Stromal Cells in Chronic Inflammation and Tertiary Lymphoid Organ Formation

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Annu. Rev. Immunol. 2015. 33:715–45

The *Annual Review of Immunology* is online at immunol.annualreviews.org

This article's doi: 10.1146/annurev-immunol-032713-120252

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Keywords

stromal cells, fibroblast, chronic inflammation, persistence

Abstract

Inflammation is an unstable state. It either resolves or persists. Why inflammation persists and the factors that define tissue tropism remain obscure. Increasing evidence suggests that tissue-resident stromal cells not only provide positional memory but also actively regulate the differential accumulation of inflammatory cells within inflamed tissues. Furthermore, at many sites of chronic inflammation, structures that mimic secondary lymphoid tissues are observed, suggesting that chronic inflammation and lymphoid tissue formation share common activation programs. Similarly, blood and lymphatic endothelial cells contribute to tissue homeostasis and disease persistence in chronic inflammation. This review highlights our increasing understanding of the role of stromal cells in inflammation and summarizes the novel immunological role that stromal cells exert in the persistence of inflammatory diseases.

WHAT IS INFLAMMATION?

Inflammation is the physiological response to a pathological stimulus. It evokes responses from both tissue-resident cells and cells of the immune system with the aim of allowing cells and proteins in the vasculature to enter damaged or infected tissues and facilitate repair. The inflammatory cascade allows the selective recruitment of immune cells from the blood to the damaged tissue and encourages the turnover of cells and soluble factors at sites of infection and damage.

Inflammatory mediators, including prostaglandins and cytokines, rapidly precipitate inflammatory events, often by acting directly on the inactive components of the plasma enzyme cascades or by directly activating effector leukocyte populations. Inflammatory mediators are extremely diverse and can act at nearly every stage of an immune response. Different inflammatory mediators are released during distinct phases of inflammation depending on the nature and duration of the inflammatory insult (1, 2).

Early-phase mediators are released at the initial stages of inflammation and often exist as precursors ready for rapid release or effect. Their effect is localized, triggering activation of the clotting or fibrinolytic pathways. Late-phase mediators such as the prostaglandins and leukotrienes have to be synthesized upon inflammation from arachidonic acid, as opposed to existing as proforms that can be immediately activated. Late-phase proteins modulate the extent of inflammation and regulate the balance of inflammatory and noninflammatory events.

Despite the diversity of stimuli and inflammatory mediators that can trigger the inflammatory response, this process can be divided into distinct phases that involve different cells of the immune system as well as stromal cells (endothelium, epithelium, fibroblasts, and nerves) that represent the structural components of the tissue. The acute vascular response that involves mainly the endothelium occurs within seconds of tissue injury and is aimed at inducing vasodilation and increased permeability of the capillary wall. This leads to increased blood flow (hyperemia), increased redness (erythema), and the entry of protein-rich fluid into the tissue (edema) where tissue-resident fibroblasts and epithelial cells are activated. These events result in the localized accumulation of fluid and the characteristic swelling associated with inflammation at the site of injury or infection. The increased flow and permeability also contribute to an increase in cellular traffic of polymorphonuclear leukocytes and release of noxious mediators and subsequent loss of function of the affected organ. These physiological responses all lead to the cardinal clinical features of inflammation: hot, red, swollen, painful tissues that do not function properly.

The acute cellular response occurs over a period of minutes to hours and is characterized by the appearance of leukocytes, particularly neutrophils, at the site of injury, where they participate in direct microbial killing. The chronic cellular response involves the recruitment of different leukocyte cell populations, including monocytes and lymphocytes from the blood, that interact with tissue-resident stromal cells. Once tissue injury has been repaired and infectious agents eliminated, tissue homeostasis requires the removal of effector cells via phagocytosis and lymphatic drainage. Final wound repair entails the removal of any blood clots (fibrinolysis) and the migration of fibroblasts and endothelial cells into damaged or missing tissue to restore tissue architecture (1, 2).

Chronic inflammation occurs in a wide range of disabling diseases, including rheumatoid arthritis (RA), psoriasis, and inflammatory bowel disease (IBD). Whereas episodes of self-limiting inflammation are physiological and require pathogen clearance, the persistence of inflammation is deleterious for the organ where it occurs as well as for the systemic repercussions occurring to other organs. For example, in RA, the continuous inflammation drives the process of accelerated atherosclerosis and endothelial dysfunction (3).

Robust evidence highlights how blood, lymphatic endothelium, and stromal cells such as fibroblasts critically contribute to the persistence of the inflammatory process. Indeed, the cross talk

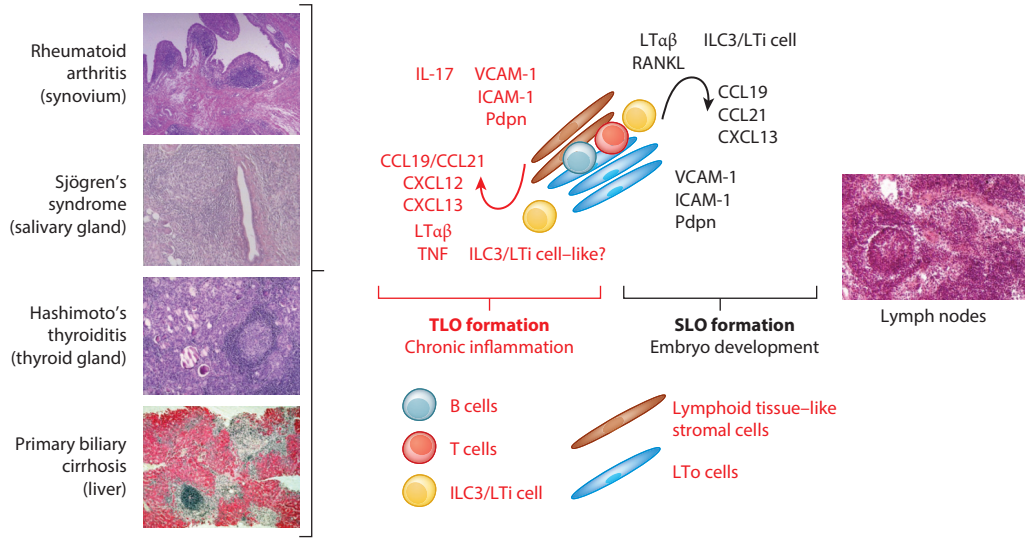

Figure 1

Model of SLO and ectopic TLO formation. SLOs develop during embryogenesis as a result of the interactions between LTi cells and stromal cells that become LTo cells following stimulation by the lymphotoxin β receptor. LTo cells express chemokines and cell adhesion molecules that recruit and organize the B and T cell areas of the organ. During chronic inflammation, immune cells recruited to the site of injury express inflammatory cytokines that, upon prolonged stimulation of resident stromal cells, will induce the latter to express chemokines and cell adhesion molecules that will recruit lymphocytes and organize TLOs. Modified from Reference 4. (Abbreviations: ICAM-1, intercellular adhesion molecule-1; ILC3, type 3 innate lymphoid cell; LTi, lymphoid tissue inducer; LTo, lymphoid tissue organizer; LTαβ, lymphotoxin αβ; Pdpn, podoplanin; RANKL, receptor activator of nuclear factor-κB ligand; SLO, secondary lymphoid organ; TLO, tertiary lymphoid organ; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.)

between immune and stromal cells mediated by soluble molecules and membrane-attached ligands and their receptors ultimately determines whether the inflammation will be acute or result in a chronic process leading to the formation of ectopic lymphoid tissues (**Figure 1**).

CHRONIC INFLAMMATION: DEFINED BY PERSISTENCE, SITE SPECIFICITY, AND THE FORMATION OF ECTOPIC LYMPHOID TISSUE

Whereas acute inflammation is dominated by the role of inciting agents and a program that is ultimately designed to resolve the inflammatory cascade, chronic inflammation has two unique characteristics: persistence and site specificity. Persistence results from the imbalance between inflammatory cell recruitment and clearance of the immune infiltrate. Local cell proliferation and apoptosis also influence this phenomenon, regulating the size and nature of the local inflammatory infiltrate. The molecular and cellular basis for site specificity has, until quite recently, remained enigmatic.

A series of molecules, deregulated in their temporal or spatial expression, are involved in the process of persistence of the inflammation. For example, ectopic expression of B cell survival factors, such as BAFF (B cell–activating factor/TNFSF13B), the chemokine CXCL12, or interferon β within the synovial membrane in RA patients, contributes to the phenomenon of leukocyte recruitment and persistence in the tissue. Stromal resident cells are functionally involved in the

production of these factors, ultimately influencing the aberrant accumulation of immune cells in the inflamed organ (5, 6).

The recruitment and positioning of cells to appropriate niches within immune organs are driven by a complex interaction between chemokines, often secreted by stromal cells, and their receptors that are expressed by leukocytes. Chemokine–chemokine receptor interactions influence tissue ontogeny and development (7). They also shape the spatial organization of cellular infiltrates within different organs, in both physiological and pathological conditions. Within tissues undergoing chronic inflammation, for example, infiltrates that are often organized into well-defined lymphoid tissue–like structures or tertiary lymphoid organs (TLOs) are frequently observed (8). These ectopic organs are characterized by aberrant expression of homeostatic chemokines and cytokines, similar to those observed in secondary lymphoid organs (SLOs) (8) (**Figure 1**). A series of physiological TLOs develop after birth in response to the presence of microbiota or during immune responses and are not associated with pathology. These TLOs are described in the section titled "Physiological Tertiary Lymphoid Organ Formation Is Associated with the Expression of Homeostatic Chemokines and Organizing Molecules." Understanding the physiological mechanisms that drive SLO formation and regulate the expression of lymphoid factors, ectopically expressed in chronic inflammation, is very likely to inform our understanding of the pathogenic relationship between chronic inflammation and TLO formation.

WHAT ARE STROMAL CELLS AND WHAT IS THEIR IMPORTANCE IN CHRONIC INFLAMMATION?

Stromal cells encompass numerous cell types traditionally considered the structural components of organs. These include fibroblasts, blood and lymphatic endothelial cells, pericytes, and epithelial cells. The architectural role of stromal cells is well recognized, and stromal cell specialization is known to critically regulate tissue function, e.g., hepatocytes in the liver and type 1 and 2 alveoli in the lungs. Emerging evidence now points to an intriguing conclusion that stromal cell function exceeds its architectural role and that these cells play a key role in choreographing and orchestrating immune responses and defining disease persistence. This process is not generic but is contextual to the target organ.

Fibroblasts: Ubiquitous Cells with Regional Identity

Despite compelling genetic and therapeutic evidence for a dysregulated immune system in the pathogenesis of many chronic immune-mediated inflammatory diseases, the cellular mechanisms responsible for disease persistence and tissue tropism remain poorly understood (9). Researchers have assumed that inflammation is a stereotyped response that reflects a common set of shared pathways leading to endothelial cell activation, leukocyte infiltration, and tissue repair. However, some features of the inflammatory response remain unique or private to the tissue where inflammation occurs (10, 11). Fibroblasts play a major role in defining the site-specific features of many organs, including the synovium and salivary glands (12). In the synovial joint, specialized mesenchymal cells termed fibroblast-like synoviocytes (FLSs) are responsible for integrating the many stimuli that promote disease (13). FLSs amplify inflammation and tissue damage through their production of chemokines, cytokines, and proteases, which affects the recruitment, retention, and differentiation of infiltrating leukocytes (14). Unlike fibroblasts from other sites, FLSs express high levels of vascular cell adhesion molecule-1 (VCAM-1), hyaluronan, lubricin, and cadherin-11 (15). The seminal work showing that the genetic deletion and therapeutic manipulation of cadherin-11 in mouse models of inflammatory arthritis reduced inflammation and protected against cartilage

erosion provides proof of principle that targeting mesenchymal cells can have a profound impact on both inflammation and tissue destruction (16).

Fibroblasts are ubiquitous cells identified by morphology, ability to adhere to plastic, and lack of other cell lineage markers such as those expressed by epithelial cells, vascular cells, and leukocytes (17, 18). Despite not being fully characterized in molecular terms, synovial fibroblasts at certain sites have been identified by the expression of a combination of markers such CD90, CD44, decay accelerating factor, VCAM-1, uridine diphosphoglucose dehydrogenase, and prolyl-4-hydroxylase (19). Within the same tissue, fibroblasts can also acquire certain phenotypic characteristics that contribute to define their function. For example, cadherin-11 is expressed by fibroblasts in the lining layer, where it mediates homotypic synovial fibroblast adhesion. Overexpression of cadherin-11 in cultured RA synovial fibroblasts increases their invasiveness in vitro (20).

Fibroblasts provide the structural components of the tissue and are primarily responsible for the synthesis and remodeling of extracellular matrix (ECM) components. Fibroblasts regulate the homeostasis and architecture of adjacent epithelial and endothelial structures, secreting and responding to trophic factors present in the local microenvironment (17); they thus play a critical role during tissue development, differentiation, and repair. A specific population of cancer-associated fibroblasts (CAFs) is able to influence cancer survival and spread, regulating growth, survival, and metastasis of malignant cells (21, 22). Injection of a mixture of CAFs with breast cancer cells into mice showed that the presence of CAFs accelerates tumor growth (23, 24). Recent work has enlightened the protective, immunosuppressive role that fibroblast activation protein $(FAP)^+$ CAFs play, modulating the immune response in the reaction toward cancerous cells (25).

Fibroblasts play a significant role in fibrosis. Accumulation of myofibroblasts [α-smooth muscle actin $(\alpha SMA)^+$ fibroblasts] leads to the formation of granulation tissue and hypertrophic scars by excessive ECM production. This phenomenon and the subsequent rarefication of the microvasculature occur in diseases such as kidney or liver fibrosis and scleroderma (26). Myofibroblasts in fibrotic tissue have been shown to be resistant to apoptosis and are able to promote their own survival by killing surrounding lymphocytes through a Fas-mediated mechanism (27, 28).

Fibroblasts isolated from different anatomical sites (synovium, skin, bone marrow, and lymph nodes) display topographic differentiation and positional memory (29, 30). The functional consequences of these anatomical differences have been explored using models of leukocyte-fibroblast and leukocyte–endothelial cell–fibroblast cocultures (31, 32). Investigators have recently demonstrated that fibroblasts isolated from different locations (synovial versus dermal fibroblasts) influence differently the behavior of endothelial cells in terms of leukocyte recruitment (33). While reinforcing the concept that fibroblasts convey site specificity to the immune response, these data highlight the active role played by the stroma on endothelial cells from the earliest phases of the response.

RA synovial fibroblasts are involved in different phases of the immune response (34). Fibroblasts can directly modulate recruitment of leukocytes by secreting high levels of proinflammatory chemokines such as CXCL1, CXCL8, CXCL5, CXCL16, CCL2, CCL4, and CCL5 (35–40). In different circumstances, for example, in the synovium during RA, fibroblasts can promote inflammation persistence, generating a prosurvival, anti-apoptotic microenvironment for the recruited immune cells, by secreting survival factors in response to cytokines or innate receptor stimulation (29, 35, 41–45). The role of stromal cells in the organization in the chronic inflammatory infiltrate is discussed below.

Fibroblasts are key mediators of tissue destruction, both directly via secretion of matrix metalloproteinases, cathepsins, inflammatory cytokines, and chemokines (46) and indirectly via regulation of monocyte differentiation to osteoclasts (47). Evidence of the direct erosive ability of fibroblasts derives from in vivo studies. In the rheumatoid joint, activated fibroblasts attach to and overgrow

the cartilage surface, then invade and destroy cartilage and induce bone reabsorption. Remarkably, fibroblasts maintain this destructive phenotype even after multiple passages in culture, allowing experiments to be performed in vitro with cells that are functionally representative of their in vivo counterparts (48). Furthermore, researchers have demonstrated that the invasiveness of fibroblasts in vitro correlates with rates of bone erosion in individual patients (49). Strikingly, cultured RA synovial fibroblasts (but not normal or osteoarthritis synovial fibroblasts) attach to and invade coimplanted human cartilage even after multiple passages in vitro, indicating that this invasive phenotype is both stable and disease specific (50). Fibroblasts can also regulate local bone resorbing by secreting receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL/TNFSF11), a key factor involved in osteoclast differentiation (51).

The role of fibroblasts in the generation of the microenvironment that leads to lymphoma genesis, a long-term complication of chronic inflammation, is discussed in the section titled "Tertiary Lymphoid Organ Formation in Human Disease." Functional similarities between fibroblasts and mesenchymal stem cells (MSCs) have been highlighted recently, suggesting a close relationship between these two types of stromal cells. MSCs share with fibroblasts the ability to adhere to plastic and the pluripotential capacity to differentiate down osteogenic, chondrogenic, and adipogenic lineages (52). The phenotypical and functional differences between MSCs and fibroblasts have been reviewed elsewhere (53). The functional role of MSCs in inflammation is still debated. Data from animal models and early-phase clinical trials have proved that MSCs induce immunosuppression (54). However, researchers have also described a proinflammatory phenotype for MSCs (55). In fact, the administration of murine MSCs in models of arthritis has led to divergent results, highlighting the need for a better understanding of MSC and fibroblast origin and function (56, 57). Such diversity in cell subsets and function is a well-accepted paradigm in leukocyte biology, where, for example, regulatory as well as inflammatory subsets exist within the same family of cells (58). Whether similar diversity exists within the fibroblast family of cells has not yet been adequately explored. We propose that fibroblasts exist in discrete subsets, some of which are proinflammatory, whereas others, more functionally similar to MSCs, are anti-inflammatory and regulate tissue homeostasis and organ repair. Therefore, an understanding of the origins and functional consequences of fibroblast heterogeneity will provide an important scientific basis for cell-based therapies in different rheumatic diseases.

Vascular Cells

Lymphatic endothelium. Lymphatic vessels (LVs) represent a critical component of tissues. In resting conditions, LVs drain and transport extracellular fluid and macromolecules into the systemic circulation. During inflammation, the lymphatic bed delivers activated dendritic cells (DCs), macrophages, and antigens to draining lymph nodes and regulates interstitial fluid drainage to avoid excessive swelling of the inflamed organ. This process is achieved, in most cases, by expansion of the existing, resting lymphatic network by local lymphangiogenesis (59). A recent report showed that the atypical chemokine receptor CCRL1 is expressed in lymphatic endothelial cells of the ceiling of the subcapsular sinus of lymph nodes and by scavenging chemokines creates a gradient of CCL21 that facilitates the migration of DCs from the afferent lymph into the lymph node paracortex (60).

LVs are highly dynamic structures that interact intimately with their surrounding microenvironment. During ontogeny, LVs sprout out from the cardinal vein in a process that is orchestrated by the homeobox genes Prox1 and Sox18, vascular endothelial growth factor C (VEGFC), and its receptor VEGFR-3. Recent studies have shown that LV formation also

requires interaction between podoplanin (Pdpn, also known as gp38) on LVs and platelet-derived Clec-2 for separation of LVs from the blood endothelial vessels (61, 62).

Within immunized lymph nodes, a dramatic expansion of LVs occurs that coincides with an increased influx of immune cells, thereby maximizing the potential for interaction between different cell types and enabling an effective immune response. This process is very tightly regulated by lymphotoxin αβ–lymphotoxin β receptor (LTαβ-LTβR/TNFRSF3) signaling and remodels the lymph nodes in a manner that allows an effective balance between the input and output of the cells. This in turn helps the lymph nodes recover to homeostatic conditions (63). In addition, the expression of sphingosine 1 phosphate (S1P) by efferent LVs supports the egress of S1P receptor-1^+ lymphocytes from lymph nodes, thus contributing to the return to homeostatic conditions of these organs (64).

LVs in lymph nodes not only play a mechanical role in cell delivery and egress but also have immunological functions. Investigators have demonstrated that LVs induce peripheral tolerance by expressing tissue-restricted antigens under the control of the autoimmune regulator (AIRE) and secreting immunoregulatory factors, which dampen T cell proliferation to avert exacerbated immune responses (65).

In inflammatory conditions, a similar expansion of the vascular bed occurs in peripheral tissues. Lymphangiogenesis has been shown to occur in various inflammatory conditions, including acute and chronic infections, autoimmune diseases such as RA, Crohn's disease, wound healing, cancer, and transplant rejection (66). Neolymphangiogenesis has long been associated with inflammation, and recent studies have shown that induction of the NF-κB pathway by inflammatory stimuli can activate the transcription factor Prox1. Subsequently, NF-κB and Prox1 synergistically activate the VEGFR-3 promoter, which leads to increased receptor expression in lymphatic endothelial cells, enhancing the responsiveness of preexisting lymphatic endothelium to VEGFR-3 ligands, VEGF-C, and VEGF-D (67). Pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF/TNFSF2) are known to induce VEGF-C/D in various infiltrating and tissue-resident cell types such as macrophages, DCs, mast cells, and fibroblasts at the inflamed site (68–71). The cytokine lymphotoxin α (LTα/TNFSF1), mainly secreted by lymphocytes at the site of inflammation, has also been documented to support inflammatory lymphangiogenesis (72). Taken together, these data suggest that the signals that activate the induction of the inflammatory process also program its resolution.

Data from inflamed corneas in mice and renal transplant inflammation in humans have shown that inflammation-mediated lymphangiogenesis occurs not solely by proliferation or continuous sprouting of existing LVs but also by incorporation of bone marrow–derived lymphangiogenic progenitors (such as $CD11b^+$ macrophages) into the existing or growing lymphatics. $CD11b^+$ progenitors can transdifferentiate into Lyve-1^+ vessels under pathological conditions and thus contribute to increases in LV density observed at the site of inflammation (73–75). In inflamed tissue, LVs seem to play an immunoregulatory role. CCBP2/D6, a decoy receptor expressed on lymphatic endothelium, can reduce the inflammatory response in various organs by scavenging inflammatory CC chemokines (76). In addition, inflamed lymphatic endothelium is reported to suppress DC maturation via CD11b interaction with the intercellular adhesion molecule-1 (ICAM-1) receptor on lymphatic endothelial cells (77). Therefore, efficient and productive lymphangiogenesis appears to represent a potent tool of the immune system to contribute to tissue clearance and resolution of the inflammatory process.

Researchers believe that such a coordinated series of events is altered in chronic inflammation, in which the number of infiltrating leukocytes overcomes the drainage capacity of the newly developed LVs and failed lymphangiogenesis at peripheral sites contributes to chronicity of the inflammatory process. Inhibition of neolymphangiogenesis by VEGFR-blocking agents exacerbates

pulmonary edema, prevents resolution of the inflammatory cells, and causes chronic *Mycoplasma pneumoniae* infection (78). Evidence also suggests that aberrant lymphatic return due to reduced lymphoneogenesis or dysfunctional hyperplastic LVs could be one of the factors that supports persistence of ectopic germinal centers (GCs) within TLOs in chronic inflammatory diseases such as psoriasis, and Crohn's disease (79–81).

In TLOs, lymphangiogenesis is believed to represent a productive attempt to resolve inflammation; however, it is not clear whether the newly formed LVs are able to establish a viable connection with the draining lymph node. This phenomenon of failed drainage could favor the persistence in the tissue of antigens and activated DCs, macrophages, and lymphocytes. The inability of LVs to deliver antigens to the draining lymph node could indeed favor the persistence of TLOs, where an excess of antigen is presented in structures whose stromal component is potentially unable to exert the tolerogenic activity attributed to lymph node stroma (82). In this context, the ectopic expression of CCL21 on freshly formed but nonfunctional LVs that compete with functional lymphatics may impair the trafficking of DCs and leukocytes to the draining lymph node, thus favoring this pathogenic process (83, 84). Furthermore, within TLOs, ectopic CCL21 expression on LVs could unintentionally contribute to persistence of the inflammatory process, attracting $CCR7^+$ naive T cells and DCs from the circulation to the inflamed tissue (83, 84).

Lymphangiogenesis is considered a deleterious mechanism in other human pathologies. Metastatic cells also use LVs to spread cancer. Tumor cells enter lymphatic vasculature, invading preexisting LVs or stimulating neolymphangiogenesis by inducing VEGF expression on CAFs and macrophages (85, 86). A high density of LVs is known to correlate with a high incidence of lymph node metastasis and poor prognosis in some cancers (87, 88). CCL21 expression on lymphatic endothelial cells is also believed to contribute to the spreading of $CCR7^+$ malignant cells (89). Similar to cancer, lymphangiogenesis has also been found to play a pathogenic role in transplantation biology in that it sustains delivery of donor antigens to the recipient lymph node, ultimately favoring the generation of an immune response against the transplanted tissue (90). These data suggest a key role for LV function and homeostasis in the regulation of the balance between immunity and tolerance as well the persistence of inflammation compared to its resolution.

Blood endothelium. Similar to fibroblasts and LVs, blood endothelial vessels undergo remodeling during inflammation. These cells change their structure and phenotype and participate in the inflammatory response, mainly by regulating leukocyte recruitment into tissues (91). After remodeling of the vascular structure, a newly formed extravascular matrix supports leukocyte extravasation and is associated with the expression of leukocyte adhesion molecules such as E-selectin, VCAM-1, and ICAM-1 (92, 93). Inflammatory stimuli such TNF, IL-1, certain bacteria and viruses, physical and oxidative stress (91), and antiendothelial cell antibodies (found in systemic inflammatory diseases such as vasculitides) (94) all elicit nuclear translocation of the NF-κB proteins and subsequent activation of blood endothelial cells (91, 95, 96). Failure to restore homeostasis of the blood endothelium contributes to chronic inflammatory disease and edema.

Activated blood endothelial cells synthesize cytokines such as IL-6, which regulates the acute phase response, and chemokines such as CXCL8/IL-8 and CCL2/MCP-1. The latter help to establish the chemotactic gradient necessary for the influx of various inflammatory cells into peripheral tissues (97). Blood endothelial cells can also act as antigen-presenting cells, expressing class II human leukocyte antigen molecules, in a phenomenon that has been shown to contribute to transplant rejection (98). Expression of costimulatory molecules such as OX40L/TNFSF4 and ICOSL/CD275, known to be important in the formation and activation of memory T cells, has

been documented in activated human endothelial vessels (99), 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 Sjögren's syndrome (SS), thyroiditis, and RA. Chronically inflamed organs frequently develop TLOs. This process is often accompanied by conversion of flat venular endothelial cells into tall and plump endothelial cells that closely resemble the high endothelial venules (HEVs) normally found in the T cell–rich area of the lymph nodes. TLO-associated HEVs are characterized by expression of the lymph node trafficking code, called peripheral node addressin (PNAd), which binds L-selectin expressed on naive/central memory T lymphocytes and mature DCs. This homing machinery, supported by the ectopic expression of CCL21, allows the HEVs to misguide the influx of $CCR7^+$ memory T cells into the TLOs within the inflamed tissue, leading to amplification and maintenance of chronic inflammation (100–102). Signaling through LTβR is essential for the development of HEVs in SLOs and likely plays a similar role in TLOs (103–105).

Growth of new blood vessels from existing ones is a very important feature of chronic inflammation and cancer (106). Angiogenesis in these conditions ensures continuous oxygen and nutrient supply to pathogenic cells, thus sustaining their growth and survival. Several cell types, including malignant cells, synovial fibroblasts, keratinocytes, and monocytes and macrophages, can produce classic angiogenic factors [such as VEGFA, angiopoietin, and platelet-derived growth factor (PDGF)] when the environment becomes hypoxic. Moreover, inflammatory cytokines such as IL-1, TNF (low dose), and CXCL8 have been reported to be proangiogenic, thus supporting this process while exerting other proinflammatory activities. However, blood vessels formed during pathological angiogenesis are often structurally and functionally abnormal. Tumor vasculature is highly disorganized: Vessels are tortuous and dilated, with uneven diameters, excessive branching, and shunts that lead to chaotic and variable blood flow, often resulting in the establishment of hypoxic and acidic areas in the tissue (107, 108). Similarly, the new vessels formed at the site of inflammation can exhibit structural and functional abnormalities (109). In RA, for example, the vascular network is reported to be dysfunctional and unable to restore tissue oxygen homeostasis that is disrupted during inflammation; as a consequence, rheumatoid joints are markedly hypoxic (110). In both RA and cancer, impaired angiogenesis supports the selection of cells that are metabolically resistant to low oxygen levels, potentially reducing the effectiveness of therapy aimed at disturbing the neoangiogenic process.

STROMAL CELLS IN LYMPHOID TISSUE NEOGENESIS AND CHRONIC INFLAMMATION

To understand the essential role of stromal cells and TLO formation in chronic inflammatory diseases in humans, we will first discuss the role of these cells and the signals involved in SLO development. Animal models have been critically important for dissecting the signals involved in lymphoid tissue neogenesis, and the next section mainly describes the evidence derived from these studies.

Stromal Cells in Secondary Lymphoid Organ Development

SLOs such as lymph nodes and Peyer's patches develop during embryogenesis and early postnatal life in rodents and humans. A key step in their development is the interaction between mesenchymal stromal cells and fetal liver-derived lymphoid tissue inducer (LTi) cells. LTi cells belong to the family of type 3 innate lymphoid cells (ILC3). Their development is dependent on the transcription factors RORγt and Id2 and the TNF family ligand-receptor pair RANKL-RANK/TNFRSF11A

(111–115). CXCR5^{+} LTi cells in the mouse embryo are recruited to the sites of lymph node anlagen formation by stromal cells that, upon receiving a neuron-derived retinoic acid signal (116), express the CXCR5-specific ligand, the chemokine CXCL13 (117). Clustering of LTi cells expressing both RANKL and RANK induces signaling through the latter that results in increased levels of the TNF family ligand LTαβ on the surface of these cells (118–121).

Binding of LTαβ on LTi cells to its receptor LTβR on stromal cells induces a gene expression program by activating the NF-κB transcription factors through canonical and noncanonical pathways, resulting in the expression of the chemokines CXCL13, CCL21, and CCL19 and cell adhesion molecules that are essential for the recruitment and clustering of a progressively larger number of LTi cells (122). The cross talk interactions between LTi cells and stromal cells establish a positive feedback loop, as binding of CXCL13 and CCL21 to their receptors on LTi cells induces further expression of LTαβ on their surface that ultimately sustains the enlargement of the anlagen (123).

LTi cells are essential for delivering LTαβ to engage LTβR in stromal cells, as lack of either the former cells or this ligand-receptor pair leads to a block or impaired maturation of stromal cells to VCAM^{high}ICAM^{high}MAdCAM-1^{+} lymphoid tissue organizer (LTo) cells and reduced proliferation and increased apoptosis that results in the absence of lymph nodes (124–126). LTi cells also deliver the ligand to LTβR on endothelial cells of the developing lymph node anlagen to induce the growth of the vasculature and full maturation of HEVs that facilitate the entry of lymphocytes to the adult organ (103–105). The origin of the mesenchymal stromal cell precursor that gives rise to the LTo cells has not been fully elucidated. Adipocyte progenitor cells present in the fat pads that surround the developing lymph nodes have been shown to migrate to the anlagen to differentiate into the stromal cell lineages in a process dependent on LTβR (127). The different steps involved in lymphoid tissue development and the multiple roles of stromal cells, not only during development but also during immune responses, have been discussed extensively in excellent reviews (128, 129).

After birth, LTo cells undergo further differentiation to become the stromal cell subsets present in adult lymph nodes such as fibroblastic reticular cells (FRCs) of the T zone, follicular dendritic cells (FDCs) present in B cell follicles and GCs, and marginal reticular cells (MRCs) adjacent to the subcapsular sinus (130). LTβR, TNF-receptor I (TNFRSF1A), and NF-κB signaling are strictly required for the differentiation of these stromal cell subsets, as shown by defects in spleen and lymph nodes of *Tnf*$^{-/-}$, *Tnfr1*$^{-/-}$, and *Ltβr*$^{-/-}$ mice. Moreover, Chai and colleagues (131) have shown that LTβR signaling is required for the full maturation of FRCs to support immune responses to viral infections. FRCs express CCL19, CCL21, and IL-7 to organize and sustain the T cell area by attracting CCR7^{+} DC and T cells (132, 133). FDCs express CXCL13, BAFF, and the complement receptors CR1 and CR2 that are essential for organizing the B cell follicles and GCs by recruiting and selecting CXCR5^{+} B cells (134). The role of MRCs is less clear; these cells express high levels of CXCL13 and RANKL and have been recently demonstrated to give rise to the FDCs in lymph nodes (135). Investigators have recently identified a new stromal cell type called versatile stromal cells (VSCs) that reside in the T cell area of lymph nodes (136). During immune responses, B cell follicles expand into the T cell area, and VSCs cells respond to the LTαβ^{+} B cells by upregulating CXCL13^{+} expression. HEVs are present in the T cell zone and act as entry points for lymphocytes and DCs that will further migrate to the B cell follicles and T cell areas. Recruitment of DCs to lymph nodes appears to be crucial for the growth and maintenance of the differentiated phenotype of HEVs (providing VEGF and LTαβ to endothelial cells) as well as to induce the expression of CCL21 in FRCs (104, 137, 138).

The stromal cell networks present in adult lymph nodes are essential for guiding lymphocyte entry and migration to the specific areas as well as for generating and maintaining the conduit

system for the transport of chemokines and low-molecular-weight antigens (139–143). This network interfaces with the complex vascular system that regulates lymphocytes and DC recirculation in lymph nodes.

Physiological Tertiary Lymphoid Organ Formation Is Associated with the Expression of Homeostatic Chemokines and Organizing Molecules

The similarities between lymph node development during embryogenesis and inflammation-induced TLO formation in adults suggest that identical signaling pathways are involved in both processes. A series of physiologically induced lymphoid tissue structures that are considered TLOs develop in mice and humans, such as isolated lymphoid follicles (ILFs) in the small intestine, nasal-associated lymphoid tissues (NALTs), inducible bronchial-associated lymphoid tissues (iBALTs), tear duct–associated lymphoid tissues, and milky spots of the omentum. Importantly, development of these TLOs takes place after birth and requires the presence of the intestinal microbiota (ILFs) or inflammation or infection, in the case of iBALT, that will induce activation of stromal cells and subsequent recruitment of immune cells. These TLOs appear to be essential for immune homeostasis (ILFs) and the development of local immune responses (ILFs, iBALTs, and omental milky spots) (144). More recently, the presence of lymphoid aggregates in the fat surrounding the blood vessels of the mesenteries called fat-associated lymphoid clusters has been demonstrated in adult mice and humans (145). The development of these structures may be related to the presence of type 2 ILCs that express high levels of Th2 cytokines such as IL-5, IL-9, and IL-13. A distinctive feature of these TLOs with respect to SLOs is the lack of any well-defined capsule.

The developmental requirements for the formation of this group of TLOs have not been fully investigated. ILF formation is dependent on ILC3/LTi cells, LTαβ/LTβR, and Toll-like receptor (TLR) signaling (146). In contrast, iBALTs, tear duct–associated lymphoid tissues, and NALTs appear to develop independently of LTαβ/LTβR and TLR, whereas omental milky spot formation is dependent on LTα (147–150). Interestingly, initial expression of CXCL13 in iBALTs and omental milky spots is independent of LTα. However, LTαβ/LTβR signaling is necessary to organize the architecture of the B and T cell areas by maintaining the expression of lymphoid chemokines and the differentiation of FRCs, FDCs, and endothelial cells in HEVs.

A key step in the development of TLOs is the activation of resident stromal cells toward a lymphoid tissue stroma-like phenotype that can be triggered by different pathways, depending on the presence of commensals, pathogens, or an inflammatory insult. A lipopolysaccharide-induced model of iBALT formation in neonatal mice showed that IL-17 is required for the expression of CXCL13 and CCL19 independently of LTα (147). $CD11c^+$ DCs are necessary for the maintenance of iBALTs in this model and in virus-infected adult mice (151). Fleige et al. (152) have shown recently that infection with a vaccinia virus strain induced iBALT-containing B cell follicles, $CXCL13^+$ FDCs, and other stromal cells expressing CXCL12. In contrast, *Pseudomonas aeruginosa* infection induced the accumulation of $IL\text{-}17^+$ γδ T cells into the lungs, triggering stromal cell differentiation into $Pdpn^+CXCL12^+$ cells that recruited B cells to the iBALTs, whereas $CXCL13^+$ FDCs did not develop (152). The capacity of iBALTs to sustain an antigen-specific response in the absence of secondary lymphoid tissues has been elegantly addressed by Moyron-Quiroz et al. (153) in spleen-, lymph node–, and Peyer's patch–deficient (SLP) mice. This study demonstrated that iBALTs that formed in the lung of SLP mice upon influenza virus infection were able to generate a robust primary T and B cell response to the virus and clear the infection. Influenza-specific $CD8\alpha^+$ T cells found in the iBALTs, together with $GL7^+PNA^+$ GC B cells, were able to support the production of virus-specific antibodies. Interestingly, SLP mice were able to survive higher doses of virus compared to their wild-type counterparts (153).

iBALTs have a structure very similar to SLOs containing segregated B and T cell areas, FDCs, HEVs, and lymphatic vasculature. In contrast, milky spots contain few T cells, and the CXCL13$^+$ stromal cells show a distinct pattern when compared to their counterparts in SLOs. Despite these histological differences, milky spots in SLP mice were able to support immune responses against protein antigens in the peritoneum (154).

Tertiary Lymphoid Organ Formation in Human Disease

TLO development occurs only postnatally and is associated with inflammation, cancer, and chronic antigen stimulation. During chronic inflammation, the resident stroma in target organs profoundly modifies its phenotype and function. Persistent antigenic stimulation, in the presence of a highly inflammatory cytokine milieu, enables the conversion of inflammatory stromal cells to a lymphoid tissue like–phenotype. This lymphoid tissue–like stroma is one of the key drivers of TLO organization.

TLOs are found in a wide variety of autoimmune diseases (e.g., RA, SS, and Hashimoto's thyroiditis) and in the target organs of a few chronic infections, such as *Helicobacter pylori* (inducing gastritis in the stomach), chronic hepatitis C virus (liver), Lyme disease (joint), and influenza A virus (lung) (8, 155–157). TLOs have also been described in chronic allograft rejection, in the local immune response to metal hip implants, in atherosclerosis, in allergic lung disease, and in cancer (8, 157, 158). TLO organization varies depending on the organ and site of the inflammatory disease. Broadly, human TLOs are organized with B cell follicles and, in some cases, distinct T and B cell areas. In a variable percentage of cases, depending mainly on the disease and site of formation, B cell follicles can evolve to fully formed GCs containing FDCs. TLO formation is most prominent in Hashimoto's thyroiditis and, to a lesser extent, Graves' disease (159–162); SS and RA exhibit a much lower frequency of GCs—with only 17% and 10–35%, respectively (8).

Analyses of chronically inflamed tissues from patients with SS or primary biliary cirrhosis have shown these tissues contain T cell areas with reticular networks of Pdpn$^+$ cells believed to share functional and phenotypical features of FRCs. The close association of FRC-like cells with ECM components such as collagen fibers, laminin, and fibronectin, within areas that costain for CCL21 and contain DCs, is indicative of the presence of a conduit system similar to that described in human thymus and lymph nodes, which carry blood-borne molecules and chemokines (163, 164).

Vascular structures within the T cell area of the TLOs differentiate into vessels that resemble HEVs. Those acquire the functional ability to facilitate the recruitment of CCR7$^+$L-selectin$^+$ naive T cells by upregulating peripheral node addressins (PNAd).

Similar to SLOs, chronically inflamed tissues that contain TLOs are characterized by ectopic expression of cytokines and homeostatic lymphoid chemokines such as LTα, LTβ, CXCL13, CCL21, CCL19, and CXCL12. Researchers have reported a correlation between expression of these cytokines and chemokines and an increase in TLO size and lymphoid organization in human autoimmune lesions, supporting a causative involvement of these mediators in TLO formation (8, 100, 101, 155, 165–170). Although different patterns of lymphoid arrangements usually coexist in the tissues of the same patient, TLOs harboring highly organized ectopic lymphoid follicles tend to express significantly higher levels of LTα, CXCL13, and CCL21 than those with diffuse lymphoid infiltrates (100, 101, 155, 165, 168, 171, 172). In fact, the expression levels of CXCL13 and LTβ/TNFSF3 may be highly predictive of the presence of ectopic GCs in synovial biopsies of patients with RA and SS (100, 155, 171, 173).

The persistent antigen presentation in the chronically inflamed sites seems to play a critical role in TLO formation and maintenance. This is shown by the absence of primary follicles (consisting

only of naive B cells) in inflamed areas and the fact that local production of immunoglobulins is restricted to a specific antigen (8).

The clinical relevance of TLOs in human disease is still unclear. Early studies performed on the synovia of RA patients showed that follicular synovitis was characterized by a more aggressive behavior and associated with a unique cytokine profile (high production of IFN-γ, IL-1β, and TNF) (174, 175). Researchers have revised this model recently, and they have not confirmed a clear association between TLO formation and poor disease outcome (176). The levels of the chemokine CXCL13 appear to correlate with disease severity and persistence of subclinical synovitis, as assessed by ultrasound (177). TLOs have also been found in the subchondral bone in erosive RA and implicated in local osteoclast activation and tissue damage (178).

In other autoimmune conditions, the persistence of TLOs has been associated with the development of locally arising lymphoma. Chronic antigenic stimulation seems to be able to induce the neoplastic transformation of lymphocytes recruited into ectopic GCs, leading to marginal zone lymphoid tissue–associated lymphoma in *H. pylori* gastritis and SS (179, 180).

During inflammation, lymphomas form preferentially within the TLOs and at very low frequencies within SLOs. This suggests that the combination of inflammation and ectopic lymphoid tissue neogenesis either supports the conditions for the generation of aberrant B cell clones or impairs the function of checkpoint mechanisms and structures that physiologically prevent lymphoma development in SLOs. Within SLOs, B cell expansion and maturation are tightly regulated. In contrast, diverse factors present in TLOs, such as continuous and excessive antigen/self-antigen presentation by nonspecialized antigen-presenting cells, aberrant expression of homeostatic chemokines, and prosurvival cytokines, may influence the maturation of autoreactive B cells and favor the escape of B cell clones characterized by abnormal proliferating features.

Although investigators have described ectopic lymphoid structures in various inflammatory disorders, TLOs are more frequently observed in chronic diseases affecting epithelial or mucosal tissues. Ectopic lymphoneogenesis has been described in the thyroid gland during Hashimoto's thyroiditis, in the intestine of IBD patients, and in the thymus of patients with myasthenia gravis (8). These data strongly advocate a role for the epithelium in TLO formation. In SS, the epithelium provides the early chemoattractive stimuli for the recruitment of immune cells in the glands (181). Nonetheless, epithelial activation alone is not able to promote TLO formation; indeed, there is no TLO formation in the skin, and the formation of TLOs in the kidneys of patients with lupus is debated (8). Taken together, these findings suggest the presence in the body of permissive and nonpermissive sites for TLO generation. To date, there is no agreement about the factors that regulate tissue permissiveness. The type of antigen and its presentation, the contribution of the local microenvironment and cytokine milieu, and the degree of T cell activation might all contribute to this phenomenon.

A series of reports provide evidence that synovial B cells from RA patients or B cells infiltrating salivary glands of SS patients have undergone somatic hypermutation in situ. This supports the idea that TLOs contain permissive environments for B cell differentiation into plasma cells that perpetuate the disease (158, 182, and references within).

In human pathology, it is not possible to dissect whether, as observed in SLOs, the increased chemokine expression by the stromal cell network is influenced in its function by the expanding number of $LT\alpha\beta^{+}TNF^{+}$ immune cells or whether the expansion of the lymphoid stroma precedes the recruitment of lymphocytes to the tissue. Critically, where established TLOs were formed in the tissue, treatments aimed at depleting lymphocytes have often failed to induce long-term remission (183), thus implying an active role for stromal cells in this phenomenon.

Animal Models of Tertiary Lymphoid Organ Formation

Analyses of TLOs in patients give a snapshot of these structures once they are fully formed. Several animal models that ectopically express various cytokines and chemokines in nonlymphoid tissues have been generated in order to understand the mechanisms and kinetics underlying TLO formation. These models have been very instructive and, surprisingly, have showed that expression of a single proinflammatory cytokine, homeostatic chemokine, or both is indeed sufficient to induce formation of TLOs.

Seminal work by Ruddle and her group (184–186) showed that ectopic expression of LTα under the control of rat insulin promoter (RIP) led to the formation of inflammatory lesions organized like lymphoid tissues containing mature stromal cell networks, separate T and B cell areas, FDCs, and HEVs in the pancreas and kidney of RIP-LTα transgenic (Tg) mice. LTα formed trimers that, upon binding to TNF-RI, induced the expression of CXCL13 and CCL21 that organized the lymphocyte areas of the TLOs in this mouse model. A similar process was observed in RIP–TNF Tg mice. Moreover, these TLOs appeared to be functional, as they responded to antigen, formed GCs, and promoted local isotype switching (184–186). Taken together, these models suggest that signaling through TNF-RI is sufficient to initiate TLO formation. Overexpression of LTα and LTβ in the pancreas under the control of the same promoter (RIP-LTα/LTβ Tg mice) results in the formation of the LTα/β ligand that, upon binding to LTβR, induces high expression levels of CXCL13, CCL19, and CCL21 and the formation of significantly larger TLOs than in RIP-LTα mice (187). In contrast to the RIP-LTα mice, RIP-LTα/β mice were characterized by luminal PNAd expression, thus maximizing the signals for naive T cell and DC recruitment in the aggregates (187). These studies represent the first line of evidence proposing the notion that TLO formation during inflammation involves the same signaling pathways driving the physiological development of SLOs during embryogenesis in a sterile environment.

Similarly, constitutive expression of homeostatic chemokines in pancreatic β cells results in TLO formation. Cyster, Luther, and colleagues (164, 188) generated RIP-CXCL13 Tg mice that developed ectopic lymphoid structures characterized by segregated B/T cell zones, $CD11c^+$ DCs, and a dense network of $BP\text{-}3^+$ ($CD157^+$) stromal cells that contained HEVs. Remarkably, CCL21 was detected on HEVs and in the stromal cells of the T cell area. TLO formation in this model is dependent on the presence of B cells, LTαβ, and LTi cells (164, 188). Likewise, pancreatic islets in RIP-CCL21 Tg mice contained infiltrates that consolidate into organized lymphoid tissues, with T cell/DC clusters and surrounding B cells, a mature stromal network, and vasculature that resembles HEVs (189, 190).

Lira and colleagues (191) demonstrated that ectopic expression of CCL21 in the thyroid gland was sufficient to induce TLO formation that resembles the structures seen in Hashimoto's thyroiditis and Graves' disease. The lymphoid structures in the thyroid of these mice were similar to the ones formed by expression of this chemokine in the pancreas. TLO formation in this model is independent of all ILCs (including LTi cells), as shown by crossing these mice in the $Id2^{-/-}$ background. $CCR7^+CD4^+$ T cells are the first cells to infiltrate the thyroid gland and appear to be essential for TLO formation as they cluster with host DCs. The cross talk between resident stroma and T cells appears to be critical for the recruitment of migratory DCs and clustering in the developing TLO. The growth of a lymphatic network in this model is dependent on LTβR signaling (192).

Importantly, neither of the two CCL21 Tg models described presents evidence for the development of $CD35^+$ FDCs or $CXCL13^+$ stromal cells, suggesting that CCL21 overexpression alone is not able to induce complete lymphoid tissue neogenesis (189, 190). Similarly, ectopic expression of CCL19 under the cytokeratin promoter was not sufficient to induce fully formed

lymphoid tissue, and RIP-CCL19 Tg mice developed TLOs that are smaller and less frequent than those seen in RIP-CCL21 Tg mice. RIP-CXCL12 Tg mice presented with small and unorganized infiltrates in the pancreas that were enriched in DCs, B cells, and plasma cells and contained relatively few T cells (193). Thus, CXCL13, CCL21, CCL19, and CXCL12 are not equal in their ability to promote TLO neogenesis, which may be due, in part, to their differential capacity to promote LTαβ expression on lymphocytes. CXCL13 has been shown to trigger LTαβ expression on B cells, whereas CCL21 and, to lesser extent, CCL19, but not CXCL12, can promote expression of this ligand in $CD4^+$ T cells. Interestingly, LTαβ expression can also be induced on naive T cells by IL-7 and IL-4, suggesting that there may be multiple ways to engage the LTαβ/LTβR/NF-κB/chemokine axis and trigger the formation of TLOs (193).

Coexpression of IL-6 and IL-6R under the MHC class I and β-actin promoters leads to the formation of iBALTs in the perivascular and peribronchial regions of the lung (155). Overexpression of IL-5 in the respiratory epithelium also results in development of organized iBALTs. However, unlike the models of homeostatic chemokines or TNF-family ligands mentioned above, which do not develop diabetes or thyroiditis despite TLO formation, the IL-5-dependent induction of iBALTs leads to epithelial hypertrophy, goblet cell hyperplasia, accumulation of eosinophils in the airway lumen and peribronchial areas, and focal collagen deposition, which are all signs of severe lung pathology (155).

Using a T cell Tg model of experimental autoimmune encephalomyelitis (EAE) that mimics multiple sclerosis, Peters and colleagues (194) demonstrated that Th17 cells induce ectopic lymphoid tissues in the central nervous system (CNS). The formation of these TLOs is partly dependent on IL-7 and Pdpn, which are both expressed by T cells. Th17 cells become $Pdpn^+$ once they enter the CNS, where they induce CXCL13 expression that organizes TLOs. Interestingly, blocking Pdpn in this model reduces the number of TLOs in the CNS, suggesting that both IL-17 and Pdpn may be therapeutic targets for the treatment of autoimmune diseases.

Collectively, these results indicate that ectopic expression of inflammatory cytokines or homeostatic chemokines in a permissive tissue is sufficient to induce TLO formation but not autoimmunity, tissue destruction, or both unless additional signals such as cognate antigen presentation, costimulation, and cytokines are provided to activate the infiltrating T cells. Evidence suggests that in some conditions, TLO formation is beneficial for disease outcome. Tg overexpression of LTα and the LTβR ligand named LIGHT (an acronym for homologous to lymphotoxins exhibiting inducible expression and competing with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes)/TNFSF14 driving TLO formation in animal models of melanoma and fibrosarcoma has been shown to promote tumor regression rather than spreading (195, 196). Similarly, there is no evidence that TLO formation during infection is deleterious. TLOs in infection may simply represent the immune system's attempt to deal with the pathogen locally in a contained and efficient manner.

Animal Models of Chronic Disease and Tertiary Lymphoid Organ Formation

Features of ectopic lymphoid neogenesis have been detected in animal models of autoimmunity. In nonobese diabetic (NOD) mice, highly organized TLOs form in the pancreas during the chronic phase of the disease. Formation of these TLOs follows a highly regulated process, with lymphoid aggregates characterized by T and B cell segregation, FDC networks, and differentiation of GC B cells during the progression from peri- to intrainsulitis in early diabetic mice. This process is preceded by local upregulation of LTαβ, LIGHT, and the chemokines CXCL13 and CCL19 (197). Moreover, researchers have found TLOs to be functional in supporting in situ autoreactive B cell differentiation, indicated by the expression of activation-induced cytidine deaminase, the

enzyme required for B cell affinity maturation and class switching, and the presence of plasma cells displaying anti-insulin reactivity (198).

In mouse models of atherosclerosis that develop adventitial aortic tertiary lymphoid organs (ATLOs), aorta smooth muscle cells acquired features similar to LTo cells and expressed VCAM-1, CXCL13, and CCL21 upon activation of the LTβR and TNF-RI-signaling pathways. Importantly, interruption of the LTβR signaling axis by administration of the LTβR-immunoglobulin fusion protein that serves as a receptor decoy suppressed CXCL13 and CCL21 expression, reduced HEV formation, and disrupted the structure and maintenance of ATLOs (199, 200).

Investigators have demonstrated that LTα expression in tumor cells leads to the formation of intratumoral lymphoid tissue able to sustain an efficient immune response, indicating that LTα-LTβR signaling plays a central role in the induction of functionally competent TLOs (158). Increased expression of CXCL13 and BAFF has been found in the meninges-associated ectopic GC formation in a mouse model of CNS inflammation (8) and in a spontaneous mouse model of autoimmune gastritis (155). Wengner et al. (201) have demonstrated in a mouse model of chronic antigen-induced arthritis that the development and organization of TLOs are impaired in the absence of CXCR5 and CCR7.

TLO functionality has been addressed in several murine models. Circumstantial evidence of naive T cell priming at the sites of TLOs includes the presence of a restricted T cell repertoire in melanoma-associated TLOs and T cell epitope spreading in the CNS during EAE. Where a correlation has been found between autoantibody serum levels and the presence of ectopic GCs, it has raised the possibility that TLOs can contribute directly to the disease process, independently of SLO activation (8, 155, 157, 158, 182).

What Are the Role and Origin of Stromal Cells in Secondary and Tertiary Lymphoid Organs?

Taking into consideration all the evidence mentioned above, one can state that stromal cells, fibroblasts in particular, contribute critically to the microenvironment in chronic inflammation, providing prosurvival and retention signals such as B cell survival factors (BAFF), IL-7, and inflammatory and homeostatic chemokines (e.g., CXCL8, CCL5, CXCL1, CXCL12, CCL21, and CXCL13). Pathogenic tissue fibroblasts therefore play a key role not only in immune cell recruitment but also in aggregate organization (202). This phenomenon, which leads to the generation of TLOs, potentially represents the attempt of targeted stromal cells to respond locally and efficiently to the increased request of survival factors for the incoming lymphocytes.

The central role of stromal cells in TLO formation has been shown in two different systems. Cupedo, Mebius, and colleagues (203) showed that intradermal injections of single-cell suspensions of neonatal lymph nodes in newborn and adult mouse hosts resulted in the formation of ectopic structures with induction of lymphoid tissue–like stromal cells that recruited host-derived lymphocytes and organized them into distinctive areas. Interestingly, however, injections of cell suspensions from adult lymph nodes failed to generate such structures unless a costimulatory signal or inflammatory stimuli were given. Likewise, grafting of stromal cell scaffolds under the kidney capsule of adult mice resulted in the generation of artificial lymph node–like structures that recruited lymphocytes and organized them into B/T cell segregated areas able to support immune responses (204). In both systems, the cross talk between donor immune cells and the recipient stroma is essential for lymphoid structure formation.

Peduto and colleagues (205) elegantly demonstrated that local resident fibroblasts gave rise to $Pdpn^+$ lymphoid tissue–like cells in experimental models of cancer or local inflammation induced by mechanical and inflammatory stimuli. Intriguingly, the induction of lymphoid tissue–like

stromal cells was shown to be independent of TLR or TNF-R signaling but linked to the presence and activation of polymorphonucleated cells in the initial phases of the inflammatory process. Resident fibroblasts undergo high levels of proliferation to create a stromal cell network expressing IL-7, CXCL13, CCL19, CCL21, CXCL12, RANKL, VCAM-1, BP-3, and Pdpn. Reticular networks of ERTR7$^+$, Pdpn$^+$, and BP-3$^+$ stromal cells, suggested to differentiate from tissue-resident myofibroblasts, have also been observed in pancreatic infiltrates of RIP-CXCL13 Tg, RIP-LT$\alpha\beta$ Tg, and NOD mice (164, 184, 187, 188).

Altogether, these results indicate that under inflammatory conditions, stromal cells undergo a profound phenotypical and functional modification that changes their tissue specialization to a lymphoid tissue–like characteristic able to provide local structure and survival mechanisms to the incoming immune cells. It is not clear whether this phenomenon occurs only on a small population of precursor cells in permissive tissues, such as myofibroblasts, that already express some markers of lymphoid tissue stromal cells and are stimulated to expand upon exposure to inflammatory cytokines. Alternatively, the local resting stroma or adipocyte precursors or MSCs/stromal cells may be the cells responding to a long exposure to cytokines and other factors expressed by a critical mass of immune cells recruited to the inflamed area; they may do so by differentiating to become the TLO stroma compartment. The evidence shown by Peduto and colleagues (205) strongly argues in favor of resident cells having the central role in TLO formation. We refer to the cells responding to the inflammatory stimuli to form TLOs as resident stromal cells.

Recently, Krautler et al. (206) showed that PDGFRβ^+ stromal-vascular cells from nonlymphoid organs can differentiate into FDCs upon LTβR and TNF-R triggering, suggesting that a similar population may represent the source of FDCs in TLOs. These stromal vascular cells localize in the same area as the adipocyte progenitor cells that become lymphoid tissue stromal cells upon LTβR signaling and the putative FRC precursor cells (127, 207).

Interestingly, a fully developed FDC network is not present in all human TLOs, and tissue permissiveness in these cases also influences the degree of specialization of the resident stroma. For example, in SS, only 20–40% of the inflammatory foci develop a fully formed GC, whereas in RA synovium, this percentage seems even smaller. The size of the lymphocytic aggregate may influence the differentiation of the resident stroma. Indeed, in human TLOs, researchers have demonstrated that the size of the ectopic aggregates correlates with their degree of organization. Moreover, the level of chemokine expression influences the degree of organization among aggregates of a similar size (100). Critically, treatments aimed at depleting lymphocytes in human conditions have partially failed where established chronic TLOs were present, thus suggesting that both stromal and leukocyte components should be targeted in TLO-associated pathologies (202).

Researchers have convincingly associated the expansion of the resident lymph node stroma with the development of a functional immune response against invading pathogens (208). Conversely, disruption of the stromal cell network has been observed upon severe chronic viral infection as well as the need for LTi cells to interact with stromal cells to restore lymphoid tissue architecture after infection (209, 210). A recent report (211) has identified the precursor of the LTo cells in spleen that gives origin to FRCs, FDCs, MRCs, and perivascular cells following the destruction of the stromal network as a consequence of the immune response to a viral infection.

Nonetheless, the functional consequences of the loss of stromal cell organization have been appreciated only recently. Fearon's group (212) has elegantly shown that conditional deletion of Pdpn$^+$FAP$^+$ FRCs in the early (but not established) phases of the response to influenza severely compromises the CD8-mediated host response. Similarly, deletion of LTβR in lymph node FRCs results in impaired immune responses to viral infections (131, 213). More recently, Turley and colleagues (214) demonstrated that FRCs contribute to adaptive immunity by regulating the niche

for B lymphocyte survival. The use of a similar strategy of conditional deletion of FRCs in TLOs would be highly informative of the role of lymphoid tissue–like stroma in chronic inflammation.

Stromal cells can influence the size and shape of the immune repertoire, not only by modulating the availability of the survival factors but also by inducing deletion or expansion of specific cell clones. This process, ultimately aimed at inducing peripheral tolerance, is achieved by stromal cell presentation of a range of peripheral tissue–restricted antigens (65, 215–217). Investigators have described a similar phenomenon in lymph node LVs, known to express the melanocyte-associated enzyme tyrosinase (65) and able to induce deletion of self-reactive $CD8^+$ naive T cell clones. Two reports have also shown that lymph node stromal cells also induce $CD4^+$ T cell tolerance by presenting with high levels of peptide-MHC-II complexes that appear to be acquired from DCs (207, 218). In addition, recent evidence indicates that stromal cell presentation of self-antigen in the context of MHC-II molecules is required to provide survival signals to antigen-specific Tregs (219).

Taken together these results demonstrate that induction of tolerance toward different peripheral tissue antigens is achieved not by a single cell type but by different stromal cells and by a variety of mechanisms. In addition, lymph node FRCs, upon inflammation, can produce nitric oxide (NO), a natural immunosuppressant, therefore influencing T cell expansion and priming (220–222).

Stromal cells also contribute to the tolerogenic microenvironment of the gut by providing the initial retinoic acid (RA) signal to induce the local generation of Treg cell–promoting DCs. This phenomenon has been observed specifically in mesenteric lymph nodes and in the gut lamina propria, where a nonepithelial $Pdpn^+CD31^-$ stromal cell population constitutively expresses the RA-converting enzymes, retinal dehydrogenase (Raldh) 1, 2, and 3 (223, 224). Expression of RA and granulocyte-macrophage colony-stimulating factor by stromal cells is sufficient to induce the conditioning of DCs that will result in both induced Treg generation and imprinting of gut-homing molecules on T cells (225). Peripheral conditioning of DCs has also been shown to be induced by intestinal epithelial cells through the production of RA and transforming growth factor β (226).

TLOs require chronic antigenic stimulation for their maintenance, and tolerance seems not to occur, suggesting that locally activated lymphoid tissue–like stromal cells in ectopic lymphoid tissues either have a different origin than their lymph node counterparts or have lost the capacity to present antigens and express NO to induce tolerance and immunosuppression. The mechanisms of this differential regulation are not known.

What Cells and Signals Are Required by Stromal Cells to Cooperate in Tertiary Lymphoid Organ Formation?

Similar to lymph node development during embryogenesis, TLO formation requires cross talk between immune and stromal cells. The evidence from different animal models of inflammation and TLO formation indicates that ILCs, including ILC3s/LTi cells, are not essential for the formation of ectopic lymphoid aggregates (144, 227, 228). A recent report has shown that TNF–TNF-RI/II–NF-κB signals are sufficient to induce TLO formation and the development of several lymph nodes, independently of the presence of ILC3/LTi cells and, to a certain extent, LTβR signals (227). However, the LTαβ/LTβR–NF-κB (p52/RelB) pathway is essential for chemokine expression and the corresponding lymphocyte recruitment and organization of B and T cell areas (227). In addition to TNF–TNF-RI/II and LTαβ/LTβR signals, other pathways can induce resident stromal cells to undergo a phenotypic change toward lymphoid tissue–like cells with a role for IL-17 in iBALT formation (147, 152, 205).

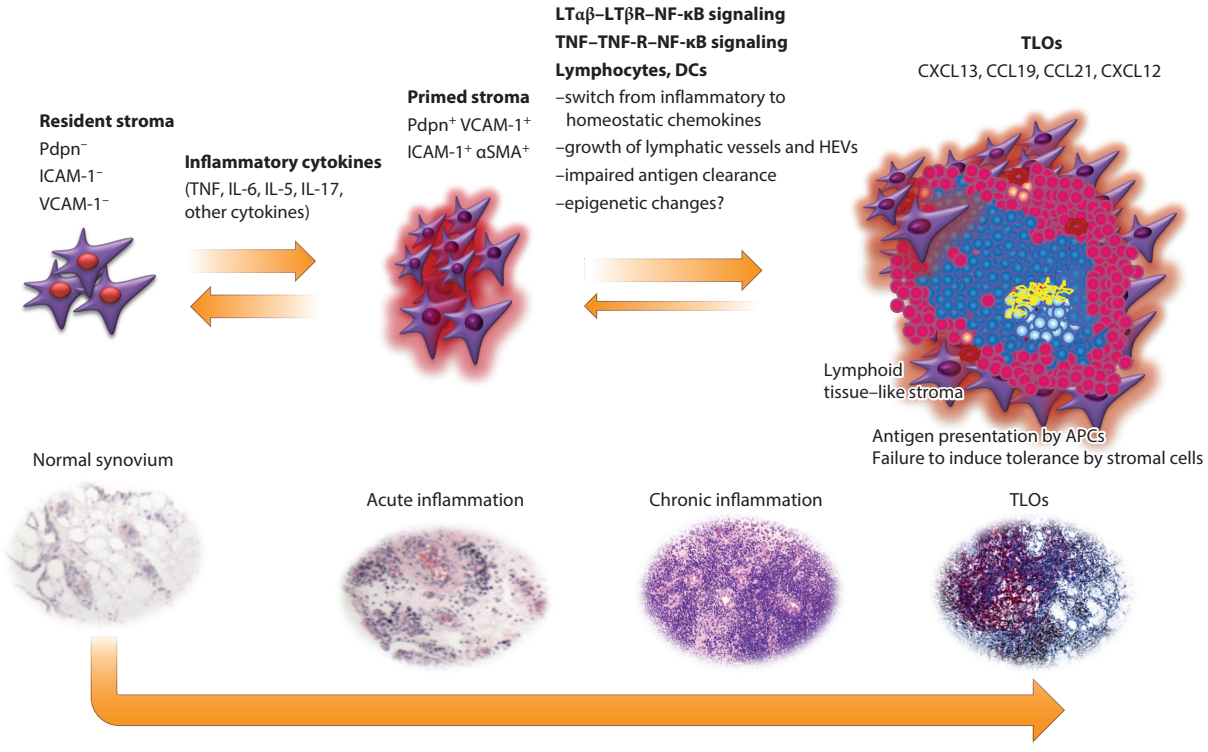

Figure 2

Model of stromal cell differentiation during chronic inflammation. During inflammation, resident stromal cells respond to inflammatory cytokines and express chemokines and cell adhesion molecules that attract neutrophils and macrophages to the site of injury or infection. Following antigen clearance, the cells return to their original phenotype. However, prolonged inflammation induces the primed stromal cells to acquire changes in their phenotype and become lymphoid tissue–like stromal cells, resulting in the formation of ectopic TLOs. (Abbreviations: αSMA, α-smooth muscle actin; APC, antigen-presenting cell; DC, dendritic cell; HEV, high endothelial venule; ICAM-1, intercellular adhesion molecule-1; LTαβ, lymphotoxin αβ; LTβR, lymphotoxin β receptor; NF-κB, nuclear factor-κB; Pdpn, podoplanin; TLO, tertiary lymphoid organ; TNF, tumor necrosis factor; TNF-R, TNF receptor; VCAM-1, vascular cell adhesion molecule-1.)

We propose a two-step model to describe the changes in stromal cells during inflammation leading to TLO formation. Inflammation induces the recruitment of immune cells to the target tissue. The presence of immune cells and the inflammatory cytokine milieu will induce resident stromal cells to become primed and express inflammatory chemokines. This step is reversible, and the stromal cells will return to their homeostatic status upon resolution of inflammation. Extended exposure to antigens and failure to resolve inflammation could trigger the second phase, in which contact of the primed stroma with a critical mass of LTαβ$^+$ immune cells induces a series of epigenetic changes in the latter that result in a switch to a functional lymphoid tissue–like phenotype in which homeostatic rather than inflammatory chemokines (as in the early phases of the inflammatory response) are secreted (see **Figure 2**). This phenomenon of lymphoid conversion of the stroma occurs alongside the recruitment of DCs, necessary for the expansion of the local lymphatic vasculature (151). Chronic antigenic stimulation or other mechanisms still under investigation then contribute to the stabilization of the TLOs in a process that appears to be very difficult to revert.

Lymphoid tissue stromal cells were viewed until recently as just providing support for the proper interactions of immune cells among themselves. In the last few years several studies have shown that stromal cells provide support and guidance for the migration of immune cells in lymphoid tissues and regulate immune responses through direct and indirect interactions with the latter cells. Increasing our understanding of the function of stromal cells in the homeostasis of lymphoid tissues and their role in regulating immune responses is essential to unravel their role in supporting TLO formation during chronic inflammatory diseases. In this regard, a series of questions remain to be answered:

- What specific markers identify stromal cells in TLOs?
- Do adipocyte progenitors, mesenchymal stem cells, and/or perivascular cells migrate and contribute to the formation of the stroma in TLOs?
- Are neurons and/or retinoic acid involved in inducing the expression of CXCL13 and other chemokines that contribute to TLO formation?
- What is the role of IL-7 and IL-17 in TLO formation? Some cases suggest a role for both cytokines. Are other cytokines involved in TLO formation and maintenance?
- What signals mediate the interactions between endothelial cells and fibroblasts during chronic inflammation? Can these signals be used as therapeutic targets to block or resolve TLO formation in chronic inflammatory diseases?
- What signaling pathways can be manipulated in TLO stromal cells to unravel their antigen-presenting properties to induce tolerance, generation of regulatory T cells, and NO expression?
- Can stromal cells in TLOs be induced to support antitumor immune responses?

SUMMARY POINTS

1. Given that all inflammatory reactions take place within a defined background of specialized stromal cells, understanding the biology of these cells and how they relate to stromal cells in lymphoid tissues is critical to appreciating how immune cell infiltrates become established and persist in chronic immune-mediated inflammatory diseases.
2. Populations of leukocytes recruited to sites of inflammation should not be considered in isolation but in conjunction with stromal cells that provide survival, differentiation, and positional cues upon which the formation and persistence of immune cell infiltrates depend.
3. Inflammation is a contextual, not a generic, process, and the divergent responses of different inflammatory diseases to therapy are likely to be due to the intrinsically distinct origins and gene expression programs that will direct the behavior of fibroblasts within microenvironments.
4. TLOs are commonly found in chronic autoimmune diseases; in some studies of these diseases they are associated with worse outcome and comorbidity development. Given their critical role in TLO development and maintenance, we propose that stromal cells should be targeted alongside the leukocyte component in TLO-associated pathologies.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The work was supported by a BBSRC grant, the EU FP7 integrated project INFLACARE, and the College of Medical and Dental Sciences at the University of Birmingham (J.C.), and by the Wellcome Trust (F.B.).

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