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A stromal address code defined by fibroblasts

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Abstract

To navigate into and within tissues, leukocytes require guidance cues that enable them to recognize which tissues to enter and which to avoid. Such cues are partly provided at the time of extravasation from blood by an endothelial address code on the luminal surface of the vascular endothelium. Here, we review the evidence that fibroblasts help define an additional stromal address code that directs leukocyte behaviour within tissues. We examine how this stromal code regulates site-specific leukocyte accumulation, differentiation and survival in a variety of physiological stromal niches, and how the aberrant expression of components of this code in the wrong tissue at the wrong time contributes to the persistence of chronic inflammatory diseases.

The recruitment of cells into tissue occurs across vascular endothelium and requires a combinatorial set of cellular interactions involving capture receptors (selectins), activation molecules (chemokines) and adhesion receptors (integrins). Collectively, these comprise an endothelial address code, which provides a regulatory mechanism for organ-specific trafficking of leukocytes (reviewed in Ref. [1]). Although there is overwhelming experimental support for this site-specific recruitment model, it does not address how leukocyte navigation is regulated beyond the vascular endothelium, within the tissue itself.

Once a cell has been recruited into tissue, it must make several decisions – should it briefly scan the stromal environment on its way out via draining lymphatic vessels or should it remain in the interstitium for an extended period? Should it differentiate, proliferate or die? Recent evidence suggests that stromal cells, such as fibroblasts, directly affect the behaviour of infiltrating cells by providing retention, differentiation and exit codes analogous to the endothelial entry code.

Vascular address codes

Naïve lymphocytes continuously cycle through the vascular and lymphatic circulation until they encounter their cognate antigen, displayed by professional antigen-presenting cells, in lymph nodes. They gain entry to lymph nodes because they express the correct address code: high levels of L-selectin and the chemokine receptor CCR7 [2-5]. These molecules recognize ligands, which are found on the lumenal side of lymph node high endothelial venules (HEVs). CCR7 engagement causes cells to adhere firmly to the HEVs through integrins, enabling their transit through the endothelium into the lymph node (Figure 1).

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Antigen-experienced B cells become unresponsive to lymph node cues and cease to recirculate, partitioning into specific niches, such as the red pulp of the spleen and the bone marrow [6]. By contrast, central memory T cells (which retain CCR7 expression) continue to recirculate like naïve cells to lymph nodes, whereas effector memory T cells traffic through tissues [7]. Some memory T cells preferentially recirculate to the tissue in which they were initially activated – a site where they are most likely to re-encounter their cognate antigen. However, such well-defined patterns of homing have thus far only been described for the skin and the gut [8] (Figure 1).

Dendritic cells (DCs) communicate tissue identity to T cells

There is now good evidence to suggest that T cells are imprinted with an endothelial address code as they undergo activation in the lymph node, and that a cognate interaction with interdigitating DCs is sufficient for this process (reviewed in Ref. [9]).

DCs isolated from various lymphoid organs all share the ability to activate $CD8^+$ T cells but only those taken from Peyer's patches (PPs) imprint them with the gut-homing code ($\alpha_4\beta_7$, $CCR9^+$) [10]. The positional identity of DCs appears stable enough to withstand *ex vivo* culture. Furthermore, Stagg *et al.* showed that DCs possess an instructive accessory component that is not dependent on cognate T-cell receptor (TCR)–MHC–peptide interaction and can act *in trans* [11]. Recently, Iwata *et al.* have suggested that retinoic acid might be such an accessory signal generated by intestinal DCs to induce a gut-homing phenotype in T cells [12].

An elegant study by Dudda *et al.* demonstrated that bone marrow-derived DCs injected intracutaneously can induce $CD8^+$ T cells that express the cutaneous vascular entry code [CLA^+ (cutaneous leukocyte antigen)]. T cells induced after intraperitoneal injection of the same bone marrow-derived DCs, however, expressed the intestinal entry code (integrin $\alpha_4\beta_7$) [13]. Although the possibility that endogenous tissue resident cells have a direct role in imprinting T cells to return to the injection site cannot be formally excluded, this strongly suggests that DCs are plastic enough to acquire tissue-specific properties from their local microenvironment. So what tells a DC, or any other bone marrow-derived cell, that it is in a particular tissue microenvironment?

Address codes are sequentially encountered

Leukocytes arriving in interstitial tissue exhibit different properties to their blood-borne counterparts. In particular, their ability to respond to stromally derived cues for proliferation, differentiation, positioning and survival is likely to be radically altered [14]. Despite the inherently sequential nature of leukocyte trafficking, the effects of transendothelial migration on leukocyte behaviour within tissues remain poorly characterized.

For obvious experimental and technical reasons, *in vitro* studies of leukocyte-stromal interactions have neglected the effects of prior vascular and lymphatic endothelial transit on the subsequent behaviour of leukocytes. Likewise, the cellular conversations that take place between endothelia and underlying stroma remain largely uncharacterized, although *in vivo* data suggest that they could be crucial to defining the vascular address code, by transcytosis and vascular expression of stromally derived chemokines [15]. There is now a large body of evidence to support the concept that the proinflammatory behaviour of endothelial cells is affected by the type of matrix on which they grow and the nature of the underlying stromal cells (reviewed in Ref. [16]).

Rainger *et al.* have recently developed a novel flow-based co-culture system, which directly allows the effects of stromal cells on overlying endothelium to be described [17]. This

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model has shown that stromal cells, such as smooth muscle cells and fibroblasts, prime the overlying endothelium and lead to an alteration in the pattern of leukocyte recruitment across the endothelium [16,17].

Leukocyte migration from peripheral tissues to local lymph nodes requires movement through the lymphatic endothelium in a baso-lumenal direction. The important discovery of unique markers for lymphatic endothelium, such as the hyaluronic acid receptor LYVE-1 and vascular endothelial growth factor receptor 3 (VEGFR3), has facilitated the isolation and molecular characterization of lymphatic endothelial cells [18,19]. This now provides an opportunity to further investigate their contribution to stromal address codes experimentally, and to determine if an exit code exists for the lymphatic system analogous to the entry code on vascular endothelium (reviewed in Ref. [20]) (Figure 2).

Fibroblast diversity, autonomy and positional identity

Fibroblasts isolated from different tissues display different functional properties (reviewed in Ref. [21]). Consistent with the varying biophysical requirements of different tissues, phenotypic differences in the well-known structural functions of fibroblasts, such as migratory capacity, extra cellular matrix (ECM) production and degradation and contractility, have been reported. The less well-known immunomodulatory functions of fibroblasts are also known to vary according to anatomical site and disease status [22].

The molecular basis for such fibroblast diversity has only recently been addressed. Global transcriptome analysis of a panel of human fibroblasts has been performed by several independent research groups [23,24]. Comparative analysis revealed that the transcriptional profiles of fibroblasts can be clustered into well-defined groups defined by anatomical site. Furthermore, specific homeobox (HOX) gene expression is very strongly associated with the cluster groups [24]. HOX genes encode important transcription factors that confer regional identity to tissues [25]. This provides support for the concept that fibroblasts possess positional identity that is transcriptionally imprinted, and mechanistic evidence to explain long-standing observations of stable fibroblast diversity in the adult, even within a single tissue type.

Such diversity is not unique to the fibroblast element of tissue stroma. Vascular endothelial cells cultured from different anatomical sites also show a wide diversity of transcriptional and proteomic profiles [26,27]. In addition, a similar approach has revealed important biological differences between vascular and lymphatic endothelium [28]. Other stromal elements, such as epithelial cells, smooth muscle cells and pericytes, are likely to be as varied as fibroblasts.

It seems plausible that tissue-transiting cells, such as lymphocytes and DCs, receive instruction about their anatomical position, at least in part, from endothelial cells, stromal fibroblasts and their secreted products. Equally, tissue-specific epithelia and other specialist cells with positional identity, such as macrophages, might contribute to the instructional process. Fibroblasts are particularly well-suited to define positional identity because they exhibit considerable autonomy. Members of the fibroblast family are known to function as key organizers at the heart of the immune system, for example, during lymph node development and the presentation of antigen by follicular DCs in germinal centers [29].

Stromal address codes in leukocyte development

Fibroblast-like stromal cells are required to support effective haematopoiesis and help define the bone marrow stromal niche [30]. Recent work has revealed that the expression of a few crucial molecules can support leukocyte survival, regulate leukocyte position and control

leukocyte differentiation in a variety of stem-cell niches, including the bone marrow, thymus and lymph node. These molecules appear to constitute part of a stromal address code that consists of a homeostatic or constitutive chemokine {CXCL12 (SDF-1), CXCL13 [B cell-attracting chemokine-1 (BCA-1)], CCL19 [EBI1 ligand chemokine (ELC)] or CCL21 [secondary lymphoid-tissue chemokine (SLC)]} together with VCAM-1 (CD106), in the presence of a cytokine or growth factor [(interleukin-6 (IL-6), IL-7 and fibroblast growth factor-7 (FGF-7) or FGF-10] (Figure 3).

(i) Bone marrow

In the bone marrow, CXCL12 is expressed by a subset of fibroblasts that co-express VCAM-1 but not IL-7 [31]. The interplay of CXCL12 and VCAM-1 is essential in regulating the release of B cells into the bloodstream and their maturation. Genetic ablation or functional blockade of the unique CXCL12 receptor, CXCR4, leads to premature release of both granulocyte and B-cell precursors [32,33], whereas genetic deletion of the α_4 integrin subunit prevents early B-cell lymphopoiesis, suggesting that VCAM-1 is also likely to be essential in retaining cells in the bone marrow [34].

Tokoyoda *et al.* have elegantly demonstrated that multipotent haematopoietic stem cells and pre-pro-B cells associate specifically with the $CXCL12^+$ fibroblast subset and that CXCR4 engagement on the pre-pro-B cells increases their adhesiveness to VCAM-1 through $\alpha_4\beta_1$ integrin [31]. Interestingly, $CXCL12^+$ and $IL\text{-}7^+$ stromal subsets are spatially separated but do not have a clearly compartmentalized distribution. Upon maturation from pre-pro-B cell to pro-B cell, migration away from $CXCL12^+$ towards $IL\text{-}7^+$ stromal cells is observed. A similar role for $CXCL12^+$ in the retention of immature neutrophils and the subsequent recall of senescent neutrophils to the bone marrow has recently been demonstrated [33].

The $CXCL12^+$ bone marrow fibroblast niche also provides a resting place for terminally differentiated B cells (plasma cells). Long-lived plasma cell survival and function are highly dependent on their close association with bone marrow fibroblasts [35]. An $\alpha_4\beta_1$ integrin-mediated interaction, which does not appear to use VCAM-1 as the ligand, is reported as essential to the secretion of IL-6 from fibroblasts. Together with engagement of plasma cell CD44, a receptor for the extracellular matrix component hyaluronic acid (HA), IL-6 promotes the antibody-secreting function of long-lived plasma cells. CXCL12 is responsible for attracting the plasma cells back to their particular stromal niche, and like IL-6, is a potent survival factor for them [36].

(ii) Thymus

Mesenchymal fibroblasts are now known to have at least two vital roles in thymic function. First, they are required to support the proliferation of thymic epithelial cells through the release of FGF-7 and FGF-10 [37]. Second, in early T-cell development they are necessary for the appropriate presentation of IL-7 on the extracellular matrix [38].

In the postnatal thymus, $CXCL12^+$ and $VCAM\text{-}1^+$ cortical fibroblasts are crucial for attracting and supporting the migration of early T-cell progenitors from the thymic medulla to the cortex [39,40]. Repositioning of thymic progenitors is necessary for their maturation, which relies on sequential signals from phenotypically distinct thymic epithelia. After positive selection in the cortex, T cells in neonatal mice depend on CCR7 ligands to reverse their direction of movement, back into the medulla where negative selection can take place [41]. Thus, stromal fibroblasts and epithelia provide adhesive substrates, chemotactic cues, differentiation stimuli and proliferative signals to developing T cells. The positional identity of these cells is clearly pivotal to compartmentalizing the cortical and medullary microenvironments of the thymus.

(iii) Peripheral lymph nodes

Although the fundamental role of the fibroblast as choreographer is similar, the stromal address code necessary for the generation of secondary lymphoid tissue differs mechanistically from that required for leukocyte retention and differentiation in primary lymphoid tissues, such as bone marrow and thymus. VCAM-1^+ $LTbR^+$ fibroblastic 'organizer' cells secrete the homeostatic chemokine CXCL13, attracting and activating $\alpha_4\beta_1$ integrin on $CXCR5^+LT\alpha_1\beta_2^+CD3^-CD4^+CD45^+$ 'inducer' cells. When these cells come into contact a positive feedback loop is initiated whereby lymphotoxin-β (LT-β) receptor engagement, stabilized by VCAM-1 ligation, leads to upregulation of CXCL13 and VCAM-1 expression, and consequently an increased recruitment of inducer cells [42-44] (reviewed in Ref. [29]).

Transgenic mouse studies have revealed a hierarchy among the homeostatic chemokines in their ability to support lymphoid neogenesis. Ectopic expression of CXCL13 in pancreatic islet cells suggested it was the most efficient, followed by CCL21, CCL19 and finally CXCL12 [45]. However, the choice of tissue for ectopic expression affects the outcome, suggesting that certain microenvironments (pancreas) are more plastic than others (brain and skin) [46,47].

Pathological stromal codes

An increasing number of studies suggest a key pathological role for the ectopic temporal and spatial expression of cytokines and chemokines in diseases, such as rheumatoid arthritis (RA), autoimmune liver disease, thyroid disease and diabetes [48-50]. These chronic immune-mediated inflammatory conditions are characterized by the abnormal persistence and continued episodic recruitment of infiltrating inflammatory cells, which is accompanied by a local expansion and activation of fibroblasts in the diseased tissue [48]. The fibrotic tissue gradually acquires an extremely stable state that leads inexorably to loss of tissue function and in several diseases the tissue becomes organized into lymphoid-like structures that have been called 'tertiary lymphoid tissue' [51].

These observations, in conjunction with data from transgenic mice that overexpress chemokines involved in lymphoid organogenesis, have led to the proposal that chronic inflammation is lymphoid neogenesis in the wrong place at the wrong time [52,53]. Applying this concept more globally predicts that the aberrant expression of other components of the stromal address code involved in defining other stromal niches, such as the bone marrow and thymus, might also contribute to pathogenesis in chronic inflammation.

Many research groups have now reported findings that support this concept of an ectopic, aberrant stromal code. Fibroblast-derived CXCL12 and CXCL13 are overexpressed in the rheumatoid arthritis joint [53,54] and in a range of other chronic inflammatory immune mediated diseases [55-57]. Transforming growth factor-β (TGF-β) found at high levels in the inflamed synovium, induces the expression of CXCR4 on highly differentiated T lymphocytes and this might provide a mechanism for their retention at this site [54]. The prolonged survival of these highly differentiated T cells is promoted by the secretion of type-I IFNs by synovial fibroblasts and macrophages [58]. Of interest, synovial fibroblasts isolated from the rheumatoid synovium also express high levels of VCAM-1 and IL-6 [59,60].

Dysregulated expression of constitutive chemokines has also been reported in the salivary glands in Sjogren's syndrome, in the liver in cirrhosis and in the thyroid in Hashimoto's thyroiditis [49,61,62].In a considerable proportion of cases, organized lymphoid aggregates

with lymphocytes expressing the appropriate cognate chemokine receptor are also found. In other words, the diseased stromal tissue expresses an inappropriate lymphoid stromal code. Why this occurs remains unclear but recent advances in our understanding of the origins of fibroblasts have begun to provide some tantalizing clues.

Origins of fibroblasts: mesenchymal proliferation, local transdifferentiation from epithelium or seeding from bone marrow?

Despite increasing evidence for their crucial immunomodulatory functions, fibroblasts are still widely thought of as primary mesenchymal cells that are deposited in tissue interstitia, simply as a consequence of organ development. It is not disputed that their developmental origin is the primary mesenchyme, and that fibroblasts can proliferate to generate new fibroblasts, however, emerging evidence suggests that immature fibroblasts are not phenotypically equivalent to primary mesenchyme. This might imply that they are not functionally equivalent either.

After unsuccessful attempts to identify fibroblast-specific markers by a conventional antibody approach, an elegant subtractive hybridization strategy using transcripts from fibroblasts and epithelial cells from the same microenvironment, identified fibroblast-specific protein (FSP1), also called S100A4 (Mts1) [63]. Iwano *et al.* demonstrated that $FSP1^+$ fibroblasts can be derived from local secondary epithelia in response to physical injury or inflammation in adult mice; a phenomenon well known to developmental and cancer biologists, called epithelial to mesenchymal transition [64]. This has led this group and others to propose that, in several tissues (lung, gut, kidneys), new fibroblasts can arise from epithelial structures.

A third pathway by which fibroblasts can be generated is from precursor cells called fibrocytes. These represent a small population (~0.5%) of circulating blood cells that co-express markers of the haematopoietic ($CD34^+CD45^+$) and mesenchymal (vimentin$^+$ collagen I^+) lineages and appear to be derived primarily from $CD14^+$ peripheral blood monocytes [65]. Fibrocytes rapidly gain entry to sites of tissue damage, where they exhibit the contractile function of myofibroblasts and secrete extracellular matrix proteins to promote wound healing [14,65-67]. *In vivo* the homeostatic chemokines CCL21 and CXCL12 regulate fibrocyte traffic into tissues in the context of wound healing and pulmonary fibrosis [65,68]. Interestingly, there is substantial evidence to suggest that fibroblast proliferation and matrix deposition occur independently in idiopathic pulmonary fibrosis. This is also the case in rheumatoid arthritis, suggesting that tissue damage and inflammation can be uncoupled (reviewed in Refs [48,69]).

Therefore, it is now clear that fibroblasts can be derived in at least three different ways: locally (by proliferation or transdifferentiation) and distally (from the bone marrow). In some organs and models of inflammation (e.g. experimentally induced renal fibrosis), all of these processes appear to contribute to the pathological accumulation of fibroblasts [64]. These findings need to be reconciled with the work of Chang *et al.*, who found stable transcriptionally imprinted positional identity in fibroblasts from normal human tissue [25]. Do fibroblasts from different sources have intrinsic positional imprints or are they adaptable to new microenvironments, as DCs appear to be? As the lineage relationships and differentiation potentials of human fibroblasts become more clearly defined, it will be important to determine whether distally or locally derived fibroblasts predominate in different fibrotic diseases, and whether this depends on the presence or absence of epithelium in target organs.

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Concluding remarks

The prevailing paradigm accounting for the accumulation of specific leukocyte subsets in an inflamed tissue is based on endothelial selectivity at the point of recruitment. However, this ignores the role of selective retention within the tissue, mediated by a stromal address code that is defined, at least in part, by fibroblasts. A combinatorial stromal address code involving homeostatic or constitutive chemokines (CXCL12, CXCL13, CCL19, CCL21), adhesion molecules (VCAM-1) and cytokines (IL-6 and IL-7) is found at key sites of the immune system, in the bone marrow, thymus and lymph node, where it regulates leukocyte maturation and lymphoid organogenesis. The inappropriate expression of some or all of the components of this physiological stromal address code is likely to be involved in the switch from resolving to persistent inflammatory disease. Fibroblasts are able to impose a stromal address code on tissue-infiltrating leukocytes, which governs their accumulation, survival and differentiation. Exactly how the stromal address code becomes stably altered is unclear, although it is possible that transdifferentiating epithelial cells and infiltrating fibrocytes mistakenly acquire the stromal address code of lymphoid tissues, rather than the appropriate code for the microenvironment they have entered. Episodes of injury and inflammation are traumatic times for fibroblast regional identity – particularly if the appropriate stromal area code is not re-established correctly.

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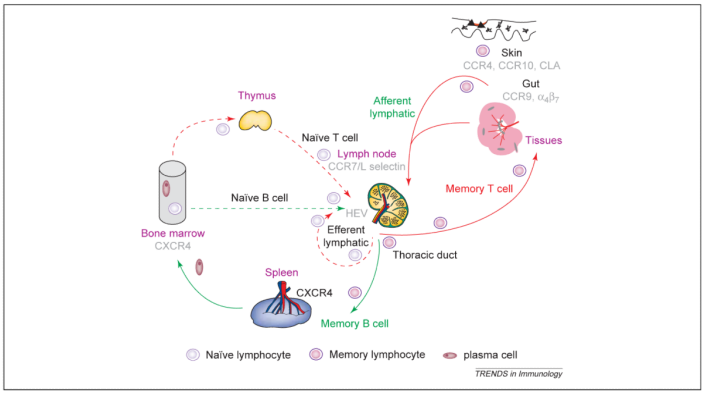

Figure 1.

Vascular codes provide for compartmentalization within the immune system. During the development of an immune response, immature naïve T cells (red dotted arrows) and B cells (green dotted arrows), which have been educated in the thymus and bone marrow, respectively, enter lymph nodes through the high endothelial venules (HEVs) using the endothelial entry code CCR7 and L-selectin (area codes are shown in grey). Antigen-experienced B cells (green arrows) leave the lymph node and migrate into specific niches, such as the red pulp of the spleen and the bone marrow, using the endothelial entry code CXCR4. Antigen-experienced central memory T cells continue to recirculate back to the lymph nodes via the efferent lymphatics and thoracic duct (red arrows; dot and dash). Effector memory T cells preferentially recirculate to the tissue in which they were initially activated; for example, if they are activated in a lymph node draining the skin they acquire the skin entry code CCR4, CCR10, CLA (grey) and if activated in a lymph node draining the gut they acquire the gut entry code CCR9, $\alpha_4\beta_7$. At the end of immune surveillance within tissues, memory T cells can recirculate back to the lymph nodes via the afferent lymphatics (red arrows).

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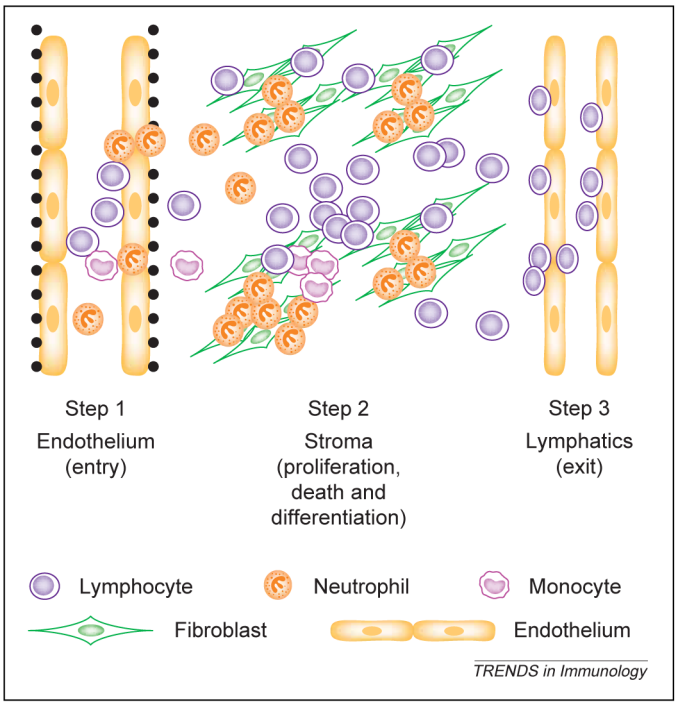

Figure 2.

Leukocyte–stromal interactions are involved at every stage in lymphocyte recirculation. The molecular basis by which leukocytes leave the circulation and migrate across endothelium has been well studied (step 1, endothelium). Leukocytes also interact with the basal lamina, matrix proteins and pericytes surrounding the endothelium (black dots), before entering interstitial tissue (step 2, stroma), where they interact with tissue stromal cells, such as fibroblasts. How leucocytes exit tissue into the lymphatics (step 3, lymphatics) remains poorly understood. Each step in the process delivers different types of instructions to the leukocyte. Step 1 regulates entry; step 2 regulates proliferation, survival and differentiation and step 3 regulates exit.

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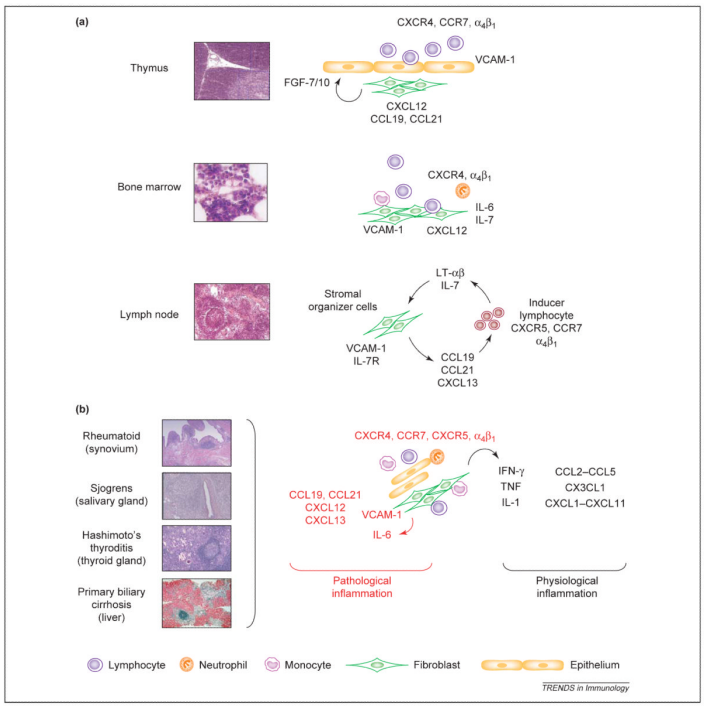

Figure 3.

(a) Stromal area codes regulate leukocyte accumulation, differentiation and survival in the thymus, bone marrow and lymph node. Homeostatic chemokines (CXCL12, CXCL13, CCL19, CCL21), adhesion molecules [vascular cell adhesion molecule-1 (VCAM-1)] and cytokines and growth factors [interleukin-6 (IL-6), IL-7, fibroblast growth factor-7 (FGF-7), FGF-10] are components of the stromal code that help define stromal niches (bone marrow, thymus and lymph node) involved in leukocyte accumulation, differentiation and survival. Fibroblasts produce the appropriate cytokine or chemokine and express the appropriate adhesion receptor that is recognized by cognate receptors on the infiltrating leukocytes. In the case of the developing lymph node, 'inducer' lymphocytes produce lymphotoxin-α and IL-7, which induce the secretion of constitutive chemokines from the stromal 'organizer' cells, leading to the development of lymphoid aggregates with a lymph node structure. (b) Components of the stromal area code are aberrantly expressed in disease. During physiological inflammation, inflammatory chemokines (CCL2-CCL5, CX3CL1 and CXCL1-CXCL11) and inflammatory mediators, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-1, are produced by stromal cells and lead to the recruitment of inflammatory cells (lymphocytes, neutrophils and monocytes). During the resolution phase of inflammation, these proinflammatory stimuli are removed. However, in chronic persistent inflammation (pathological inflammation), stromal cells in tissues, such as the synovium, salivary gland, thyroid and liver, begin to aberrantly produce or express components of the physiological stromal code normally associated with primary and secondary lymphoid tissue, leading to the generation of pathogenic tertiary lymphoid tissue.