanin is bound to CD44 both proteins become confined to cholesterol-rich lipid rafts and this inhibits contractility induced by either one (50).

The critical switch to drive PDPN into lipid rafts and thereby inhibit podoplanin-driven contractile activity is delivered by DCs in the form of CLEC-2. As already mentioned, CLEC-2 is upregulated on DCs following inflammation and helps promote DC motility(16). But CLEC-2 is also a potent, short-acting inhibitor of PDPN (45, 46, 51) an unusual example of reciprocal signaling that puts in question traditional definitions of ligand and receptor. Upon binding to CLEC-2, PDPN becomes clustered and sequestered into cholesterol-rich lipid rafts where it is rendered inactive (45, 52). This mechanism has also been shown in the skin, where CLEC-2^{+} platelets inhibit podoplanin-driven migration of keratinocytes (51). The biological consequences of this unusual mode of regulation are dramatic. Increased availability of CLEC-2^{+} DCs in the lymph node at the outset of an immune response results in an abrupt loss of tension through the actomyosin cytoskeleton of the FRCs (45) effectively allowing FRC network stretching and lymph node expansion. Indeed, genetically engineered mice in which CLEC-2 expression is selectively ablated on DCs ($CD11c^{Cre}Clec1b^{fl/fl}$ mice) show significantly reduced lymph node expansion following immunization compared to controls (45, 46). Similarly, lasting inhibition of PDPN activity using anti-PDPN antibody treatment *in vivo* results in increased lymph nodes following immunization and an increase in proliferation of antigen-specific T cells(46).

These findings indicate that PDPN activity is an important factor in the regulation of lymph node size and potentially the scale of adaptive immune responses.

6. DCs in the expansion of the stromal network

Despite the remarkable flexibility of the stromal cell network, there comes a point, between 2-5 days after initiation of a response, where stretching is no longer sufficient to support lymph node expansion. Beyond this stage, an increased number of fibroblastic stromal cells are required. There are many potential cues to initiate proliferation of the existing FRC network. Perhaps proliferation is initiated as the physical limit of stromal cell length is maximised and the point at which the interconnected network is in danger of becoming detached and disconnected. Podoplanin activity may also play a role in controlling the proliferation of FRCs as FRCs from $PDPN^{-/-}$ mice proliferate more in culture that wildtype counterparts (46). As PDPN signaling is inhibited by CLEC-2, it is reasonable to suppose that CLEC-2^{+} DCs not only provide the signal that causes FRC network stretching but also promote FRC proliferation. Consistent with that notion, increases in FRC number following LPS challenge in vivo are attenuated in $CD11c^{Cre}Clec1b^{fl/fl}$ mice (46). The number of CLEC-2^{+} DCs is at its peak during the acute phase of FRC stretching but it has also been reported that CLEC-2 can be cleaved and retain bioactivity in soluble form (53) so that there are potentially additional sources of CLEC-2 at later phases of LN expansion, including soluble CLEC-2 released from platelets. As FRCs relax their cytoskeleton and stretch to accommodate increased lymph node size, they increase in size (44, 45). This

change may be part of the set of cues that initiates the proliferation and expansion of the FRC network. FRCs may also respond directly to inflammatory stimuli prior to stretching priming them to proliferate, although it may also be possible that even a small change in physical tension at these early stages is the initiating factor for proliferation.

We know that fibroblastic stroma in the steady state can derive from a variety of sources (54-56) but the relative contribution of each of these sources to lymph node expansion during adaptive immune responses is unclear. Proliferation of pre-existing FRCs can be documented by uptake of EdU or similar assays that identify proliferating cells (44). Other data suggest a contribution from recruited percursors (57). In particular, the adipose tissue of the fat pad, within which most lymph nodes are found embedded has been shown to act as a source of stromal precursors, both in development (58) and during lymph node expansion in adult mice (54). In development, it has been described that a common mesenchymal precursor cell is able to give rise to both fibroblastic stroma in the developing lymph node and to adipocytes (59). The fate of these stromal precursors is dependent on the balance of signals received by the differentiating cells. LTβR stimulation promotes their differentiation into lymphoid tissue stromal cells (59). As DCs provide a rich source of ligands for LTβR, they could play a role in the conversion of adipose-derived, mesenchymal-precursors into new FRCs during lymph node expansion (54). In a series of transplant experiments, adipose-derived infiltrating stromal cells invaded deeply into the lymph nodes and positioned themselves in close proximity to the FRC network, as determined by PDPN and ER-TR7 staining (54). It is therefore possible that these cells might

combine with the existing reticular network to extend and expand the stromal architecture to allow further lymph node expansion.

There may also be a conversion of other stromal cell subsets in order to expand the stromal networks during lymph node expansion. In peripheral tissues such as the skin, gut, and in tumours, inflammation can induce the conversion of resident fibroblast populations into podoplanin-expressing stromal cells (60, 61), expressing IL-7, CCL19 and CCL21. This conversion was found to be dependent on $Gr1^+$ myeloid cell populations (60). There is therefore a precedent for the conversion of mesenchymal cells into FRC-like fibroblasts, and potentially signaling from myeloid cells such as DCs that drive this differentiation. Similar mechanisms may be in action within lymph nodes.

There are significant similarities in gene expression between FRCs and a proposed lymph node specific pericyte population termed ‘double negative’ (DN) cells, for example, the expression of chemokines such as CXCL14, cytokines such as Flt3L, and markers of fibroblastic function such as αSMA (19), potentially making DN cells a pre-FRC. Fibroblasts throughout different tissues display remarkable plasticity and ability to respond to the local microenvironment (62-64). It is conceivable that DN cells under the right conditions could therefore differentiate and contribute to the growing FRC network.

There is also precedent for the proliferation, conversion and differentiation of neighbouring cells in increasing FDC numbers during expansion of B cell follicles. Lineage tracing experiments revealed that marginal reticular cells (MRCs) (55) residing immediately below the subcapsular sinus, contributed to

the expanding FDC population. Like other stromal cell subsets, MRCs express high levels of PDPN, CXCL13 and RANK but, unlike T zone FRCs, they also express MAdCAM1. During lymph node expansion, MRCs proliferate and migrate into the follicles where they adopt an FDC phenotype (55). Interestingly, the proliferation of MRCs still occurs in Rag-/- mice, meaning that the increasing number of B cells is most likely not the stimulus for MRC proliferation (55). preFDCs can also be found in the form of PDGFRβ^{+} pericytes (56). PDGFRβ-Cre reporter mice showed labeling of FDCs in B cell follicles of the spleen, whereas deletion of PDGFRβ^{+} progeny resulted in a loss of FDCs (56). PDGFRβ^{+} pericytes in other tissues can also convert to FDC, able to support the accumulation and organization of B cells during the formation of tertiary lymphoid structures at inflammatory sites (56).

There is some evidence that like FDCs, FRCs could also be derived via the conversion of another stromal subsets. Endosialin/TEM1 (CD248) is a fibroblast marker (65, 66) observed in inflammatory settings such as rheumatoid arthritis (67). In resting lymph nodes CD248 expression is confined to the capsule but, following immunization, it is observed throughout the lymph node although, interestingly, shows little or no colocalisation with podoplanin (68). This lymph node-wide expression is relatively short-lived and disappears within 2-3 weeks of immunization. The identity and origin of the appearing CD248+ cells is still unclear, however, CD248 knockout mice exhibit significantly attenuated lymph node expansion at 5-7 days post immunization (68). CD248 expression is regulated by hypoxia (69) and in vitro at least has significant effects to promote proliferation and motility of mesenchymal cells (65, 68). CD248+ cells might be fibroblasts from the

capsule, double negative (DN) stroma or an infiltrating precursor population that have not yet acquired PDPN expression, but may with additional signals from the leukocyte population, contribute to the expanding FRC network.

7. DCs in recovery of normal lymph node architecture

In addition to promoting the production of VEGF by FRCs, LTβR ligands secreted by DCs have a profound influence on FRC viability, survival and adhesion (39). LNs lacking DCs lose FRC network integrity and fail to maintain adequate FRC numbers (39). This tells us about the steady state of LN architecture but also provides potential mechanisms to resolve the issue of reducing the numbers of FRCs as the immune response and the inflammation are resolved and the lymph node shrinks back to the original size. Recent work has also shown that even quick resolution of acute infection can leave a long lasting 'scar' in local tissue and unbalance future immune responses (70). This may involve damage to the local lymphatic system with lymphatic vessels that become chronically 'leaky' and lead to redirection of afferent lymph cells, including DCs, into adipose tissue (70), This immunological scarring may contribute to the development of chronic inflammatory diseases. Indeed, in the case of chronic infections such as HIV, lymph node damage and fibrosis are commonly observed (71, 72). Reduced numbers of T cells diminishes T-cell derived LTβ, which, like that of DCs, helps maintain the FRC network. Loss of T cells during the infection therefore causes a corresponding loss of FRCs. Since FRCs produce IL-7 to promote survival of T cells, a negative spiral of loss of lymphoid tissue function ensues. To make matters worse, increased TGFβ signaling from regulatory T cells induces the

diminishing population of FRCs to overproduce extracellular matrix proteins which, over time, causes tissue fibrosis, further restricting access of T cells to the source of IL-7 (3). Numbers of DCs tend to also be reduced in HIV infection (73). Knowing that DCs play important roles in the maintenance of the FRC network (39), therapeutic strategies to rescue the DC population may have significant benefit.

8. Concluding remarks

DCs have traditionally been viewed as the sentinels of the immune system. The transport and presentation of antigen by DCs provides the critical signal for the initation of adaptive immune responses. Our understanding of the role of DCs is now significantly broadened. The same sentinels communicate directly with the stromal cells of lymph nodes, maintaining their integrity and "priming" their ability to accommodate lymph node expansion. We understand some of the molecular players involved in the crosstalk between DCs and stroma, in particular the axes of LTβ/LTβR and CLEC-2/PDPN signaling. There are likely to be several others and their elucidation will help our understanding of a process that is emerging to be just as critical to immunity as the delivery of antigen and the trafficking of leucocytes.

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