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Ectopic lymphoid organs and immune-mediated diseases: molecular basis for pharmacological approaches

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Abstract

Chronic inflammation is the result a persistent increase in the expression of several pro-inflammatory pathways with impaired inflammatory resolution. Ectopic lymphoid organs, abnormal lymphoid annexes, emerge during chronic inflammation, and contribute to the physiopathology of chronic inflammatory disorders. This Review discusses the available data about the pathophysiological role of ectopic lymphoid organs in the progression of immune-mediated inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel and respiratory diseases, atherosclerosis, Sjögren syndrome. The molecular pathways underlying the emergence of ectopic lymphoid organs are of interest for the development of novel pharmacological approaches for the management of chronic inflammatory diseases.

Inflammation is the body's defense mechanism resulting from the coordinated activation of immune and non-immune signaling pathways triggered by harmful stimuli (i.e. infections, tissue injury, toxic compounds or irradiation)(Medzhitov, 2010). Once the harmful stimulus is eliminated by the inflammatory process, a coordinated process involving different type of cells, cytokines and repairing mechanisms restores the organ's homeostatic status, a process known as the resolution of inflammation (Medzhitov, 2010). Disturbances in the resolution of inflammation can contribute to a variety of chronic inflammatory diseases, collectively named **immuno-mediated inflammatory diseases (IMIDs)** (Scrivo et al., 2011). Indeed, a number of heterogenic disorders, such as multiple sclerosis (MS), rheumatoid arthritis (RA), uveitis, myasthenia gravis, psoriasis, scleroderma, systemic lupus erythematosus (SLE), glomerulonephritis, chronic obstructive pulmonary disease (COPD), asthma, inflammatory bowel diseases (IBDs) are associated with impaired inflammatory resolution, leading to amplification and perpetuation of inflammation in target organs, as well as a plethora of systemic effects (David et al., 2018). However, recent evidence suggests that the classification of chronic inflammation dependent from inability to remove the inflammatory trigger or from a deficit of the resolution process might represent an excessive simplification as there are forms of chronic inflammation where the inflammatory trigger is effectively removed but the inflammatory system maintain memory of its presence and is continuously stimulated to the activation (Sugimoto et al., 2016).

Over the years, increasing evidence has accumulated to show that IMIDs are often associated with peculiar histological appendages, consisting of abnormal lymphoid annexes, later referred to as ectopic or tertiary lymphoid organs, which originate and develop at sites of inflammation in target tissues of the IMIDs (see Table 1) (Corsiero et al., 2016). Ectopic lymphoid organs, analogous to secondary lymphoid organs, are organized aggregates of lymphocytes also containing antigen-presenting cells, lymphatic sinuses, high endothelial venules, follicular dendritic cells, and **fibroblastic reticular cells** (Furtado et al., 2014). However, such ectopic lymphoid annexes differ

from normal lymph nodes since they generally do not have a capsule, are not confined to a fixed location in the body, develop post-natally and exhibit plasticity (Corsiero et al., 2016). In parallel, the lack of a solid stromal cell infrastructure in these abnormal annexes do not allow an adequate anatomical compartmentalization of cells of the immune system, thus subverting immune cell homeostasis (Barone et al., 2016). Indeed, the presence of a microenvironment, enriched in survival factors and pro-inflammatory cytokines, but missing in key checkpoints for autoreactive cell screening, seems to be at the core of a machinery for local generation of pathogenic autoantibodies, thereby contributing to the development and progression of many IMIDs (Carragher et al., 2008). In addition, there is emerging evidence that the condition of **inflammaging**, a chronic, sterile, low-grade inflammatory condition, which contributes to the pathogenesis of several age-related disorders, seems to be sustained also by such lymphoid tissue abnormalities (see BOX 1).

The availability of experimental animal models of IMIDs contributes to better understand the mechanisms underlying these diseases, to identify immune cell subsets involved in the modulation of ectopic lymphoid organs, and to improve our knowledge on the role of these structures in the inflammatory processes (Jones et al., 2016). Given the fact that all of the cell types present in ectopic lymphoid organs are also important for host defense against invading microorganisms, helminths, tumor cells and other noxious stimuli, one of the major challenge for future research is to identify pharmacological tools able to interfere selectively with these cells in ectopic lymphoid organs without compromising the systemic immune response and the integrity of the surrounding tissues (Hughes et al., 2016).

With this review, we intend to summarize the main determinants for ectopic lymphoid organ onset and development, pointing out their relevance in various immune-inflammatory diseases, and identifying molecular targets useful to develop novel therapeutic interventions in ectopic lymphoid organ-associated diseases.

Secondary lymphoid organs: the hubs of adaptive immune responses

The immune system is functionally compartmentalized into primary (bone marrow and thymus) and secondary lymphoid tissues (lymph nodes, tonsils, spleen, Peyer's patches and mucosa associated lymphoid tissue) where immune responses are initiated and maintained (Zhao et al., 2012). In particular, secondary lymphoid tissues are the hub of adaptive immune responses wherein rare cognate lymphocytes encounter dendritic cells bearing antigen from peripheral tissues and differentiate into effector and memory cells responsible for eliminate antigen (Ng and Chalasani, 2010).

During the initiation phase for the development of all lymphoid annexes, retinoid acid produced by nerve endings at predetermined anatomical sites, induces the expression of the chemokine CXCL13 by the mesenchymal cells (Kain and Owens, 2013)(Figure 1). Thus, the CXCL13 gradient leads to attract in the area the $CD3^{-}CD4^{+}CD45^{+}$ precursor lymphoid tissue inducer (LTi) cells, which undergo toward the differentiation and maturation into $LT\alpha_1\beta_2$-expressing LTi cells, characterized by the presence of the chemokine receptors CXCR5 and CCR7 (Vondenhoff et al., 2007) (Figure 1). The $LT\alpha_1\beta_2$-expressing LTi cells act on stromal cells via LTβ receptors, up-regulating their expression of adhesion molecules (ICAM-1, VCAM-1, MAdCAM-1) and promotingtheir release of chemokines such as CXCL13, CCL21, and CCL19 (Figure 1) (Onder et al., 2017). These chemokines, eliciting the attraction of other LTi cells as well as of other hematopoietic cells, trigger a rapid development of a highly organized and compartmentalized lymphoid tissue (Vondenhoff et al., 2007) (Figure 1). Cytokines, adhesion molecules and chemokines are active players in promoting the recruitment of of NK cells, DCs, B and T cells, whereas $LT\alpha_1\beta_2$ appears to be essential in shaping the processes of clustering and organization of the lymphoid structure (Jackson, 2019).

In this context, structural cells such as fibroblastic reticular cells hold a relevant role. Indeed, besides acting as a physical scaffold for the lymph node, they provide specialized microenvironments ideal

for cellular interactions (T or B cell zones or subcapsular regions) (Novkovic et al., 2016, Perez-Shibayama et al., 2019). Fibroblastic reticular cells promote and maintain the survival and expansion of immune cells whose role is to maintain a homeostatic pool size, shaping the composition and activity of T and B cells. This occurs through the coordinated and tailored release of paracrine signals, such as cytokines (i.e. IL-2, IL-6, IL-7, IL-15, BAFF, TNF, IFN-γ), chemokines (CCL19, CCL21, CCL25, CXCL12, CXCL13) (Lo et al., 2003, Soldevila et al., 2004) and nitric oxide (Brown and Turley, 2015, Fletcher et al., 2015, Griffith et al., 2014, Jameson, 2002). In addition to the coordinated release of cytokines by fibroblastic reticular cells, cytokine-mediated signals are also shaped by T_H and **T regulatory (T_{Reg}) cells** through an efficient cellular cytokine uptake system (Busse et al., 2010), which restricts the range of action of cytokines. Naïve T cells and dendritic cells are in continuous physical contact with fibroblastic reticular cells, where T cells and dendritic cells migrate along the fibroblastic reticular cell network and where they establish contact with each other (Fletcher et al., 2015). It is worth noting T cells contribute to the maintenance of the structure of the fibroblastic reticular cell network via the release of **lymphotoxin B** (Zhao et al., 2014).

Ectopic lymphoid organs: the hubs of an altered immune communication

Mounting evidences pointed out that during chronic inflammation there is an unsettlement of several signals comprising cytokine receptors, kinases and transcription factors, flowing to an increased expression of several pro-inflammatory genes (Wu et al., 2014). In parallel, an impairment of the main pro-resolving molecular pathways (i.e. lipid mediators, cytokines, metabolic factors, neurotransmitters) supports the perpetuation of the inflammatory process (Sugimoto et al., 2016). Set of preclinical and clinical studies allowed to demonstrate the onset of a "pathophysiological network" between immune cells involved in the development and perpetuation of the chronic inflammation (Pawelec et al., 2014, Wu et al., 2014). In this context, the presence of ectopic lymphoid annexes is emerging as a distinctive trait, common to a series of chronic inflammatory diseases, such as Sjögren syndrome, Hashimoto thyroiditis, myasthenia gravis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, atherosclerosis, primary sclerosis cholangitis, indicating a potential role of these structures in the physiopathology of the disorders (Barone et al., 2016, Drayton et al., 2006).

From an anatomopathological point of view, the tertiary lymphoid organs are characterized by a localization into organs or tissues not predisposed embryologically to allow the development of lymphoid tissues, such as the meninges, salivary glands, kidneys, blood vessels, heart, synovium pancreas or liver (Golub and Cumano, 2013).

Set of preclinical experiments performed in murine models of chronic inflammation allowed to demonstrate that the onset of ectopic lymphoid organs is driven, at least in part, by the same factors governing the development of secondary lymphoid organs (Kain and Owens, 2013).

However, it is worth to note that on the contrary to what observed in the development phases of the normal lymphatic tissues, the onset of tertiary lymphoid organs is initiated also in the absence of precursor **LTi cells** (Cherrier et al., 2012). In particular, it has been observed that the initiation phase

for ectopic lymphoid tissue generation is triggered by the presence of local inflammatory microenvironment, supported by immune cells, such as $CD4^+$ cells secreting IL-17, NK cells, neutrophils, γδ T cells, ILC3s (Jones et al., 2016) (Figure 1). Following their accumulation at sites of inflammation, these pioneering immune cells establish a tight interaction with fibroblasts, perivascular myofibroblasts, resident mesenchymal and stromal cells, releasing cytokines such as interferon-γ, interleukin 1β and TNF (Jones et al., 2016). These cytokines promote the synthesis of adhesion molecules and chemokines (i.e. CXCL12, CXCL13, CCL19 and CCL21), which provide pivotal cues for an optimal spatial organization of the cells into the lymphoid annexes (Jones et al., 2016) (Figure 1).

Subsequently to the accumulation of tertiary lymphoid organ-initiating immune cells at sites of inflammation, the process of lymphoid neogenesis is supported by the continuous release of critical survival signals (i.e. BAFF, IL-7, and CXCL12) useful for incoming lymphocytes and for the differentiation of long-lived plasma cells (Aloisi and Pujol-Borrell, 2006, Jones et al., 2016) (Figure 1). In this context, it has been observed that a chronic antigen stimulation determines a persistent activation of innate and adaptive immune cells in the inflamed tissue with an increased expression of lymphotoxin $\alpha_1\beta_2$ by activated B and T cells, as well as of lymphoid chemokines by resident stromal cells, infiltrating macrophages, dendritic cells and other parenchymal cells (Aloisi and Pujol-Borrell, 2006) (Figure 1). The **follicular dendritic cells** (FDCs), a unique population of cells essential for efficient germinal centre formation and for the production of high-affinity antibodies (Heesters et al., 2014), are believed to provide a uniquely long-lasting "depot" of antigen that can be accessed by B cells well beyond clearance of the initial infection or injury from which the antigen was acquired (Hughes et al., 2016). They are thought to be important in the affinity maturation of the B-cell receptor. Only B cells expressing a receptor of high enough affinity will be successful in acquiring sufficient antigen from the FDC to in turn present the antigen to their cognate T cell and receive survival signals (Hughes et al., 2016). A study demonstrated that disruption of the FDC network in a

model of arthritis led to reduced germinal center formation in lymphoid follicles, impaired recruitment of follicular helper T (T_{fh}) cells into B cell areas, diminished autoantibody production, and attenuation of disease (Victoratos and Kollias, 2009). Although presently, the source of FDCs within ectopic lymphoid organs is still debated, several studies indicate that B cell production of LTα1β2 is a pivotal factor for differentiation of FDCs within ectopic lymphoid organs (Hughes et al., 2016). The recruitment of B cells, T cells and dendritic cells to tertiary lymphoid organs is thought to be facilitated by acquisition of a high endothelial venule-like phenotype by activated endothelial cells (Jones et al., 2018). During embryonic and neonatal life, the high endothelial venules develop in all secondary lymphoid organs and are critical for initiating and maintaining immune responses since they extract naïve and memory lymphocytes from the bloodstream, regardless of antigen receptor specificity, and deliver them to antigen-presenting cells inside lymph nodes (Jones et al., 2018). It has been observed that during chronic inflammation high endothelial venule also develop postnatally in ectopic lymphoid annexes mainly driven by the release of CCL19 and CCL21 from stromal cells (probably fibroblasts or fibroblast precursors) (Link et al., 2011). In particular, the CCL21, a homeostatic chemokine produced in secondary lymphoid organs, is aberrantly produced by endothelial cells and high endothelial venules in inflamed peripheral tissues, favoring an anomalous distribution of the naïve T cells at inflammatory sites thus triggering the early events of lymphoid neogenesis (Manzo et al., 2007). Interestingly, such increased CCL21 production involve specific endothelial differentiation or activation pathways not shared with the secondary lymphoid organ, representing a useful target for selective therapies (Manzo et al., 2007).

In parallel, it is emerging that also ectopic lymphoid organs are characterized by lymphangiogenesis process (Tammela and Alitalo, 2010). Indeed new lymphatic vessels have been observed within organized lymphoid aggregates in autoimmune thyroiditis, rheumatoid arthritis, and Sjogren's syndrome (Muniz et al., 2011). CCL21 displayed to hold a relevant role in the formation of lymphatic vessels in inflamed tissue (Muniz et al., 2011). Muniz et al. (2011) reported that the

191 infiltration of $CD4^+$ T cells into the CCL21-expressing tissues, was followed by an up-regulation of
192 the dendritic cell-attracting inflammatory chemokines (i.e. CCL2, CXCL10 and CXCL9)(Marinkovic
193 et al., 2006). Such chemokines deriving from incoming $CD4^+$ T cells, from stromal cells, or from the
194 endothelium, elicited the recruitment of dendritic cells into the tissue, a pivotal event for the formation
195 of new lymphatic vasculature in tertiary lymphoid organs (Muniz et al., 2011). The experimental
196 depletion of dendritic cells was followed by a marked inhibition of the lymphangiogenesis, thus
197 suggesting that a deepen characterization of the molecular mechanisms underlying the influx of
198 dendritic cells into the inflamed tissue, could represent a novel therapeutic target to blunt the
199 lymphangiogenesis. Of note, the dendritic cells are also central for retention of B and T cells in the
200 tertiary lymphoid organs, through a mechanism involving lymphotoxin β production
201 (GeurtsvanKessel et al., 2009).
202 Over the years, increasing evidences are pointing out a certain degree of heterogeneity about the
203 structural organization of tertiary lymphoid organs in various immune-inflammatory diseases, as
204 described below.

205

206 ***Multiple sclerosis***

207 Multiple sclerosis (MS) is a chronic autoimmune, progressive demyelinating and
208 neurodegenerative disease of the central nervous system (CNS), which gradually results in severe
209 neurological deficits (Dargahi et al., 2017). In this context, there is the presence of an immuno-
210 inflammatory infiltrate characterized by the presence of T cells, accompanied by a variable number
211 of B cells, monocytes and occasional plasma cells (Matthews, 2019). Recent evidences described the
212 presence of tertiary lymphoid organs in the meninges of MS patients, leading to the hypothesis that
213 differentiation and maturation of autopathogenic B and T cells may partly occur inside the CNS
214 (Mitsdoerffer and Peters, 2016). In this regard, additional investigations are needed to further support

215 the role of such lymphoid annexes in orchestrating the complex network of cells and cytokines in MS
216 lesions.

217 At present, the experimental evidences obtained in a murine model of experimental autoimmune
218 encephalomyelitis (EAE), allowed to understand the role played by ectopic lymphoid annexes in the
219 pathogenesis of such immune-mediated demyelinating disease (McCarthy et al., 2012). Indeed, in
220 agreement with the data from MS patients, the tertiary lymphoid annexes were consistently found in
221 association with meninges also in EAE mice (Columba-Cabezas et al., 2006, Magliozzi et al., 2004,
222 Pikor et al., 2015).

223 In this pre-clinical model, Columba-Cabezas et al. (2003) described the expression of lymphoid
224 chemokines CCL19, CCL21 and CCR7 in EAE lesions, thus suggesting their possible involvement
225 in promoting cellular interactions, prodromal for the expansion of autoreactive lymphocytes within
226 the inflamed CNS (Columba-Cabezas et al., 2003). At present, no evidences were provided about the
227 effect of the pharmacological blockade of such lymphoid chemokines as a viable therapeutic approach
228 to blunt the EAE progression. In this context, Peters et al. (2011) recognized an active role for the
229 Th_{17} cells in the formation of ectopic lymphoid organs in the CNS during EAE (Peters et al., 2011).
230 In particular, it emerged that the specific expression of podoplanin on Th_{17} cells, together with the
231 presence of Th_{17}-specific cytokines like IL-17, may act in concert to promote the lymphoid
232 neogenesis in the CNS (Peters et al., 2011). Since it is recognized that podoplanin is crucial for the
233 development of lymph nodes and other secondary lymphoid structures, it was hypothesized that this
234 trans-membrane glycoprotein was coopted by Th_{17} cells to form tertiary lymphoid structures in the
235 target tissues during autoimmune inflammation (Peters et al., 2011). On these basis, IL-17 and
236 podoplanin may provide important targets in regulating tissue ectopic lymphoid follicle generation
237 (Peters et al., 2011).

238 Despite a number of data highlighted that MS was a primarily T cell-mediated disease, ample
239 evidence revealed that B cells and autoantibodies are crucially involved (Kuerten et al., 2012). In this

regard, it has been observed that B cell aggregates represent an early event in EAE onset in CNS tissue of mice, whereas in the chronic phase of EAE such B cell aggregates acquired the phenotype of tertiary lymphoid organs (Kuerten et al., 2012). Recently, it has been observed that the treatment with the S1P1 receptor modulator fingolimod, decreasing the egress of $B220^+$ B cells from secondary lymphoid organ, blunted the evolution of CNS B cell aggregates into ectopic lymphoid organs (Bail et al., 2017). These intriguing results corroborate the critical role for B cell aggregates in the onset of such abnormal annexes, and suggests the pharmacological modulation of B cell as viable way to counteract these pathological lymph nodes.

Chronic obstructive pulmonary disease and asthma

The presence of tertiary lymphoid tissue is often associated with chronic obstructive pulmonary disease (COPD), a chronic lung condition mainly related with smoking (Yadava et al., 2016).
One of the first reports describing the presence of B-cell follicles in COPD dates back to 1992, when Bosten et al. described an increase of small airways containing B-cell follicles in patients with severe COPD compared to normal subjects and patients with mild-to-moderate COPD (Bosken et al., 1992). A subsequent study provided the first evidence about a correlation between COPD disease severity and the incidence of tertiary lymphoid organs (Hogg et al., 2004). Set of experiments, aimed at understanding the pathophysiological significance of ectopic lymphoid annexes, allowed to demonstrate that in patients with COPD these structures influence the IFN-γ production in the lung (Briend et al., 2017). In particular, in patients affected by severe COPD IL-18^+ macrophages and dendritic cells were localized in the lung lymphoid aggregates, influencing the IFN-γ release from closely-situated lymphocytes mainly NK cells, contributing to pulmonary tissue destruction (Briend et al., 2017).

Preclinical models of COPD provide detailed information about the molecular mechanisms underlying the development of ectopic lymphoid follicles. In particular, Rangel-Moreno et al. (2011)

proposed a novel model of follicle formation, starting from the release of IL-17 by non-LTi cells (i.e. T cells), which, spurring the expression of CXCL13 and CCL19, promoted B and T cell infiltration and supported actively to the structure of follicular dendritic cell networks (Rangel-Moreno et al., 2011). Of note, when the inflammatory response has been resolved , the expression of CXCL13 and CCL19, as well as the overall architecture of the bronchus associated lymphoid tissue, was maintained by the interaction of LT-expressing lymphocytes with LTβR-expressing stromal cells or vascular endothelial cells, suggesting a critical role for these cells in consolidating the ectopic lymphoid structures (Rangel-Moreno et al., 2011). In such ectopic lymphoid annexes, plasma cells expressing IgM, IgG, or IgA were located closely to lymphoid aggregates (Morissette et al., 2014), indicating these structures as a site of B cell activation, leading to plasma cell differentiation and antibody release within the lungs (Morissette et al., 2014).

Mice exposed to cigarette smoke, analogously to what observed in patients with COPD, showed a strong positivity for the B cell–attracting chemokine CXCL13, deeply involved in the onset of tertiary lymphoid organs (Bracke et al., 2013). In this context, mice administered with a monoclonal antibody against CXCL13, either in a prophylactic (during the entire course of the chronic exposure to cigarette smoke) or therapeutic (starting from established inflammation at 3 months of cigarette smoke exposure) manner, displayed almost a complete disruption of the peribronchial and parenchymal lymphoid follicles (Bracke et al., 2013). The absence of these lymphoid follicles attenuated inflammatory cell recruitment in the broncho-alveolar lavage and partially protected the mice against destruction of alveolar walls upon the chronic cigarette smoke exposure (Bracke et al., 2013). However, no effect was observed on the development of airway wall remodeling (Bracke et al., 2013).

Recent studies have highlighted the presence of lymphoid tissues in allergic airway inflammation. T helper (Th) 2 cells and type 2 innate lymphoid cells play central roles in the pathogenesis of allergic airway inflammation such as asthma. In this context, the IL-5-producing memory-type pathogenic Th2 cell population (Tpath2) subset plays a key role. This memory-type subset can be detected in

various allergic inflammatory lesions. Potential mechanisms involved in the maintenance of these cells at the local inflammatory site has been recently proposed by Hirahara et al. Using mice model for chronic airway inflammation they demonstrated that inducible bronchus-associated lymphoid tissue (iBALT) was shaped during chronic inflammation in the lung and that memory-type Tpath2 cells are highly maintained within iBALT. Furthermore, the maintenance of the Tpath2 cells within iBALT seems to be supported by IL-7-producing lymphatic endothelial cells (LECs) within these structures. Furthermore, in human samples from patients with eosinophilic chronic rhinosinusitis ectopic lymphoid structures, consisting of memory-type $CD4^+$ T cells and IL-7^+IL-33^+ LECs, accumulated in nasal polyps, contributing to the persistence of local airway inflammation (Hirahara et al., 2018).

Inflammatory bowel diseases

The inflammatory bowel diseases (IBDs), an umbrella term comprising the Crohn's disease and ulcerative colitis, are inflammatory diseases affecting the gastrointestinal tract characterized by a chronic remittent clinical course, with alternating bouts of remission and flares of active inflammation (Pagnini et al., 2019). Increasing evidences described the presence of a dysfunctional lymphatic system and the development of ectopic lymphoid tissue as peculiar a hallmark in IBDs (McNamee and Rivera-Nieves, 2016). However, the effective role of such abnormal lymphoid annexes in the pathophysiology of IBDs deserve further investigations and a more thorough characterization.

Recently, a comprehensive analysis of the ectopic lymphoid structures has been performed in order to clarify the mechanisms leading to their formation in the mesentery of Crohn's disease patients, with particular regard for the preferential development of such lymphoid annexes in the mesenteric creeping fat, an adipose tissue wrapping the inflamed gut typically observed in Crohn's disease patients (Guedj et al., 2019). It has been observed that the localization of these annexes in the fat

315 tissue, was ascribable to the local expression of high levels of CXCL13, CXCL16, CCL19, CCL20
316 and CCL21 chemokines, produced by the adipocytes in the vicinity of ectopic lymphoid organs
317 (Guedj et al., 2019).
318 Since the mesenteric segments involved the development of such abnormal lymphatic annexes were
319 anatomically close to ulcerations and fistulae, the authors hypothesized that the exposure of the
320 mesenteric tissue to inflammatory mediators such as TNF and bacterial components (i.e. LPS) trigger
321 the production of chemokines by the local adipocytes (Guedj et al., 2019). This evidence was
322 corroborated by a set of *in vitro* experiments performed on 3T3-L1 adipocyte cells displaying a
323 synergistic effect of LPS with TNF in eliciting the expression of the full panel of chemokines required
324 to recruit the different cell populations involved in the formation of tertiary lymphoid structures
325 (Guedj et al., 2019). In addition, the performance of functional chemotaxis assays showed that the
326 supernatant of 3T3-L1 cells stimulated with TNF and LPS, induced the recruitment of several
327 subpopulations of T and B cells, not observed with the supernatant of unstimulated cells (Guedj et
328 al., 2019).
329 Despite further investigation are needed, a deleterious role of tertiary lymphoid structures is emerging
330 in Crohn's disease. Indeed, the presence of these annexes were associated with a more severe
331 phenotype of the disease, whereas the surgical removal of the mesentery during ileocolic resection
332 reduced post-operative recurrence (Guedj et al., 2019).
333 Preclinical models of intestinal inflammation provided useful information regarding the molecular
334 mechanisms underlying the onset of ectopic lymphoid aggregates. In a murine model of dextran
335 sulphate sodium (DSS) chronic colitis, allowed to highlight a relevant role of the autonomic nervous
336 system for the formation of tertiary lymphoid organs (Olivier et al., 2016). Indeed, the formation and
337 maintenance of the lymphoid annexes during colitis was dependent by the intestinal vagal innervation
338 projecting to neurons in the inflamed areas (Olivier et al., 2016). In this model, the surgical vagal
339 denervation determined a reduction of CXCL13 and CCL20 production, thus affecting the onset and

development of tertiary lymphoid organs (Olivier et al., 2016). Of note, the formation of such lymphoid annexes resulted independent from the $LT\alpha_1\beta_2$- LTβR signaling axis, despite this axis appeared relevant for the formation of FDCs (Olivier et al., 2016).

At present, the effective role of tertiary lymphoid organs in IBD onset and development is still scarcely characterized, with limited human data. A better characterization about the involvement of such abnormal lymphoid annexes in the pathophysiology of IBD remains a critical point in our understanding of intestinal immunity.

Atherosclerosis

Atherosclerosis represents the leading cause of the coronary artery disease, peripheral vascular disorders, and cerebrovascular diseases (Ilhan and Kalkanli, 2015). From a pathophysiological point of view, atherosclerosis is a chronic inflammatory condition initiated by the retention, oxidation, and modification of lipids (specifically low-density lipoproteins), followed by an intense immunological activity (Insull, 2009). Indeed, it has been observed that such condition is likely to be initiated by the activation of endothelium with the expression of adhesion molecules, leading to the adhesion of leukocytes, mainly monocytes and T-cells, to the endothelium and subsequently migration into the subendothelial layer of the vascular wall (the intima) (Wolf and Ley, 2019). However, the presence of dendritic cells, neutrophils and B cells has been also documented in atherosclerotic plaques (Wolf and Ley, 2019).

An increasing number of clinical (Dutertre et al., 2014, Houtkamp et al., 2001) and preclinical (Grabner et al., 2009) studies highlighted the presence of organized ectopic lymphoid structures in the adventitia of atherosclerotic aortas. These annexes are commonly localized in the connective tissue that surrounds the inner atherosclerotic lesions, suggesting a role in perceiving and in mounting immune responses against plaque antigens (Michel et al., 2007). Indeed, the immune effectors

generated within these structures could hence be self-reactive participating to arterial tissue destruction, and thus to pathophysiological mechanism of atherosclerosis (Michel et al., 2007).

A recent study by Akhavanpoor et al. (2018) firstly identified that, the previously described cellular infiltrations within the adventitia observed in coronary arteries, were structurally ectopic lymphoid annexes. In particular, the authors, by means of immunohistochemical investigations, observed that the adventitial cellular infiltration typically observed in atherosclerotic lesions were positive for lymphoid tissues (Akhavanpoor et al., 2018). In parallel, it has been also demonstrated a direct correlation between the size of tertiary lymphoid organs with the plaque size and the lesion instability (Akhavanpoor et al., 2018). The pathophysiological relevance of the ectopic lymphoid annexes was supported by the evidence that the lesions leading to fatal myocardial infarction were strictly related with a loss of cellular and structural composition of the lamina media next to these lymphoid aggregates and to the cellular invasion of the adjacent atherosclerotic lesion (Akhavanpoor et al., 2018).

By means of hypercholesterolemic ApoE knockout mice, which models the familial hypercholesterolemia in humans (Getz and Reardon, 2016), it has been possible to better understand the molecular mechanisms underlying the formation of tertiary lymphoid organs in atherosclerotic vessels (Grabner et al., 2009), delineating a possible role for these lymphoid structure in atherosclerosis. In particular, the vascular smooth muscle cells of these mice beneath the atherosclerotic plaques, activated through LTβR and **TNFRSF1A** signaling, expressed CXCL13, CCL21 and CCL19, providing the base for lymphoid neogenesis (Grabner et al., 2009). Further investigations allowed to identify a critical role for M1 macrophages in orchestrating this process (Guedj et al., 2014b). Indeed, M1 macrophages, expressing higher LT-α and TNF levels than non-polarized M0 or reparative M2 macrophages, appear to be relevant LTi candidates, which confer a LTo phenotype to the vascular smooth muscle cells (Guedj et al., 2014b).

389 ***Rheumatoid arthritis***

390 Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease affecting the lining of the
391 synovial joints with consequent destruction, leading to progressive disability, premature death, and
392 socioeconomic burdens (Guo et al., 2018). The clinical manifestations of symmetrical joint
393 involvement include arthralgia, swelling, redness, and even carrying out daily physical activities (Guo
394 et al., 2018).

395 As an autoimmune disorder, B-cells, T-cells and macrophages hold a critical role in RA onset and
396 development (Yap et al., 2018). The immuno-inflammatory cells can either reside in synovium or
397 circulate in peripheral blood. B-cells secrete autoantibodies, such as rheumatoid factors and anti-
398 citrullinated protein antibodies (ACPA), and cytokines such as IL-13, IL-14 and IL-15 (Yap et al.,
399 2018). In parallel, B-cells also mediate T-cell activation through expression of costimulatory
400 molecules. In this context, the main function of T-cells is to activate macrophages and fibroblasts and
401 transform them into pro-fibrogenic and pro-inflammatory phenothypes. (Yap et al., 2018). Together
402 to T- and B-cells, also the pro-inflammatory macrophages produce a number of cytokines and
403 chemokines to support the inflammation in the joints (Yap et al., 2018).

404 The presence of ectopic lymphoid structures resembling germinal centres, characterized by
405 follicular dendritic cell networks have been reported to occur in the synovial membrane, with a
406 variable prevalence in approximately 25% of patients with RA (Humby et al., 2009, Manzo et al.,
407 2005).

408 However, the actual pathophysiological role of these structure in promoting autoimmunity and
409 chronic inflammation is still debated and deserve further investigations (Humby et al., 2009). A
410 pioneering paper by Humby et al. (2009) provided evidence that, within the synovial membrane of
411 patients with RA, the lymphoid structures are characterized by follicular dendritic cell networks,
412 which expressed activation-induced cytidine deaminase (an enzyme essential for the regulation of B
413 cell diversification) and were surrounded by plasma cells producing ACPA (Humby et al., 2009). Of

note, the authors, by means of a human RA-SCID mouse chimera model, demonstrated that transplanted RA synovial grafts containing ectopic lymphoid structures elicited the survival and proliferation of B cells, stimulating the expression of the activation-induced cytidine deaminase, and supporting the production of ACPA (Humby et al., 2009), supporting the role of these annexes in the disease pathogenesis.

When considering the molecular mechanisms underlying the onset of tertiary lymphoid organs at synovial level, it emerged that the non-canonical NF-κB pathway, driven by its key regulator NIK, plays an important role in this context (Noort et al., 2015). Under physiological conditions this signaling is crucially involved in the normal lymphoid organogenesis (Bonizzi and Karin, 2004). Of note, it has been observed that RA patients displayed a higher level of this signaling in synovial tissues characterized by the presence of tertiary lymphoid annexes, when compared with synovial samples from patients without ectopic lymphoid annexes (Noort et al., 2015). In particular, NIK^+ endothelial cells and FDCs are abundantly present in synovial tissues containing abnormal lymphatic structures, suggesting that these cell subtypes may be critical orchestrators in sustaining the ectopic neo-lymphogenesis eliciting the CXCL13 expression (Noort et al., 2015).

Recently, it has been observed an inverse correlation between the level of IL-27 and the incidence of tertiary lymphoid organs and the gene signatures associated with their development and activity (Jones et al., 2015). By means of mice lacking for IL-27 receptors, it has been demonstrated a relevant role played by IL-27 in counteracting the synovial formation of tertiary lymphoid organs through inhibition of homeostatic chemokines, lymphoid cytokines, and transcriptional regulators involved in lymphoid neogenesis (Jones et al., 2015). In particular, such negative effects of IL-27 on neo-lymphogenesis is ascribable to its inhibitory effects on synovial Th_{17} and T_{fh} effector cytokines and peripheral $podoplanin^+$ Th_{17} cell numbers (Jones et al., 2015).

Sjögren's syndrome

Sjögren's syndrome (SSj) is a chronic inflammatory autoimmune disease of unknown origin. The hallmark of the disease is an exocrinopathy, which often results in dryness of the mouth and eyes, fatigue, and joint pain (Mariette and Criswell, 2018). Although the cause of this disorder appear still poorly understood, increasing evidences showed an exaggerated innate and adaptive immune responses at the basis of pathophysiological mechanisms (Kiripolsky et al., 2017). In particular, the B and T cells are activated and abnormalities in both populations have been observed in glandular tissue and in the circulation either in pre-clinical models as well as in patients (Kiripolsky et al., 2017). Specifically, it has been reported an alteration in normal $CD4^+$ T helper subset ratios, prodromal for the production of inflammatory cytokines, leading to the B cell differentiation and class switching (Kiripolsky et al., 2017).

Over the years, increasing evidences described the presence of tertiary lymphoid organs in patients affected by SSj (Amft et al., 2001, Aziz et al., 1997, Salomonsson et al., 2003). Amft et al. (2001) reported that the ectopic expression of CXCL13 and CXCL12, B cell–homing chemokines, were associated with the formation of germinal center- like structures in patients with SSj, but not in any of the salivary gland control samples (Amft et al., 2001). These chemokines were also expressed on endothelial structures in SSj (Amft et al., 2001). Interestingly, the receptor for CXCL13, CXCR5, was expressed mostly on cells in the lymphocytic infiltrates, mainly identified as B lymphocytes, despite occasional $CXCR5^+$ T cells were also reported (Salomonsson et al., 2002). In particular, by using a series of phenotypic markers, Salomonsson et al. (2003) identified an architecture and activity in the salivary glands of SSj patients typically found in organized secondary lymphoid tissue (Salomonsson et al., 2003). The observations of large aggregates of B and T cells and dense populations of proliferating cells in close proximity to FDCs networks suggest that ectopic lymphoid annexes are formed in patients with SSj (Salomonsson et al., 2003). These subjects displayed a high expression of adhesion molecules and chemokines, involved in attracting and organizing lymphocytes

463 in secondary lymphoid organs, as well as apoptosis and local production of autoantibodies (anti-
464 Ro/SSA and anti-La/SSB) (Salomonsson et al., 2003).
465 Alunno et al. (2015) provided evidence about a role of telocytes, a stromal cell subset involved in the
466 control of local tissue homeostasis, in SSj. These stromal cells resulted markedly reduced in minor
467 salivary glands from SSj patients when compared to normal subjects (Alunno et al., 2015). Such a
468 decrease was associated with both worsening of glandular inflammation and progression of ectopic
469 lymphoid neogenesis (Alunno et al., 2015). It is not clear if the loss of telocytes in SSj patients
470 represents either the cause or the consequence of local inflammation, it would be useful to investigate
471 if and to what extent these cells participate in the organization of ectopic lymphoid annexes. Recently,
472 Fonseca et al. (2018), investigated whether the balance of blood Tfh cells and T fr cells can provide
473 information about ectopic lymphoid neogenesis and disease activity in primary SSj (Fonseca et al.,
474 2018). The authors reported that the blood Tfr:fh T cell ratio represented a useful marker for ectopic
475 lymphoid structure formation in minor salivary glands, being strongly associated with B cell, $CD4^+$
476 T cell, and PD- 1^+ICOS^+ T cell infiltration in this context (Fonseca et al., 2018).
477 The events leading to the development of tertiary lymphoid organ patients with SSj have been
478 investigated in a murine model of in a virus-induced autoantibody formation in the salivary glands
479 (Barone et al., 2015). In this context, the authors highlighted that IL-22 is a critical cytokine to
480 promote differential expression of chemokine (C-X-C motif) ligand 12 and chemokine (C-X-C motif)
481 ligand 13 in epithelial and fibroblastic stromal cells that, in turn, is pivotal for B-cell recruitment and
482 organization of the ectopic lymphoid annexes (Barone et al., 2015). In this regard, the genetic or
483 pharmacological blockade of IL-22 with an anti-mouse IL-22 Ab-03, impaired and reversed the
484 formation of tertiary lymphoid tissues and autoantibody production, thus suggesting a rationale for
485 the use of IL-22-blocking agents in this pathological condition (Barone et al., 2015).
486 In the differential diagnosis of SSj, other IMIDs such as IgG4-related disease (IgG4-RD) must be
487 taken into consideration. This is a chronic fibro-inflammatory disease characterized by a significant

increase of serum IgG4 levels and marked infiltration of IgG4-positive plasma cells in affected organs such as lacrimal glands, salivary glands, lymph nodes, pancreas, retroperitoneum and lungs (Puxeddu et al., 2018). Dense lymphoplasmacytic infiltrates and ectopic lymphoid structures are characteristic histopathological findings in the lesions of IgG4-RD. In these structures T and B cells interaction leads to an uncontrolled somatic hypermutation, class switch recombination and differentiation of plasma cells (Pitzalis et al., 2014), leading to the progression of the disease. Recently circulating PD-$1^{hi}CXCR5^{-}$ peripheral T leper (Tph)-like cells have been found increased in IgG4-RD patients. Interestingly, these cells are able to infiltrate the affected tissues, to exert cytotoxic effects and to produce chemokines such as CXCL13, responsible for the recruitment of $CXCR5^{+}$ Tfh cells and B cells with consequent formation in the organs of ectopic lymphoid structures(Kamekura et al., 2019, Kamekura et al., 2017). These remarkably results may contribute to identify specific therapeutic targets and to develop new strategies for IgG4-RD treatment.

Concluding remarks

Since the critical role of ectopic lymphoid organs in IMIDs has been proposed, research has been focusing on the understanding the mechanisms involved in the regulation of these structures, leading to design novel pharmacological approaches to manage IMIDs (Corsiero et al., 2016).

In this context, promising results come from studies performed in animal models of EAE, SSj and IBD, in which a soluble lymphotoxin-beta receptor-immunoglobulin (LTbetaR-Ig) fusion protein is able to block some of the pathways involved in the formation of ectopic organized lymphoid tissues, with consequent improvement of the clinical symptoms (Browning, 2008).

In parallel, the chemokines CXCL13, CCL21 and CCL19, involved in cell recruitment of B and T cells in these dynamic structures, have been recognized as potential molecular targets, suggesting that their pharmacological blockade could be a promising therapeutic strategy for the IMIDs. This has been supported by in-vivo studies in animal models of RA (Zheng et al., 2005) and MS (Klimatcheva et al., 2015), in which the blockage of CXCL13, via an anti-CXCL13 antibody, resulted in an improvement of the clinical setting in both diseases.

Among cytokines, IL-23 promotes the release of IL-22, leading to increase CXCL-13 expression, relevant for the lymphoid aggregation, B cell clustering and autoantibodies production in the ectopic lymphoid tissues (Barone et al., 2015). In recent years, risankizumab and guselkumab, novel monoclonal antibodies against IL-23, are emerging in the clinical scenario as useful therapeutic strategy to control chronic inflammatory diseases, such as psoriasis and Crohn's disease.

In this context, might be interesting to evaluate the efficacy of these monoclonal antibodies in countering the inflammatory processes and the development of tertiary lymphoid organs. In light of the recent data, the pharmacological blockade of IL-22 can represent a useful tool to control abnormal lymphogenesis (Barone et al., 2015), by acting indirectly on CXCL-13 and CXCL-12 over-expression, resulting in a reduction of B-cell clustering, lymphoid aggregation and autoantibodies production. In this regard, promising results come from recent clinical studies in psoriasis

526 (ClinicalTrials.gov Identifier: NCT00563524) and RA (ClinicalTrials.gov Identifier:
527 NCT00883896), in which fezakinumab, a human monoclonal antibody against IL-22, was found to
528 improve some clinical aspects of these diseases.

529 Several cytokines, besides IL-22 and IL-23, act together for maintenance of the ectopic
530 lymphoid annexes, shaping the immune cell phenotype and activity. In particular, B cells infiltrating
531 these structures are characterized by a marked increase of $LT\alpha_1\beta_2$ driven by IL-4 signals (Ansel et al.,
532 2000). In parallel, IL-9 displayed a critical role in supporting B cell activation and differentiation and
533 IL-21 in the lymphoid structures organization (Ciccia et al., 2015, Jones et al., 2015). Up to now,
534 monoclonal antibodies against cytokines involved in ectopic lymphoid annexes, such as dupilumab
535 (anti-IL-4Rα), MEDI-528 (anti-IL-9) and NNC01140006 (anti-IL-21) are under clinical investigation
536 in some IMIDs, and preliminary results have demonstrated their potential beneficial effects in
537 controlling some aspects of these diseases. Further pharmacological approaches for targeting
538 specifically these positive feed-back loops may be effective in counteract the formation of
539 inflammatory lesions. However, there are still several aspects regulating these structures that must be
540 clarified. Thus, several outstanding questions must be addressed before developing more specific
541 disease-modifying therapies targeting ectopic lymphoid organs in IMIDs (see Outstanding
542 Questions).

Clinician's corner

- Ongoing efforts to widen the benefit-to-risk window of anti-inflammatory therapy in chronic diseases will require efforts on a number of complementary fronts, such as a greater comprehension of the pathophysiological significance of tertiary lymphoid organs.
- At present, the available therapeutic strategies aimed at counteracting immune-related disorders are far to be satisfactory, often displaying harmful effects leading to dramatic health status
- Researchers have been trying to understand the molecular details underlying the onset and development of tertiary lymphoid organs, in order to identify critical checkpoints able to shape the structural organization of such lymphoid annexes
- Next challenges will be to evaluate the prognostic and diagnostic potential of ectopic lymphoid organs in specific diseases, as well as to ascertain their potential as novel therapeutic targets, as well as to determine how diseases, characterized by a marked presence of abnormal lymphoid annexes, are sensitive to the current arsenal of biologic interventions used in routine clinical practice.

Glossary

Chemokines

are low-molecular-weight proteins that stimulate recruitment of leukocytes. They are secondary pro-inflammatory mediators that are induced by primary pro-inflammatory mediators such as interleukin-1 (IL-1) or tumor necrosis factor (TNF)

Fibroblastic reticular cells

immunologically specialized myofibroblasts of mesenchymal origin

Follicular dendritic cells

cells that is essential for efficient germinal centre (GC) formation and for the production of high-affinity antibodies. they develop from perivascular precursors of stromal cell origin that are seeded throughout the body. Follicular dendritic cell maturation requires lymphotoxin and TNF signalling through B cells, and the disruption of these pathways leads to the loss of follicular dendritic cells

Immuno-mediated inflammatory diseases (IMIDs)

umbrella term indicating a group of conditions or diseases that lack a definitive etiology, but which are characterized by common inflammatory pathways leading to inflammation, and which may result from, or be triggered by, a dysregulation of the normal immune response

Inflammaging

a chronic low-grade inflammation that develops with advanced age

Lymphoid tissue inducer (LTi) cells

583 hematopoietic cell type with critical roles in the immune system during both the embryonic and adult
584 stages of development. Their distinguishing features are expression of RORγt and IL-7Rα in the
585 absence of lineage markers (e.g. CD3, CD19, B220, CD11c, Gr-1)
586
587 **Lymphotoxin B**
588 a type II membrane protein of the TNF family
589
590 **M cells**
591 specialized epithelial cells of the mucosa-associated lymphoid tissues, characterized by their ability
592 to transport antigens from the lumen to cells of the immune system, thereby initiating an immune
593 response or tolerance
594
595 **T regulatory (T_{Reg}) cells**
596 a specialized subpopulation of T cells that act to suppress immune response, thereby maintaining
597 homeostasis and self-tolerance
598
599 **TNFRSF1A**
600 also known as tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) and CD120a, is
601 a ubiquitous membrane receptor that binds tumor necrosis factor-alpha (TNFα)

Box 1. Age-related immune cell decline: role of lymphoid organs

Immune function declines with age (immunosenescence), predisposing to increased susceptibility to infections, reduced efficacy of vaccination, and increased incidence of autoimmune diseases and malignancies(Simon et al., 2015). Immunosenescence is associated with low-grade systemic inflammation (inflammaging), which accelerates degenerative diseases, most prominently cardiovascular and neurodegenerative disorders (Goronzy et al., 2015). Thymic involution, the collapse of TCR diversity, imbalances in T cell populations, and the clonal expansion of senescent T cells releasing proinflammatory cytokines and cytotoxic mediators are all present during immunosenescence(Goronzy et al., 2015).

The lymph nodes of aged subjects display altered structural organization, decreases in T cell populations, altered lymphocyte movement and dysregulated interactions between T and B cells(Becklund et al., 2016, Turner and Mabbott, 2017). Scaffolding within the lymph node stroma, which is central to the compartmentalization of leukocytes into discrete niches, is disorganized (Turner and Mabbott, 2017). In particular, the follicular dendritic cell regions, which are important in defining B cell zones, are decreased in size and have decreased chemokine CXCL13 expression, leading to less well-defined B cell follicular regions(Turner and Mabbott, 2017). The number and CCL19 expression of fibroblastic reticular cells, which delimit the T cell zone, are reduced(Becklund et al., 2016). In addition, the stromal network appears compressed and less reticular with larger gaps in gp38 staining, a marker for the fibroblastic reticular cells (Becklund et al., 2016). Such age-dependent alterations in the lymphoid environment impair T cell homing and distribution into the lymphoid tissues and survival factor signaling, thus distorting the complex molecular equilibrium, on which immune quorum sensing is based.

624 **Table 1.** Immuno-mediated inflammatory disorders displaying ectopic lymphoid organs
625

Disease	***Target tissue***	***References***
Rheumatoid arthritis	Joints	(Shi et al., 2001)
Hashimoto's thyroiditis	Thyroid	(Aust et al., 2004, Marinkovic et al., 2006)
Myasthenia gravis	Thymus	(Cron et al., 2018, Shiao et al., 2010)
Multiple sclerosis	CNS	(Kuerten et al., 2012, Lehmann-Horn et al., 2016)
Sjogren's syndrome	Salivary glands	(Hansen et al., 2007)
Ulcerative colitis	Gut	(Weninger et al., 2003)
Crohn's disease	Gut	Weninger et al., 2003
Chronic obstructive pulmonary disease (COPD)	Lung	(Bracke et al., 2013, Yadava et al., 2016)
Atherosclerosis	Arteries	(Akhavanpoor et al., 2018, Guedj et al., 2014a)

626

627 **Figure legends**
628
629 **Figure 1.** Schematic diagram displaying the main step in the onset and development of secondary
630 (A) and ectopic (B) lymphoid organs.

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Figure

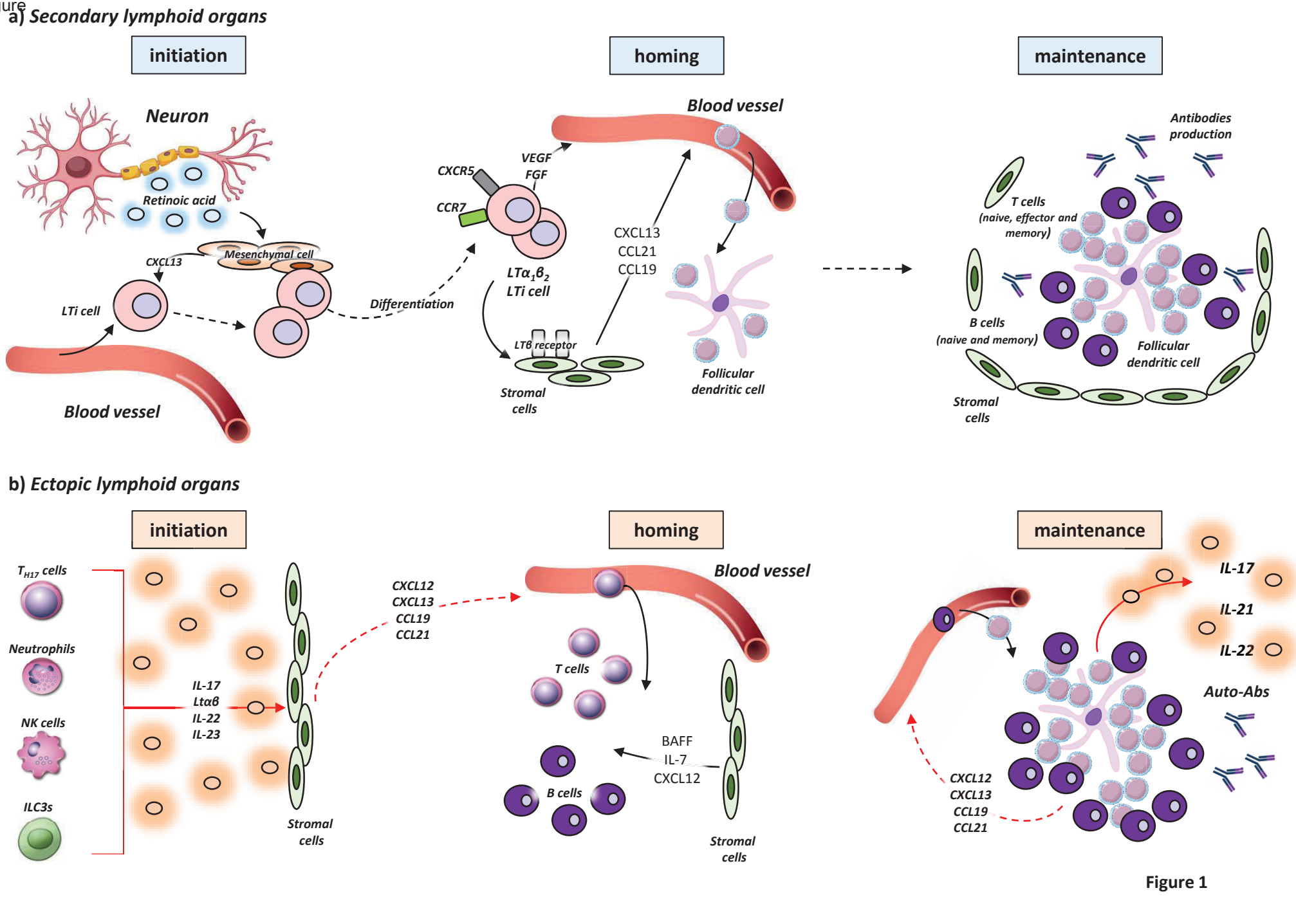

Figure 1

Outstanding questions

Retinoic acid is relevantly involved in the physiological onset lymphoid tissues formation. Is it also involved in activating LTi cells in chronic inflammatory disorders?

To what extent do tertiary lymphoid organs contribute to the ongoing inflammatory process and tissue damage in humans?

Are there differences in immune response tertiary lymphoid and secondary lymphoid organs?

Are there any peculiar cell populations in ectopic lymphoid annexes with different functions from that in secondary lymphoid organs?

Which cells and soluble mediators contribute more than other to ectopic lymphoid annexes onset and development?