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INVITED REVIEW

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Stromal cells in tertiary lymphoid structures: Architects of autoimmunity

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Abstract

The molecular mediators present within the inflammatory microenvironment are able, in certain conditions, to favor the initiation of tertiary lymphoid structure (TLS) development. TLS is organized lymphocyte clusters able to support antigen-specific immune response in non-immune organs. Importantly, chronic inflammation does not always result in TLS formation; instead, TLS has been observed to develop specifically in permissive organs, suggesting the presence of tissue-specific cues that are able to imprint the immune responses and form TLS hubs. Fibroblasts are tissue-resident cells that define the anatomy and function of a specific tissue. Fibroblast plasticity and specialization in inflammatory conditions have recently been unraveled in both immune and non-immune organs revealing a critical role for these structural cells in human physiology. Here, we describe the role of fibroblasts in the context of TLS formation and its functional maintenance in the tissue, highlighting their potential role as therapeutic disease targets in TLS-associated diseases.

KEYWORDS
tertiary lymphoid organs, fibroblasts, stromal cells, inflammatory cytokines, autoimmunity

1 | INTRODUCTION

Originally described by Louis Picker and Eugene Butcher in 1992 as the third lymphoid compartment of the immune system,[1] ectopic or tertiary lymphoid structures (ELS or TLS) are currently defined as non-capsulated anatomical entities comprised of organized aggregates of immune cells that harbor within non-immunological organs.[2,3] TLS formation is classically induced in an inflammatory microenvironment, either in response to exogenous stimuli or as a reaction of abundant expression of local antigens in the context of autoimmunity, cancer or tissue transplant.[2,4–9] TLS forming at specific sites has been assigned individual identities, and those include the following: the mucosa-associated lymphoid tissues (MALT); lymphocyte-rich clusters forming in the gastrointestinal tracts (GI); the fat-associated lymphoid clusters or FALCs, present in the adipose tissue of the mesenteries; and the nasal and inducible bronchial-associated lymphoid tissues (NALT and iBALT) that encompass the aggregates detected in the respiratory tract from the nasal cavity to the lung parenchyma.[2,10–12] Reliance on antigen exposure has been reported and believed to support the functional role of TLS in disease, as hub for affinity maturation of a humoral response against the specific antigen and, often, autoantigen.[13] While the link between antigen, antigen-specific B cell maturation and autoantibody production has not always been proved in the different

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conditions,[13] there are enough evidence to support the formation of TLS as response to local antigen displayed on both professional and non-professional antigen-presenting cells, in the context of an environment rich in inflammatory mediators, such as TNF, IL-6, IL-17 and IL-22.[10,13–15]

The molecular mediators present within a chronically inflamed microenvironment appear to be necessary but not sufficient to support TLS assembly. In chronic disease settings, TLS formation has been described in "permissive organs" such as the thyroid of patients with Hashimoto thyroiditis, the synovium of patients with Rheumatoid arthritis or the salivary glands of patients with Sjögren's syndrome.[2,13,15–18] Intriguingly all of those, except the synovium, are tissues characterized by an epithelial component that appears to play a critical role in particular in the initial release of inflammatory mediators and in the process of local antigen presentation that underpins TLS formation.[19–21]

Importantly, TLS does not form in all patients and, even within the same tissue, and have been characterized by the detection of different organizational features, suggesting a potential gradient, either in the quantity/quality of the antigen presented or in the intensity of the inflammatory response shaping TLS assembly [9,13,22] that supports the establishment of structures characterized by different degrees of organization. The mechanistic switch that drives, in certain patients, the formation of TLS has not been elucidated, and the presence of biomarkers predictive of TLS development in individuals is still being explored.

It is currently believed that TLS acts as amplifiers of the immune response and acts as hubs for survival and maintenance of pathogenic effector cells within the tissue.[3,4,10,13,14,23] In this context, the presence of TLS has been associated with poor prognosis when detected in chronic autoimmune conditions and with scarce response to lymphocyte-depleting agents.[24,25] At present, the prognostic role of TLS in cancer is debated and appears variable, based on the type of cancer within which they are found and the nature of the clinical stage of the cancer itself.[4,6,26] More recently, TLS formation in solid tumors has been associated in patients with a positive response to treatment with immune checkpoint inhibitors, opening a potential avenue of research aimed at favoring, rather than blocking, TLS development in the context of cancer.[27]

There are no indications of whether TLS has a supporting role in the catalysis of disease initiation as well as perpetuation itself. Early publications reported the formation of TLS in association with the inflammatory process, but not necessarily with the pathogenic process underpinning disease. Indeed, the presence of TLS was described by *Kratz et al* in the pancreas and kidneys of rat insulin promoter-lymphotoxin (RIP-LT) transgenic mice in the absence of pathology.[28] TLS in these mice was characterized by delineated areas of T and B cells, presence of plasma cells, primary and secondary follicles as well as high endothelial venules (HEVs). These structures possessed the ability to respond to antigen, supporting the process of local B cell affinity maturation but not tissue damage. Interestingly, the remodeling of the vascular bed observed within the TLS was also shown to be dependent on lymphotoxin (LT), as the ectopic expression of LT in *Rag2*$^{-/-}$ mice leads to vascular changes even in the absence of lymphocytes.[28] These findings, using RIP-LT transgenic mice, led to the conclusion that LT, a molecule well known for its role in secondary lymphoid organ development, was able to imprint the stromal compartment of a non-lymphoid organ to acquire morphologic and functional features of a lymph node.

Fibroblasts are the most predominant non-hematopoietic stromal cells, primarily functioning as producers of the extracellular matrix (ECM) that shapes tissue anatomy. Traditionally considered only for their plastic and architectural properties, fibroblasts have been most recently understood to play a functional role in homeostasis and disease, including supporting some of the acquired immunological functions observed in TLS.[2,15,20,29,30] Originally grouped coarsely, fibroblasts have been more recently recognized as a largely heterogeneous population defined by uniquely assigned phenotypes and functions. The use of multi-omics, followed by in vivo validation studies, has enabled several groups with the ability to demonstrate fibroblasts diversity and plasticity in different organs and in response of different conditions, unveiling the key role of fibroblasts in the process of immune surveillance, inflammation and repair.[15,19,29] In order to capture the granular landscape of the role of this compartment in organ disease, the shared and/or exclusive features of fibroblasts in different organs are currently under investigation. Our group and others have attempted to establish the specific role of fibroblasts in the context of TLS, with the aim of dissecting the contribution of this population, not only in the establishment and maintenance of TLS in the tissue, but also in supporting TLS pathogenic functions. We are going to review some of those data in the current review, mapping our work in the broader effort made by the community in trying to understand the role of fibroblasts in health and disease.

2 | PLASTICITY AND SPECIALIZATION: CRITICAL FIBROBLAST FUNCTIONS IN SUPPORT OF SLOS AND TLS FORMATION

The molecular mechanisms underpinning the formation and maintenance of TLS within a specific tissue are not completely understood. Many efforts over the years have aimed to overlay TLS and SLO developmental factors. However, it now appears that TLS aggregation is the result of a very different process from SLO assembly. While some similarities are shared, TLS formation involves a sequence of events and signaling cascades critically different from those regulating the development of lymph node (LN), spleen, or Peyer's Patches (PP).[3,15,31–36]

Mature SLOs play numerous functions in homeostatic and disease conditions, mainly acting as immunological filters, providing an adequate microenvironment to facilitate interaction between naïve cells in search for their cognate receptor antigen.[32,33,37] In this context, lymph node (LN) provides surveillance to the lymphatic system, while spleen filters the blood for bloodborne antigens.[38] While providing a hub for maturation and proliferation of autologous,

antigen-experienced immune cells, SLOs support the screening of autoreactive clones escaped from central tolerance. Key structural anatomical differences among different SLOs support different functions. LN are tightly organized with a system of lymphatic vessels and canaliculi for antigen movement from the outer to the inner part of the organ. The spleen presents a less organized structure, with lack of a capsule defining the boundary between the red and white pulp (respectively, inhabited by macrophages and lymphocytes) and the absence of antigen-delivering, afferent lymphatic vessels.[38,39] While the LN and spleen differ in their anatomical location, these SLOs share the common function of identifying potential pathogenic insult and mounting effective immune responses. SLO stroma is critically important to support these functions. SLO non-vascular stroma is largely represented by fibroblastic reticular cells (FRC), a fine network of canaliculi forming fibroblasts that display unique functional properties, including the ability to support the dramatic anatomical required to adapt SLO to the lymphocyte influx occurring in response to antigen stimulation.[40–42] The mechanism underpinning the physical ***plasticity*** of the SLO fibroblasts has been identified in the LN in the interaction between Podoplanin (PDPN), a glycoprotein broadly expressed on FRCs and CLEC-2, its receptor expressed by dendritic cells, first incomers in the SLO upon antigen exposure, during inflammation or upon immunization. Deletion of this interaction results in the significant reduction in the ability of LN expansion during an immune response.[41] The changes occurring after antigen presentation result in further anatomical modification of the LN stroma that includes the maturation of a specialized network of stromal cells, or follicular dendritic cells (FDC) in the inner part of the B cell follicle. Expansion of the FDC network is required to support the process of B cell affinity maturation.[43,44] Similar occurrence has been described in the spleen.[37,45–48]

Even during development, SLO stroma presents a large degree of plasticity. Early anlagen mesenchyme has the ability to differentiate, upon specific stimuli into diverse and highly specialized stroma, which then create functional micro domains within the lymphoid organs. The efficacy of the immune responses is highly dependent on this ***specialization*** of the SLO resident mesenchyme into these micro domains, which support the formation of diverse anatomical and functional areas that provide different lymphocyte survival and developmental needs.[32,49–52] Loss of SLO compartmentalization has been demonstrated detrimental for the immune response. Similarly, the adaptability of the stroma during the immune response has been deemed critical to enable expansion of the B follicle required to accommodate the germinal center reaction upon immunization. ***Specialization*** and ***plasticity*** of the fibroblasts in SLOs can therefore be defined as the most critical properties required to shape an efficient immune response.

It has been shown in both humans and mice that TLS lack some of the critical architectural features that secondary lymphoid organs, including the presence of a capsule and of an organized lymphatic and blood vasculature.[2,18] Furthermore, while the development of SLO is genetically programmed during development at fixed anatomical locations, TLS development occurs postnatally, in response to chronic inflammatory cues in non-immunological organs. Importantly, TLS might resolve and disappear upon antigen clearance or persist, in pathophysiological settings augmenting the process of tissue damage and aberrant antibody production.[9,53] Similar to SLOs, TLS functions are largely supported by a specialized network of stromal cells that share some of the specialization and plastic features ascribed to the SLO network.[2] However, differently from SLOs, where the general anatomy of the organ is maintained over time, the anatomy of TLS in its cellular composition is highly variable,[2,8,13,14,54] likely reflecting the different maturation stages of these structures.

We and other attempted to identify the presence of a TLS stromal cell precursor, present in non-immune organs but "preprimed" to support the development of a stromal cell network able to sustain lymphocyte migration, survival and proliferation. It is possible to speculate that the presence of this "preprimed "dormant fibroblast precursor could define the permissiveness of certain tissues to harbor TLS.[15,55,56] The signals regulating the ability to differentiate a TLS stroma precursor in response to microenvironmental cues will be later discussed in this review.

In animal models of TLS, early TLS is characterized by small T cell aggregates either surrounding epithelial or endothelial structures. Those are progressively enlarging, supporting the income of B cells that position in the inner follicle. A network of FDC and clear B cell proliferation is observed only in fully mature TLS, and the formation of a functional germinal center requires 10-15 days. This anatomical heterogeneity is underpinned by the development of differentially specialized stroma micro domains, similar to those described in SLOs.[2,13,18] Similar to what is observed in the LN, where different follicles (primary and secondary) can be found at different degree of maturation, the process of TLS development in the tissue is not homogeneous and TLS heterogeneity, both in terms of anatomical and functional maturation, has been described. *Helmink et al* early identified the presence of TLS heterogeneity within a patient biopsy whereby mature GC^{+} TLS, which harbor proliferative B cells, co-reside with immature TLS.[6] In cross-sectional studies, different degree of organization is detected in the salivary glands of patients with Sjögren's syndrome.[57] Progressively enlarged TLS in the salivary glands is associated with increased B cell number and inversion of the B/T cell ratio. Germinal center formation is only observed in large TLS inhabited mainly by B cells. Those, however, can be found in close location to smaller and less organized foci formed in the same gland.[57] The heterogeneity of the TLS within a patient warrants exploration of the question of what signals drive in the tissue the formation of more mature TLS. This is of particular interest in the clinical setting, whereby TLS supports the pathogenic process or the response to therapy.

3 | FUNCTIONAL IMPRINTING OF MESENCHYME IN SLO VERSUS TLS

The development of the TLS stromal network that ultimately supports lymphocyte organization in the tissue presents key similarities

but also critical differences with the development of SLO stroma. SLO development is programmed embryonically, TLS develops in postnatal life in response to molecular cues, mediated by pathogen associated molecular patterns, pro-inflammatory cytokines or cellular contact with activated lymphocytes, largely absent during embryonic lymphoid neogenesis.[2,7,8,13,15,35,54,58] While the mesenchymal compartment of SLOs does not have an assigned functional identity, other than to become the supportive stroma of an immunological organ, TLS formation occurs within organs harboring postnatal differentiated stroma ascribed to provide anatomical support and form the extracellular matrix of the organ. However, as mentioned, it has become recently clear that postnatal fibroblasts inhabiting mature organs can retain the plasticity to alter their functional phenotype. Our group demonstrated that in animal models of TLS, tissue-resident fibroblasts, under chronic inflammatory conditions as well as in response to pathogenic insult, are capable to acquire an *immunofibroblast* phenotype and function, including the expression of adhesion molecules, lymphoid chemokines, and lymphocyte survival factors that can then sustain B and T cell survival in the tissue. The plastic formation of this *immunofibroblast* network requires a multistep process of priming, expansion, and maturation.[15] This cascade is critically different from that underpinning SLO development.

The development of LN in mice and humans is driven by the prenatal interaction between a specific population of $CD45^+$ $CD3^-$ $CD4^+$ $CCR7^+$ $CXCR5^+$ $RANK^+$ $RANKL^+$ $IL\text{-}7R\alpha^+$ $LT\alpha_1\beta 2+$ $ROR\gamma^+$ ($CD4^-$ $RORc^+$ in humans) lymphoid tissue inducer cells (LTi) and non-hematopoietic mesenchymal or endothelial derived $CD45^-$ $CD3^-$ $CD4^-$ $PDGFR\alpha^+$ $PDGFR\beta^+$ $LT\beta R^+$ lymphoid tissue organizer cells (LTo) of the LN anlagen.[32,59–61] At first, the recruitment of LTi was thought to be driven by the lymphotoxin beta receptor (LTβR)-mediated expression of CXCL13 from mesenchymal derived LTo cells within the LN anlagen. In turn, the expression of CXCL13 by the resident mesenchyme has been shown to be induced by retinoic acid (RA) signaling cascade.[62,63] More recently, it has become clear that alternative signals determine the fate of the T and B cell resident stromal cells. Using a $YAP/TAZ^{FRC\Delta}$ mouse, *Choi et al* demonstrated that the ability of FRC to secrete CCL21 and CCL19, key chemokine involved in T cell migration is dependent on the YAP/TAZ transcription factors, downstream effectors of the Hippo pathway.[64] In absence of this signal, LN lacks lymphocyte compartmentalization. Interestingly, this signal precedes the LTβR pathway, as the shuttling of the YAP/TAZ proteins from the nucleus to the cytoplasm is observed following engagement of the LTβR pathway. This results in the suppression of the Hippo signaling pathway in the FRC but the maintenance and continued maturation of the FRC phenotype.[64]

B cell stroma differentiation relies on different signals. *Koning et al* demonstrated that embryonic mesenchymal cells treated with exogenous RA and subsequently with LTβR agonists produce CXCL13 but not CCL19 and CCL21,[62] suggesting that the engagement of the RA signaling cascade drives the stromal cell precursor to an FDC identity but the engagement of LTβR signaling is necessary for the further maturation of the FDC. Interestingly, engagement of LTβR signaling prior to the RA signaling results in upregulation of CCL19, CCL21, and IL-7, a chemokine/cytokine signature aligned with T cell supportive FRC stroma.[62] Further investigations aimed to understand how the RA and Hippo pathway interacts in SLO development are warranted.

The signaling pathways involved in the specialization of the TLS stroma into T or B cell supportive microdomains are not yet clearly known. The organization of the lymphocytes in a compartmentalized manner is orchestrated by the sequential expression of the lymphoid chemokines CCL19, CCL21, and CXCL13 in TLS harbored in several tissues of differing anatomical location, and specialization of TLS-associated vasculature has been observed with upregulation of the addressin recognizing L-selectin and peripheral node addressin (PNAd).[15,65,66] Initial formation of TLS comprises of a collection of infiltrating lymphocytes, which assume the role of LTi cells and are therefore assigned to act as lymphoid tissue initiator cells (LTi).[13,67] Early expression of CCL19 has been detected in animal models in dependency of lymphotoxin alpha simulation [15] (and unpublished work). The cellular crosstalk between the resident mesenchyme and the incoming T lymphocytes results in the progressive maturation of the stromal compartment that supports the expression of B cell chemokines and survival factors, CXCL13, and B cell activating factor (BAFF). Consequent infiltration and expansion of the TLS ultimately leads to the appearance of largely, poorly defined GCs, within the B cell area, that present some hallmarks of B cell proliferation and differentiation of FDCs.[2,13]

The spatial development of TLS has been observed in proximity to vasculature or epithelial structures, often associated with pericytes, smooth muscle cells, and myofibroblast, thus suggesting that the resident vasculature facilitates the extravasation of lymphocytes from the circulation into the inflamed or infected organ and that pericytes play a role in TLS establishment.[2,13] Our group has identified a series of sequential events underpinning the maturation of resident stromal cells of non-immune organs to acquire an *immunofibroblast* phenotype: Those include *priming*, *expansion*, and *maturation* of the harboring *immunofibroblast* network.

3.1 | Immunofibroblast priming

We and others have demonstrated that fibroblast *priming* occurs independently from the LTβR signaling cascade during TLS formation. The secretory cues involved in TLS stroma development encompass a series of cytokines belonging to the tumor necrosis factor (TNF) and interferon (IFN) family, but also IL-13, IL-1 family cytokine, IL-17, and IL-22.[15,68–73] The cellular source of these cytokines and the predominant role in *immunofibroblast* remodeling varies, depending on the tissue within which the TLS is developing and the stimuli in response to which it forms, revealing a diversity of drivers in different diseases. The upregulation of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) and PDPN defines the key *priming* phenotypical changes, supporting the acquisition of an "adhesive" phenotype that facilitates the physical interaction of primed fibroblasts with incoming lymphocytes which express complementary

integrins.[15,72] In some studies, the transient expression of lymphoid chemokines, likely responsible for the initial wave of lymphocytes infiltration has also been defined within the *priming* step.[10,69] The cellular source for priming cytokines in context of TLS formation has been actively studied. The role of $CD4^+$ T cell–derived IL-17 in *immunofibroblast priming* has been described in several models including experimental autoimmune encephalomyelitis (EAE) and iBALT formation.[10,72,73] Additionally, epithelial-derived type 1 IFN has been reported to induce *immuno-remodeling* of lung fibroblasts upon influenza infection.[69] Our group has recently demonstrated the ability of IL-13, to induce the initial *priming of* fibroblast in salivary glands during TLS formation.[15] Interestingly, this cytokine, already implicated in tumor immunosurveillance, is produced in response to pathogenic stimuli, at site of TLS formation by epithelial cells and resident ILC2, suggesting the presence of an innate process, conserved across species (data were confirmed in human and mouse setting), whereby sentinel cytokines prime the resident fibroblast to support the immune reaction required to deal locally with an immune (or autoimmune) process.

3.2 | Immunofibroblast expansion

Fibroblast priming is followed by an active phase of proliferation. Our group identified IL-22, one of the cytokines of the IL-10 family member, as major player in this phase.[14,15] *Immunofibroblasts* isolated from inflamed $IL22R^{-/-}$ and $IL22^{-/-}$ mice failed to proliferate, affecting downstream production of lymphoid cytokines/chemokines leading to TLS abrogation. Intriguingly, IL-22 appeared to play a differential role on fibroblasts and epithelial cells. On fibroblasts, IL-22Rα engagement results in proliferation and expression of early CXCL13, while on epithelial cells the same signaling pathway elicited proliferation and production of CXCL12 but not CXCL13, suggesting intracellular modulatory pathways in these two different cell type.[14] *Pikor et al* observed a similar expansion of *immunofibroblast* networks in leukocyte rich meningeal locations in a mouse model of CNS inflammation. Both IL-17 and IL-22 were implicated for this proliferation phase.[72]

In the salivary gland TLS model, IL-22 was produced by resident ILCs in the very early phase post-TLS induction and by T cells at the peak of fibroblast proliferation. Intriguingly, no significant defect in fibroblast expansion was observed in absence of IL-17 in mice, suggesting that this cytokine, despite belonging to the same family of IL-22, plays a different role, at least in the context of TLS harboring in mucosal sites.[14,15] The expansion of this *immunofibroblast* population is similar to the expansion phase of the lymphoid tissue organizer mesenchyme observed during SLO development. Importantly, in SLO, this process is dependent on lymphotoxin,[74] while in TLS we have demonstrated that this cytokine is dispensable for activated fibroblast proliferation.[14,15]

3.3 | TLS maturation

The final step identified in the acquisition of the complete *immunofibroblast* phenotype and function consists in the maturation of the primed and expanded $PDPN^+$ population of fibroblasts. *Immunofibroblast* maturation is characterized by the stable expression of lymphoid chemokines CXCL13, CCL19, CCL21, and lymphocyte survival factors such as IL-7 and BAFF.[13–15,67] Differently from the other two phases, this last step in fibroblast maturation appears to be fully dependent on both lymphocytes and LTβR signaling. As previously described, the upregulation of cell adhesion markers, ICAM-1 and VCAM-1, facilitates the stabilization of the interaction between lymphocytes and the expanded *immunofibroblast* networks. This allows for the exchange of molecular cues responsible for the broader activation and maturation of the stromal compartment and correspondingly, on the other side, of the recruited infiltrating lymphocytes.[2,3,13,20,70] The requirement of lymphocytes to induce a mature phenotype of the resident mesenchyme was demonstrated by the failure of $Rag2^{-/-}$ and $LT\beta R^{-/-}$ mice to express the lymphoid chemokines upon adenovirus infection.[15] In this stage, infiltrating activated T cells act as *initiator* cells providing secretory LTα or surface bound $LT\alpha1\beta2$, which supports maturation of the *primed* lymphoid stroma.[2,3,15] This reliance of the infiltrating hematopoietic cells on the maturation of the underlying mesenchyme has been also observed in the context of SLO stroma maturation. In $CXCR5^{-/-}$ mice, the absence of B cells, carrying the membrane bound $LT\alpha_1\beta_2$, results in poor maturation of FDCs, with inability to express the lymphoid chemokine CXCL13.[75,76] Similarly, in TLS, the absence of the bidirectional crosstalk between resident stromal cells and infiltrating B cells halted the complete maturation and acquisition of lymphoid stromal cell phenotype. *Krausgruber et al* demonstrated that fibroblasts of non-lymphoid organs possess the epigenetic machinery poised to transcribe genes downstream of proinflammatory cytokine stimulation.[19] Within this gene set, the expression of the lymphoid chemokines CCL19, CCL21, and CXCL13 and survival factors, IL-7 and BAFF, can be induced. The signals involved in the programming of these epigenetic changes and they regiospecific determination in T and B cell supporting stroma are not known.

4 | STROMAL CELL SPECIALIZATION THROUGH THE LENS OF NOVEL TECHNOLOGIES

The emergence of novel transcriptomic technologies has enabled a granular characterization of the stromal cellular compartment in both murine and human organs.

Recently, the complexity of the stromal cell compartment of the murine LN has been unraveled using single cell analysis by Rodda et al[77] This analysis unveiled an unexpected level of heterogeneity and cellular specialization uncovering numerous subsets of fibroblasts with unique phenotypical and functional signatures.[77] The ability of SLO to execute complex functions has been largely attributed to the diversity of the underpinning stroma.[32,33,36,37,49,52,60,77,78] The exclusivity of the different population of fibroblasts creates specialized microdomains within the SLO, which influences antigen presentation, cell migration, retention, activation, and survival of

T and B lymphocytes.[31,32,49,60,79] Characterized as CD45$^-$ PDPN$^+$ CD31$^-$ CCL19$^+$ CCL21$^+$ IL-7$^+$ ER-TR7$^+$, the FRCs, also known as T zone reticular cells (TRCs), exclusively reside in the T cell cortex of the LN.[31,36,76,79] Within this population, *Rodda et al* distinguished a classical CCL21$^+$ CCL19^{high} IL-7$^+$ stromal cells and a CCL21$^+$ CCL19^{low} IL-7$^+$ population with the CCL21$^+$ CCL19^{high} IL-7$^+$ subset fulfilling the classical function of recruiting and maintaining CD4$^+$ IL-7rα^+ CCR7$^+$ naïve T cells and DCs in a L-selectin (CD62L), very late antigen 4 (VLA-4), and lymphocyte function-associated antigen 1 (LFA-1) manner.[77,80] Differently, the CCL21$^+$ CCL19^{low} IL-7$^+$ population has been associated with the expression of CXCL9 and CXCL10, the two classical ligands of CXCR3 and involved in the expression of MHC genes,[81–83] suggesting the involvement of this population in the process of tolerance induction of CD4 and CD8 T cells' reactive clones.[77] The ability of FRCs to participate in the maintenance of immunological tolerance has been previously alluded, with the identification of the ability of FRC to present MHC II complexed self-peptides of DC origin leading to T cell anergy or apoptosis and the report of the tolerance regulator gene Aire (eTACs) in the T cell zone.[84,85]

While similarities in the T cell zone stroma are present in the TLS, a full characterization with single cell analysis is missing, leaving open questions on the level of functional maturity of the TLS stroma in the context of the screening of anergic clones. Intriguingly, TLS has been characterized as hub for proliferation and survival of autoreactive clones, in particular B cell clones, opening the possibility that stromal cells in the context of the TLS fibroblast compartment is not able to act as screening factor for autoreactive clones.[13,15,18,67] Of note, TLS forms in a highly inflammatory environment, characterized by strong upregulation of costimulatory molecules and survival factors, potentially biased toward favoring proliferation rather than favoring anergy or death of autoreactive cells..[13,15,18,67]

Additional distinct FRC subsets were identified within the SLO follicle T zone interface: the cholesterol-25-hydroxylase$^+$ (Ch25) CCL19^{low} characterized by high differential gene expression of the lymphoid chemokines CCL21, CXCL13, and lymphocyte survival factors IL-7 and BAFF.[77] The duplicity of both B and T cell supporting cytokine suggests, together with the anatomical placement of the subset in the interphase between the follicle and the T cell area, that this population comprises stromal cells that are not terminally differentiated, and that retain a degree of plasticity. In this context, previous fate mapping studies have identified the presence of versatile stromal cells (VSCs) [86] at the edge of the T cell-B cell zone interface. VSCs are a population of cells that can be induced to express CXCL13 by the overspill of B cells from the B cell compartment as it expands to accommodate increased B cell infiltration during an immune response.[86] This demonstrates that, while there is compartmentalization and specialization of fibroblasts within the SLOs, the stromal architecture of these organs retains its ability to adapt to the anatomical and functional changes required during the immune response. Other highly specialized fibroblasts have been identified in the B cell zone, enabling the differentiation between primary and secondary follicles. Recent transcriptomic analysis performed in mice with the aim to characterize the stromal landscape supporting the GC reaction has been able to identify two different subsets of FDC, differentiated by the expression of Cr2 and CXCL12.[52] These subsets exist in a differentiated form in non-inflammatory conditions and increased in number in response to inflammation. The characterization of the LZ and DZ stromal compartment from immunized mice, identified a differential transcriptional status of the subsets, defined by differential expression of CXCL1, CXCL16 and IL-6.[52] The topology of the light zone versus the dark zone fibroblast is governed by the functional expression CXCL12 expression. Indeed in CXCL12$^{-/-}$ mice, FDC was scattered throughout the germinal center as opposed to being concentrated in the LZ as observed in wildtype mice.[52] This suggests that CXCR4$^+$ B cells carrying surface $LT\alpha_1\beta_2$ are unable to migrate in response to CXCL12 and therefore not able to support the cellular crosstalk responsible for the maturation of the reticular network. This observation reinforces the critical importance of infiltrating lymphocytes in the stabilization and maturation of the underlying stromal compartment as discussed during TLS formation.

GC formation in TLS is indicative of a mature TLS entity and is only detected in large B cell–rich TLS in association with high expression of lymphoid chemokines, including CXCL13 and CXCL12 and HEV formation. A full compartmentalization of the dark and light zone is not often observed in TLS, unless those form in parotid gland where mature GC often form.[87–89] While compartmentalization in T and B cell areas has been identified within the TLS stroma,[15] a granular definition of the different fibroblasts populations, as described in other diseases, is still missing. Highly organized TLS with classical GC has been described in the parotids of patients with Sjögren's syndrome and mucosal associated lymphoid tissue (MALT) lymphoma.[87–89] The reactive area of TLS is observed in these cases in close proximity to malignant areas of centrocytes like B cell infiltration. The presence of TLS in MALT formation has been identified as essential pathogenetic step, suggesting that the antigen-driven B cell proliferation process harboring within the salivary gland GC represents the key event in lymphoma development. Intriguingly, those GCs anatomically closely recapitulate the GC observed in tonsil, another highly inflamed microenvironment. Nonetheless, the frequency of malignancy development associated with Sjögren's is much higher than the incidence of tonsil lymphoma, suggesting that the inflammatory and autoimmune process that shapes formation and function of salivary glands TLS is intrinsically different from that supporting GC formation in the tonsils. The identifications of these differences could pave the way to the design of novel therapeutics able to prevent the process of lymphomagenesis occurring during Sjögren's syndrome.

5 | FIBROBLAST ROLE IN THE CONTEXT OF AUTOIMMUNITY AND CANCER

The availability of advanced high-throughput techniques has unveiled novel functions and phenotypes of stromal cells also in non-lymphoid organs, broadening from the spatial arrangement of

neighboring structural cells and immune cells to their unknown immunological and nursing functions. Many of the novel functions and subpopulations of fibroblasts have been investigated at a single cell level in murine models and human patient biopsies of inflammation and cancer, with many differences and conserved similarities being brought to light which present potential novel therapeutic avenues.[2,14,15,20,27,90–93] Use of computational biology approaches applied to transcriptomic has provided in the past few years, novel cellular atlases of healthy and disease tissue, and useful gene cassettes able to identify potentially pathogenic stromal cells in the tissue of interest. Given the presence of pathogenic cellular signatures in different diseases, much interest has been given to the definition of "gene cassettes," which are able to identify fibroblasts presenting similar pathogenic features in different diseases.[94] *Muhl et al* reported the presence of a shared fibroblast cassette in murine organs identified by *Pdgfra*, *Cd34*, *Col1a1*, *Col1a2*, *Col5a1*, and *Lox1* and a mural cell cassette that included *Des*, *Mcam (Cd146)*, *Tagln*, and *Notch3*.[94] Those markers have been largely used to define distribution and expansion of fibroblast and mural cells in the context of cancer and inflammation both in humans and mice.[29,95] Diseases explored to date include rheumatoid arthritis, and inflammatory bowel disease, in particular ulcerative colitis (UC).[15,29,96–99]

The stromal compartment in rheumatoid arthritis (RA) is uniquely characterized by individually assigned phenotype and function according to their spatial location within the RA joint.[29] In a study conducted by *Croft et al*, 2 distinct populations of fibroblasts, FAPα^+ Thy1$^-$ or Fapα^+ Thy-1$^+$, were identified in both species, using single cell analysis. The differing functional effects of the two populations were characterized as either pro-inflammatory or supportive of joint damage.[29] Elegantly, Croft demonstrated that depletion of these different populations defines topographic and functional specialization of the synovium stromal compartment previously not appreciated.[29] The developmental signals driving fibroblast specialization in RA have been elsewhere explored and identified in the Neurogenic locus notch homolog protein 3 (NOTCH3).[100] *Brenner and colleagues* demonstrated that endothelial cells are able to establish a NOTCH3 gradient, which governs differentiation of the diverse fibroblast subpopulations, suggesting that fibroblast identity within a given microenvironment is governed by its spatial location and temporal interaction with different cell compartments. In contrast to RA, the pSS microenvironment has yet to be characterized at cellular level using single cell technology. However, in vivo methodologies independent of scRNAseq have identified specific *Immunofibroblast* populations characterized by the expression of ICAM-1 and VCAM-1 and their corresponding expression pattern of the lymphoid chemokines as described in the context of *immunofibroblast* maturation.[15]

In the context of inflammatory bowel disease (IBD), 3 studies have aimed to characterize fibroblast specialization.[97–99] Leveraging the comparison between human biopsies of healthy and diseased individuals these studies identified the emergence of a population of proinflammatory fibroblasts in human diseased samples, which were not identified within healthy individual tissue. *Kinchen et al* reported the presence of a population named S4, annotated as being involved in cytokine signaling pathways and positive regulation of T cell activation.[98] In support of this observation, *Smilie et al* also identified a population of "*inflammation-associated fibroblasts*," which were unique to the UC and shared gene expression signatures, similar to cancer-associated fibroblasts (CAFs).[99] In addition, *Martin et al*, focusing specifically on Crohn's disease, identified a conserved cellular module, termed *GIMATS*, characteristically defined in a subset of patients with poor responses to anti-TNF therapy.[101] Additional studies, within the skin, identified the alterations of fibroblast function in an age-dependent manner.[102] *Solé-Boldo et al* revealed the loss of the ability of fibroblast priming with age using scRNAseq studies skin biopsies from young and old male donors. A delayed transition from the G1/2 phase of the cell cycle in the older fibroblasts in comparison with the younger counterpart was observed. Further interrogation of this phenomenon lead to the identification of an age-dependent loss of cell-to-cell interaction and the development of a gene signature in older fibroblasts suggestive of an increased susceptibility of this population to the accumulation of reactive oxygen species (ROS).[102] The authors suggest the possibility that this signature could contribute to the poor immunological responses generated in the older population as a result of the inability of the "old fibroblasts" to modulate the immune response in vivo.

In cancer, hints of conserved fibroblastic signatures among several types of malignancies have been reported. Cancer-associated fibroblasts (CAFs) have been characterized in several solid tumors, using similar transcriptomic technologies to those used to interrogate fibroblasts identified in different autoimmune diseases.[90,91,93] Identified by the expression of FAP, PDGFRα/β, PDPN, CD90, and αSMA, CAFs are the most abundant stromal component in the tumor microenvironment (TME). CAFs mediate tumor persistence and grow supporting the production of immunosuppressive factors such as transforming growth factor-β (TGF-β) and indoleamine-2,3-dioxygenase 1 (IDO-1).[23,103,104] The role of CAFs in cancer progression and response to therapy has raised awareness toward this population in the general context of the TME and a series of publications have aimed to define CAF spatial location and function. In bladder urothelial carcinoma,[95] the presence of two distinct fibroblast identified as *PDGFRa*$^+$ CAFs and *RGS5*$^+$ myo-CAFs was reported. *PDGFRa*$^+$ CAFs were associated with the expression of a plethora of cytokines and chemokines including IL-6, CXCL12, and CXCL14, a phenotype similar to immunomodulatory fibroblasts identified in inflammatory conditions.[15,29,95,97–99] Using cellular interaction inference tools within the scRNAseq data, *Chen et al* identified the critical interaction of this cell population with the CXCR4$^+$ tumor-associated macrophages (TAMs). The ability to pinpoint specific molecular interactions among cell populations has been largely explored by Davidson et al[90,105] In a landmark study on a murine model of melanoma, the presence of three distinct CAF populations and the cellular machinery that they possess to support melanoma growth in an in vivo *animal* of melanoma were investigated.[90] The three CAF populations were referred to individually as immune, desmoplastic, or contractile based on their differential gene expression profile and their potential role in the organization of the immune

TABLE 1 Table illustrating current characterization of fibroblast subsets in lymph nodes, autoimmune conditions and cancer

Characterization of fibroblast subsets in LN	Function
CCL19^{high} / CCL19^{low} T Zone Reticular Cells (TRC)	• CCL19^{high} TRCs express PDPN to support DC motility and LN expansion[41,77] • CCL19^{low} TRCs at T zone interface express **cholesterol-25-hydroxylase** guide activated B cells, T cells and DCs to the follicle T zone interface
Cxcl9^{+} T Zone Reticular Cells (TRC)	• Cxcl9^{+} TRCs an activated subset of CCL19^{high} TRCs tolerize T cells in a resting LN and position CXCR3^{+} cells during an immune response • Cxcl9^{+} TRC DEGs were dominated by **IFN-γ** genes **Gbp4** and **Gbp5**[77]
Ennp2^{+} Marginal Zone Reticular Cells (MRC)	• **Ennp2^{+}** MRCs influence lymphocyte antigen interaction with **RANKL** on follicle edge of SCS[77,107]
Itga7^{+} Perivascular Cells	• Non-adventitial PvCs support multiple functions of blood vessels • PvC subset which was characterized as **Cnn1^{+} Itga7^{-} Pdpn^{-}** enriched for thrombospondin (**Thbs1**), **Il-34** and **endothelin-A receptor**, suggesting a role in endothelial support[77]
CD34^{+} Stromal Cells	• Recent work characterized **PDPN^{+} BST1^{-} ACTA2^{-} CD31^{-} CD34^{+}** SCs surrounding large vessels in the LN medullary cords as adventitial cells[108] • Enriched for **CD248** expression which is reported in the resting LN capsule and required for LN expansion after immunization[77]
Nur77^{+} Stromal Cells	• Consist of activated cells from other subsets • Distinguished by expression of early response genes downstream of TLR or cytokine stimulation and included: **Nur77, Fosb, Fos, JunB, Egr1, Ikba, Zfp36**[77]
Follicular Dendritic Cells (FDC)	• Two FDC subsets: FDC1 and FDC2 • LZ FDC1 = increased **Cr2** and **CXCL13** • DZ FDC2 = increased **CXCL12** and **IL-6**, **CXCL16** in response to inflammation[52,77,107]
Characterisation of fibroblast subsets in autoimmunity	**Function**
Fapα^{+} Thy1^{+} Synovial Fibroblasts in Rheumatoid Arthritis	• Phenotyped as the "**immune effector**" population resident within the synovial lining layer • Adoptive transfer of this population resulted in persistence in inflammatory arthritis[29]
Fapα^{+} Thy1^{-} Synovial Fibroblasts in Rheumatoid Arthritis	• Phenotyped as the "**destructive fibroblasts**" resident within the synovial lining layer • Adoptive transfer of this population resulted in damage of bone and cartilage with little modulation of the inflammatory signature[29]
PDPN^{+} CD34^{+} Salivary gland fibroblasts in primary Sjögren's Syndrome	• The compartmentalisation of function with **ICAM^{high} VCAM^{high}** expression and enrichment for the expression of the lymphocyte survival factors **IL-7** and **BAFF**[15]
PDPN^{+} CD34^{-} Salivary gland fibroblasts in primary Sjögren's Syndrome	• Displayed enrichment for the expression of the lymphoid chemokines **CCL19**, **CCL21** and **CXCL13**[15]
SOX6^{+} colonic fibroblasts in inflammatory bowel disease	• High expression of **TGFβ** ligands **BMP2**, **BMP5**, non-canonical Wnt ligands (**Wnt5a, Wnt5b**) and the secreted Wnt antagonist **FRZB** therefore involved in the mediation of repair and regeneration of damaged tissue[97,98,100]
PDPN^{+} CCL19^{+} IL33^{+} colonic fibroblasts in inflammatory bowel disease	• This stromal population was expanded in IBD disease as it was not identified in healthy colonic tissue and aligned to stromal cells underlying TLS • The inflammation associated fibroblasts in UC had a gene signature for inflammatory gene expression of **IL-1R1**, **TNFSF11, IL-13RA2**[97,98,100]
Characterization of fibroblast subsets in cancer	**Function**
PDPN^{+} CD34^{+} CAFs in melanoma	• Engages in immune cell crosstalk via upregulation of **Cxcl12, Csf1, Ccl8**, **IL6ra, Il6st,** and components of the complement cascade • Identified stromal immune interactions between **C3/CXCL12/CSF1** expressing stromal cells and macrophages, which expressed **C3AR1, CXCR4, and CSFR1**[91]
PDPN^{+} CD34^{low} CAFs in melanoma	• An intermediate population of stromal cells that expressed PDPN and PDGFRα but low expression of acta2 and CD34 • Uniquely expressed the genes **Postn** and **TNC** and was associated with a fibrotic matrix[91]

(Continues)

TABLE 1 (Continued)

Characterization of fibroblast subsets in cancer	Function
PDPN$^-$ CD34$^-$ CD146$^+$ myoCAFs in melanoma	• **Acta2^{high}** contractile stromal had pericyte associated markers **Ng2**, **CD146**, **Rgs5** • Also shared the expression of **Col1a1, Cola1a2** with the neighboring **PDPN+** fibroblasts[91]
PDGFRα$^+$ IL-6$^+$ CAFs in bladder carcinoma	• PDGFRα$^+$ express various cytokines and chemokines including **CXCL12**, **IL6**, **CXCL14**, **CXCL1**, and **CXCL2** and were termed **inflammatory CAFs** • **iCAFs** had enrichment of cytokine-cytokine receptor interaction pathway • Also had increased proliferation ability as compared to non-inflammatory CAFs[95]
PDGFRα$^-$ RGS5$^+$ myoCAFs in bladder carcinoma	• **RGS5$^+$** stromal cells have been characterized to be similar myoCAFs in melanoma[95]
Sox9$^+$ developmental CAFs in breast cancer	• dCAFs distinguished by the expression of genes related to stem cells **Scrg1, Sox9, and Sox10** • Found during early developmental stages of cancer involved in the development and morphogenesis of TME[94]

response within the context of the cancer has been described.[90] The use of a curated receptor-ligand scRNA database enabled this group to identify the receptor ligand axis of complement component C3 as key molecule involved in the crosstalk between CAFs and cancer macrophages. Pharmacologically interference with this signaling axis in an animal model was able to disrupt the immunosuppressive microenvironment and slow tumor growth in vivo.

The engagement of the C3 and C3aR complement cascade has also been described in the context of the contribution to the *priming* of immunomodulatory synovial fibroblasts.[106] The dependency of the inflammatory synovial fibroblasts on this signaling cascade was made evident with the observation of reduced tissue *priming* in the bone marrow chimera models in C3$^{-/-}$ mice.[106] This suggests that fibroblasts may be similar in phenotype, but unique in their function depending on the disease context from which they are derived. From these studies, it is made evident that the use of computational approaches, matched to single cell transcriptomic, has vastly enabled scientists to achieve a better understanding of the heterogeneous stromal compartment within disease and can be further exploited to resolve the complexity of the fibroblast landscape (Table 1).

The ability to locate conserved signatures of fibroblast populations across species (human and mouse tissues) has been also sought in order to interrogate fibroblast specification and function in the context of the dynamic changes occurring during disease pathogenesis, providing a unique insight in fibroblast plasticity in the context of disease. *Korsunky et al* investigated the presence of a conserved fibroblast populations in tissues isolated from patients with different inflammatory conditions arising from different anatomical sites such as synovium, intestine, lung, and salivary glands.[55] Two fibroblast populations were identified as CXCL10$^+$ CCL19$^+$ immune-interacting and SPARC$^+$ COL3A1$^+$ vascular-interacting fibroblasts, which were expanded in all inflamed tissues across the four diseases at different anatomical sites.[55] The origin of this population in the tissue has not been fully elucidated. An extensive study by *Beuchler et al* investigated the presence of conserved fibroblasts across species and multiple organs evaluating commonalities between human and mice in different organs in steady and perturbed states, and two universally distributed fibroblast types were differentiated by the expression of Pi16 or Col15a1.[56] The developmental link between steady state and "inflammation induced" fibroblasts in the various mouse organs was demonstrating that the inflammatory activated subsets could be traced back to arise from the steady state fibroblasts using transcriptomic trajectory analysis.[56] Altogether, those evidences suggest the presence of plastic stromal populations distributed in different tissues that function as core progenitors of inflammatory stroma in the context of proinflammatory microenvironmental cues.[56]

From a cellular perspective, the identity of the underlying fibroblast compartment, which becomes remodeled in response to inflammation to drive TLS development, has not been defined yet at a single cell level. The identification of this progenitor and the mapping of the cues implicated in its development could provide novel therapeutic avenues to favor TLS formation in conditions where their presence has been identified as beneficial, for example in cancer or block it in conditions where TLS formation has been associated with disease progression and tissue disruption.

6 | CONCLUSIONS

We have discussed the function and organization of TLS in both health and disease and the possible different cellular compartments and signals involved in their formation and development in comparison with SLOs. We have also addressed the emerging importance and nature of the stromal heterogeneity, which exists and underpins TLS development and function. In the past, it has been challenging to define the complex heterogeneity of cells within various tissue populations and disease contexts, but with the emergence of tools such as scRNAseq this issue has been circumvented. This not only facilitates our understanding of specific cellular signals and interactions exchanged amidst the crosstalk of different cell compartments. To this end, coupled with in vitro and in vivo assays, deciphering the cellular landscape of TLS-driven disease would allow us to

characterize the specific stromal subsets that may drive the overall functional heterogeneity seen in different TLS-associated diseases. Resolving the nature of this heterogeneity would allow us to therapeutically target-specific cellular components of mechanisms enabling manipulation of TLS formation to become advantageous tools in cancer therapy or dissolved in the context of transplant rejection and autoimmunity.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest in the production and completion of this manuscript.

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