Lymphoepithelial lesions in the salivary glands of primary Sjögren's syndrome patients: the perfect storm?

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ABSTRACT

In patients with primary Sjögren's syndrome (pSS), inflamed salivary gland (SG) tissue may contain lymphoepithelial lesions (LELs). LELs are histopathological phenomena whereby B cells are present in hyperplastic ductal epithelium of the SG. Despite the potential role of LELs in pSS pathogenesis, studies on their formation, detection, and prevalence in benign lesions (not complicated with lymphoma) are scarce. Recent evidence however shows that LELs are present in approximately half of the patients with pSS, both in minor and major SGs. Migration of a small number of B cells into the epithelium appears to be a critical initial step in LEL formation. These intra-epithelial B cells are proliferative, exhibit an innate-like phenotype, and may be linked to MALT lymphoma development. Alongside intra-epithelial B cells, the hyperplastic epithelial partner in LELs also engages in the local immune reaction. Epithelial cells are a source of cytokines and chemokines, with CXCL10 in particular playing a potential role in LEL formation. Importantly, LELs also have a negative impact on the maintenance of SG homeostasis by SG progenitor cell (SGPC) populations, likely due to dysregulation of SGPC lineage commitment or induction of plasticity. In conclusion, LEL formation mirrors a perfect storm of B and epithelial cell interaction culminating in increased risk of B cell derailment and SGPC dysregulation in pSS patients. We therefore argue that attenuation of LEL formation is an important treatment goal to preserve SG function and prevent B cell derailment in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease typically associated with inflammation and dysfunction of salivary and lacrimal glands. A salivary gland (SG) biopsy has an integral role in diagnosis of patients with pSS. Abnormal SG histopathology is defined by periductal infiltrates surrounding the striated and excretory ducts (focal lymphocytic sialadenitis). The infiltrates are comprised of mononuclear cells, predominantly B cells and $CD4^+$ T cells. Data would suggest that more severe SG pathology (higher infiltration grade, greater focus score) in pSS is related to a higher proportion of glandular B cells, underpinning the pivotal role of B cells in pSS pathology (1). In addition to presence as *peri*ductal infiltrating cells, lymphocytes are also found *within* the striated and excretory ducts of primary SS patients' salivary glands. This invasion of ducts by lymphocytes, accompanied by ductal cell proliferation (hyperplasia), has evolved as a broad definition of a lymphoepithelial lesion (LEL) (2). While studies in recent years are beginning to shed light on the phenotype and function of intra-epithelial B cells (3, 4), still relatively little is known about the induced changes in ductal epithelial cells in the context of LELs, besides hyperplasia. Also, the contribution of LELs to dysfunction of the glands and disease activity in general remains unclear.

In the current review, we will summarise the available evidence on LEL formation and its relation to histological and clinical features in pSS. We will expand on the immune component, B cells in particular, and we will also address changes in the ductal epithelium. Finally, we propose a chronologic model of LEL formation, from the early stage of glandular disease to lymphoma development.

Identification and scoring of lymphoepithelial lesions

LELs can be identified by an experienced pathologist based on haematoxylin and eosin staining. For the less experienced eye and in difficult cases, additional immunohistochemical staining for high molecular weight cytokeratins (hmwCKs) aids in standardised assessment of LELs to define ductal borders and prevent misinterpretation of LELs as ectopic germinal centres (2, 5, 6). While LELs are defined by epithelial hyperplasia and the presence of intraepithelial lymphocytes, the specific lymphocyte type comprising this component of a LEL has not until recently been defined. In pSS patients, T cells are found scattered throughout the ductal epithelium, both within LELs and normal (non-hyperplasic) ducts. LELs are strongly associated however with the presence of intra-epithelial B cells, to the extent that a 'pre-LEL' histopathological stage was recently suggested, defined by B cell presence intraductally, without epithelial hyperplasia. Progressing from the pre-LEL stage, three further stages of LEL development ('severity scores') have been previously proposed based on the percentage of epithelium displaying hyperplasia and presence or absence of ductal occlusion (Table I) (6, 7). As multiple striated ducts are often present in one biopsy section, a patient's score can be based on the mean stage of LELs per biopsy or the duct with the highest severity, a strategy which may be open to further standardisation. Importantly, the intra-epithelial B/T-ratio increases significantly with advanced stages of LEL development, underlining again the salience of B cells in LEL development (6).

Considering the difficulties in identification and quantification of LELs, a digital image analysis algorithm was recently developed, whereby intraepithelial B- and T-lymphocytes are automatically detected (8). To apply this algorithm, consecutive slides were stained for CD3 (T cells), hmwCK (striated ducts) and CD20 (B cells), scanned, and digitally aligned (Visiopharm Integrator System). Although validation in multiple centres is required and the digital image analysis algorithm is not (yet) able to score the amount of epithelial hyperplasia, this technique is promising for standardised assessment of pre-LELs and LELs in multiple centres.

Table I. Scoring system for the severity of lymphoepithelial lesions in salivary gland tissue of pSS patients.

LEL severity score	Presence of intra-epithelial B cells	Presence of epithelial hyperplasia	Proportion of epithelium displaying hyperplasia	Visible lumen
Pre-LEL	Yes	No	0%	Yes
1	Yes	Yes	<50%	Yes
2	Yes	Yes	50-100%	Yes
3	Yes	Yes	100%	No

Incidence of LELs in pSS

LELs are usually detected in close proximity to lymphocytic foci and are virtually absent from biopsies of non-SS sicca patients, indicating that LEL presence is highly specific for SS (6, 8). Striated ducts further removed from foci do not generally develop into LELs. Primary SS patients with LELs have a significantly higher focus score compared to those without (8), suggesting a relationship between the amount of periductal infiltration and the formation of LELs. Both labial and parotid gland biopsies can be employed as diagnostic tools in pSS and LELs can be found in both tissues. In a diagnostic cohort of pSS patients who underwent both a parotid and a labial gland biopsy, the proportion of patients with at least one LEL in their biopsy was 56% for the parotid and 42% for the labial biopsy. In addition to this higher LEL frequency in the parotid SG, the mean stage (*i.e.* severity) of LELs is also higher in the parotid compared to the labial gland (8). Although the reasons for a higher LEL frequency and severity in the parotid glands are not known, a possible explanation could be that the foci of parotid glands contain a higher proportion of B cells or that the ductal epithelium in these glands secretes higher levels of chemokines compared to the labial gland.

Relation between LELs and clinical and immunological features

When clinical characteristics of pSS patients with intra-epithelial B cells (pre-LEL and/or LEL) in their salivary gland biopsy are compared to those without, patients with (pre)LELs display higher serological activity (rate of autoantibody positivity and serum IgG level). There is a particularly strong association with RF positivity; approximately 90% of pSS patients with intra-epithelial B cells are RF-positive and vice versa also 90% of RF-positive patients have intra-epithelial B cells (8). (Pre)LEL-positive patients also show lower stimulated whole salivary flow rates and higher ocular staining scores (8). Patients with LELs also show a higher maturity of circulating antibody-secreting cells, reflecting more pronounced B cell hyperactivity (9). Thus, the presence of LELs is indicative of more severe serological abnormalities and glandular disease and could be employed to identify pSS patients with pronounced (pathogenic) B cell involvement. Patients with LELs also demonstrate significantly higher presence of hypoechoic regions of the parotid SG as identified by ultrasound (10). Considering the size of LELs however (in the µm range), compared to the very large, macroscopic nature of hypoechoic regions, LELs are unlikely to be the sole source of hypoechoic regions in the SG in pSS.

The immune component of LELs

Intra-epithelial B cells and LEL formation

Previous work from the 80's-90's showed that LELs can be associated with a spectrum of lymphoid proliferations, from benign-appearing, polyclonal B cell expansions to high-grade lymphomas (11-13). Now, we know that almost half of the pSS patients harbour LELs in their salivary glands and

only 5–10% of pSS patients will develop B cell lymphoma. So far, relatively little is known about the initial steps of LEL formation. By quantification of intra-epithelial B cells in parotid gland tissue sections, van Ginkel *et al*. identified a pre-LEL stage characterised by the presence of intra-epithelial B cell and absence of epithelial hyperplasia (6). Vice versa, ductal cell hyperplasia almost never occurred in absence of intra-epithelial B cells, suggesting that infiltration of B cells into the duct is a critical step in LEL formation. In line with this notion, B cell depletion therapy with rituximab significantly reduced the number and severity of LELs (7).

Phenotype and function of intra-epithelial B cells

Virtually all intra-epithelial B cells and a portion of the periductal B cells in pSS patients' salivary glands express Fc receptor-like protein 4 (FcRL4) (3). FcRL4, also known as CD307d or IRTA1, is a Fc-receptor like protein that can bind systemic IgA (14,15). FcRL4 expression is normally restricted to memory B cells in mucosal tissues (*e.g*. tonsil) and mesenteric lymph nodes (16, 17). Although low numbers of FcRL4$^+$ B cells can be found in peripheral blood, these numbers are not elevated in pSS patients compared with non-SS sicca controls (4). FcRL4 is thought to inhibit BCR-mediated signalling, while Toll-like receptor (TLR)-mediated signalling remains intact, permitting B cell activation in spite of BCR activity attenuation (18, 19). We previously found that epithelium-associated FcRL4$^+$ B cells are highly proliferative, but lack the germinal centre B cell marker Bcl6, and plasma cell differentiation markers (*i.e*. Blimp1 and Mum1) in parotid gland tissue of pSS patients without lymphoma (3). The proliferative capacity of intra-epithelial B cells was also demonstrated in a separate study by the presence of clonal expansions within microdissected infiltrated ducts. These clonal expansions had increasing numbers of immunoglobulin gene somatic mutations, illustrating that somatic hypermutation can occur locally in the duct and outside germinal centres (19).

To gain more insight into the phenotype and function of intra-epithelial (FcRL4$^+$) B cells, we compared the transcriptome of FcRL4-positive vs. FcRL4-negative B cells sorted from parotid gland cell suspensions of pSS patients. FcRL4$^+$ B cells exhibited an activated phenotype, characterised by higher expression of *ITGAX* (CD11c), *TBX21* (T-bet), and *TNFRSF13B* (TACI) and lower expression of *CXCR5* and *CD40* (4). A similar B cell phenotype, also characterised by low expression of CD21, has been associated with a TLR7-induced, extrafollicular B cell effector population enriched in patients with systemic lupus erythematosus (20). Expression of T-bet by pSS patients' FcRL4$^+$ B cells could play an important role in their migration towards ducts, as T-bet directly transactivates CXCR3 expression (21). *CXCR3* was indeed upregulated in FcRL4$^+$ *versus* FcRL4$^-$ B cells, although this was not statistically significant (4). CXCR3 is also expressed by a significant part of the circulating FcRL4$^+$ B cells (4).

Another relevant marker upregulated by FcRL4$^+$ B cells is TACI. Expression of TACI is strongly linked to TLR activation and is important for T cell-independent immune responses (22), in line with a more 'innate' phenotype of FcRL4$^+$ B cells. Binding of BAFF/BlyS, produced by the epithelium, to TACI could be an important stimulus for survival and proliferation of intra-epithelial B cells. Although the function of FcRL4$^+$ B cells within LELs is not fully clear, these cells may interact with epithelial cells via adhesion factors and stimulate the epithelium by expression of pro-inflammatory cytokines, such as IL-6.

B cell migration into LELs: role for CXCL10?

Although the factors responsible for the migration of B cells into the ductal epithelium remain largely unknown, chemokine secretion by the epithelium probably plays an important role. The interferon-induced chemokine CXCL10 (IP-10) is of particular interest, because it is one of the main chemokines secreted by the inflamed ductal epithelium in pSS (23), and can attract CXCR3-expressing lymphocytes including subsets of (FcRL4$^+$) memory B cells. We therefore compared levels of CXCL10 in stimulated whole saliva (SWS) and serum samples of pSS patients with and without LELs. Both SWS and serum CXCL10 levels were higher in pSS patients with LELs (Fig. 1). In particular CXCL10 levels in SWS showed notable difference in value between LEL-positive and -negative pSS patients. Whether high salivary CXCL10 levels are a cause or consequence of LEL formation remains to be established. The role of other chemokines important for B cell migration, such as CXCL12 or CXCL13, also requires further investigation in the context of LEL formation.

Relationship of LELs to MALT lymphoma

FcRL4 is expressed by nearly all mucosa-associated lymphoid tissue (MALT) lymphomas (16). We have postulated that FcRL4$^+$ B cells are a potential precursor pool for MALT-lymphoma B cells, based on their (activated) phenotype and strong association with the ductal epithelium (4). The finding that there are (relatively) more FcRL4$^+$ B cells in parotid glands compared to labial glands may help to explain why most SS-associated MALT lymphomas develop in parotid glands rather than in labial glands (3).

Although usually benign, LELs are a diagnostic feature of MALT lymphoma (24). A causal relationship between LELs and MALT lymphoma has not been formally proven, but presence of multiple LELs with high severity in a salivary gland tissue section should draw further attention to the potential presence of MALT lymphoma. In pSS patients, RF-expressing IgM$^+$ B cells are particularly prone to derailment, since 75% of SS-associated MALT lymphomas express RF-stereotypic variable region sequences (25). Although the reasons for preferential monoclonal expansion of RF-expressing B cells are not fully understood, LELs may play an important role. Via apoptosis or exosome secretion, the inflamed and hyperplastic epithelium forms a potent source of TLR ligands, including nucleic acids bound to the au-

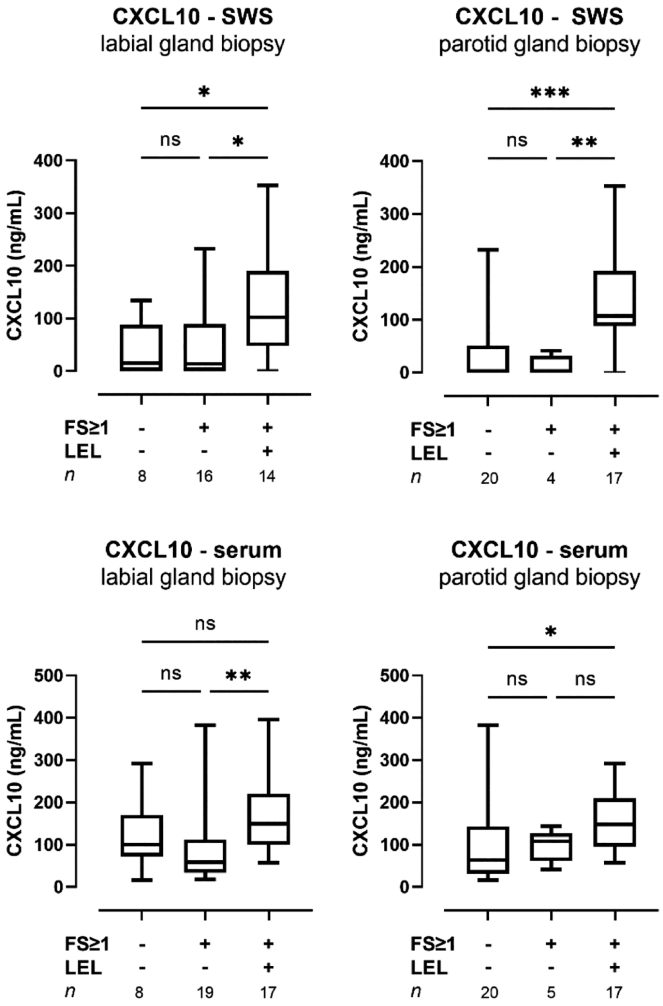

Fig. 1. CXCL10 levels in stimulated whole saliva and serum samples of pSS patients, stratified by labial or parotid gland histology. Boxplots show median and interquartile range. Patient characteristics of this diagnostic patient cohort have been previously described (8). CXCL10 levels were measured by ELISA (PeproTech, Rocky Hill, NJ).
SWS: stimulated whole saliva; FS: focus score; LEL: lymphoepithelial lesion.

toantigenic ribonucleoproteins (RNPs) Ro52, Ro60, and La (26, 27). In pSS patients, RNA-RNP particles may become part of immune complexes (ICs) containing IgG-autoantibodies. Soluble IgM-RF may also bind and promote the formation of these complexes. Vice versa, ICs are potent stimuli for RF-expressing B cells, as nucleic acid components stimulate TLRs after endocytosis, while the IgG component directly stimulates the BCR (28). Furthermore, soluble factors secreted by the inflamed ductal epithelium, such as BAFF/BlyS and other B cell-stimulating cytokines (*e.g.* type-I IFN) (29-31), promote B cell survival and proliferation (18, 24). Thus, presence of LELs as part of a pro-inflammatory tissue microenvironment appears to be an important driver of uncontrolled clonal expansion of (intraepithelial) B cells in pSS patients who develop salivary gland MALT lymphoma. As mentioned before, also in the absence of lymphoma, B cell expansions can already be found in the ductal epithelium (19).

The flip side of the coin: what effect do B cells have on the salivary gland ductal epithelium and its progenitor cells?

The immune capabilities of salivary gland epithelial cells

Through the application of adherent cultures of epithelial cells from the minor SGs, and organoid cultures from the parotid major SGs, it has become abundantly clear that the SG epithelium, or in any case those ductal components, actively contribute and encourage the inflammatory environment of the salivary glands of pSS patients. For example, the SG epithelium, once 'activated', would seem to be capable of expressing a wide range of chemokines and cytokines (*e.g.* IL-18, IL-1, IL-6, IL-7, TNF-α, BAFF, CXCL10, CXCL12, CXCL13 and CCL21) (32-45). SG epithelial expression of MHC Class I, and MHC Class II HLA-DR, CD80 and CD86 stimulatory/co-stimulatory molecules necessary for T-cell activation, as well as ability to recognise pathogen-associated molecular patterns (PAMPs) via TLRs, would all imply an ability to engage in an immune reaction. This intrinsic toolkit is what lends a fascinating angle to LEL development, from the perspective of ductal cells promoting T and B cell activity, and equally from the angle of a signalling recipient from derailed intraepithelial B cells (46, 47).

Initial observations of the epithelial component of LELs

Although also not substantial in terms of numbers of publications, available literature often focuses on the B cell compartment of LELs, in relation to the immune landscape and MALT lymphoma development in pSS. In early literature, epithelial hyperplasia in LELs was ascribed to proliferation of myoepithelial cells and the terms 'myoepithelial lesions' and 'myoepithelial islands' were used, a dogma which is no longer widely accepted, considering that myoepithelial cells envelope acinar cells, and not the ductal system (2, 48-50). What is believed more commonly is that LELs are formed by the proliferation of the basal cell layer of the striated ducts (BSD cells), a phenomenon which is intriguing in light of development in our understanding of SG homeostasis (2, 51, 52). The past ten to fifteen years of research into the SG has suggested that the SDs particularly house a population of salivary gland progenitor cells (SGPCs) (53, 54). Whether all BSD cells function as SGPCs is not clear, but at least a portion of them appear to possess potent regenerative abilities. This keratin 14^+ (KRT14) BSD cell population, including the likely SGPC subset, is anchored to the basement membrane surrounding the duct, and is normally present as a single, dispersed layer of cells (Fig. 2A). In healthy SDs, the $KRT14^+$ cell layer is distinct from the keratin 7^+ (KRT7) ($KRT14^-$) luminal SD layer. Depending on the model system used

to study them, the organism and the damage/disease context, KRT14+ BSD cells proliferate and differentiate into functionally mature cell types, including additional KRT14+ basal SD cells, luminal SDs cells, myoepithelial cells and acinar cells (54-56). This process presumably involves KRT14+ cell proliferation, migration of daughter cells out of ducts, and concomitant differentiation into relevant progeny types. In LELs, the KRT14+ cell layer is no longer a single cell layer deep (Fig. 2B). Layers of KRT14+ cells 3-4 cells deep are often present, which appear to lose their anchorage to the basement membrane, and become luminally situated within the SD (Fig. 2B). Indeed, the basal lamina loses its integrity in the SG of pSS patients, potentially contributing to this loss of K14+ BSD layer organization (57). The KRT7+ luminal SD layer, arranged in a columnar fashion and also a single layer deep in normal SDs (Fig. 2C) also appears to lose its regimented organisation, and single cell layer in LELs (Fig. 2D). These observations alone, and our previous knowledge regarding SGPCs, imply that somewhere in the development of LELs in pSS, the dynamics of SGPCs become drastically altered.

Acinar cells and dysregulated lineage commitment or progenitor cell differentiation in LELs?

Saliva is produced by acinar cells, through the secretion of water, mucous and proteineaceous components via the apical terminal. To facilitate the secretion of the aqueous component, the water channel aquaporin-5 (AQP5) is expressed on the apical membrane of acinar cells. Its expression is normally tightly regulated to this cellular location. Acinar cells do not express KRT14 or KRT7 to a high degree, and in terms of keratin expression can be more easily identified by Keratin 18 expression. Curiously, we observed ectopic AQP5 expression in cells of the striated ducts in LELs (Fig. 2B). This observation may imply dysregulated KRT14+ SGPC function, perhaps premature differentiation towards acinar cells before migration out of the SD niche. From examination of the

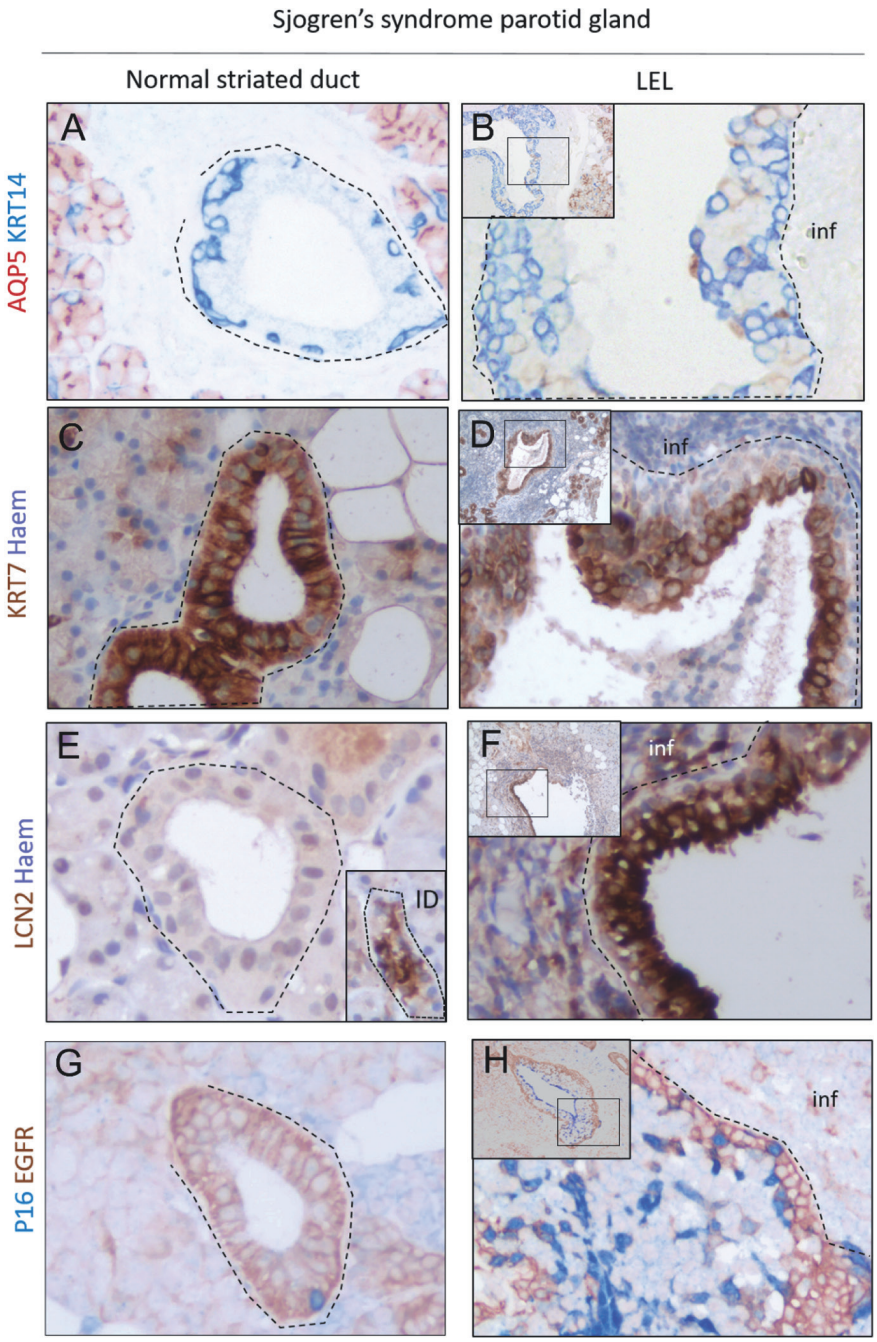

Fig. 2. Expression of marker suggestive of salivary gland progenitor and epithelial cell dysregulation in lymphoepithelial lesions. Immunostainings for: A & B) Aquaporin-5 (AQP5); C&D) Keratin 7 (KRT7); E&F) Lipocalin 2 (LCN2); and G&H) p16 and epithelial growth factor receptor (EGFR) double staining. Dashed lines outline normal appearing striated ducts, or the border of the LELs where appropriate. Inset boxes in B, D, F, H and J show low resolution image of the LEL. Images in panels A-H are counterstained with haematoxylin (blue/purple).
inf: infiltration; ID: intercalated duct.

KRT14 and KRT7 immunostainings in Figure 2A and B it would seem likely that some KRT14+KRT7+ cells in LELs may exist, although we cannot confirm this directly yet. An alternative KRT14+ cell-independent explanation for AQP5 expression by ductal cells in LELs would be direct transdifferentiation (also termed 'plasticity') between luminal SD cells into AQP5+ acinar-type cells. Plasticity of the salivary gland has been demonstrated in the mouse in response to stress and injury, whereby AQP5+ acinar cells became ductal cells (58). Although we have yet to perform AQP5 KRT7 KRT14

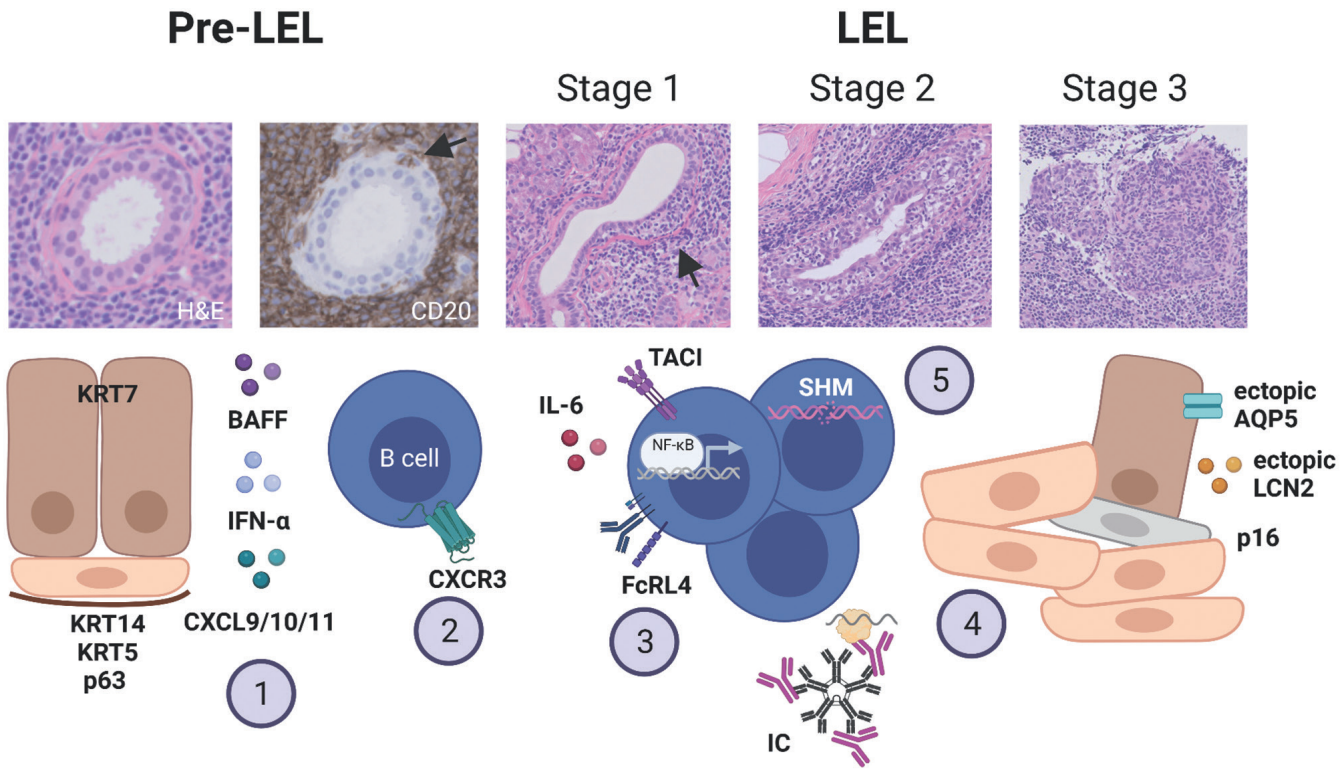

Fig. 3. Model of lymphoepithelial lesion (LEL) formation. In a pre-LEL, a small number of B cells migrate into the epithelium in response to chemokines and cytokines (step 1-2). In a stage 1 LEL, intra-epithelial B cells are further activated and increase in number, accompanied by a local cluster of hyperplastic epithelial cells. Upon activation, B cells acquire and/or maintain an activated, innate-like phenotype (step 3). In a stage 2 LEL, the majority of the epithelium shows hyperplasia and the typical layered structure is lost. This accompanied by senescene (p16 expression) and ectopic expression of acinar cell markers (AQP5) or intercalated duct markers, such as LCN2 (step 4). In this phase, extracellular presence of ribonucleoproteins (autoantigens) may fuel formation of immune complexes consisting of rheumatoid factor, autoantibodies, autoantigens, and nucleic acids. In this pro-inflammatory environment, B cells continue to receive stimulation, resulting in proliferation and somatic hypermutation (step 5). B cell expansion may ultimately lead to a stage 3 LEL, in which hyperplasia is further increased and organisation of the epithelium is compromised, resulting in loss of ductal lumen.
LEL: lymphoepithelial lesion; KRT: keratin; BAFF: B cell activating factor; IFN: interferon; TACI: transmembrane activator and CAML interactor; IC: immune complex; AQP: aquaporin; LCN: lipocalin. Created with BioRender.com.

triple labelling of a LEL, our previous work in the context of immune checkpoint inhibitor-induced SG dysfunction demonstrated presence of KRT7$^+$ AQP5$^+$ double positive cell structures, providing some foundation for this second 'plasticity'-based theory and/or an intermediate cell-type (54, 58-62).

In addition to the striated ducts, the SG also contains smaller intercalated ducts. These ductal structures contain progenitor cells, this time within the luminal layer, as opposed to the basal layer of striated ducts. Lipocalin2 (LCN2) is expressed by this luminal intercalated duct layer, in addition to KRT7. KRT7$^+$LCN2$^+$ cells are capable of replicating and differentiating into acinar cells (62). LCN2 expression is not detected in normal (appearing) basal or luminal cells of the SDs (Fig. 2E). LELs are also not associated with the intercalated ducts. Interestingly however, in LELs of pSS patients, LCN2 is expressed by luminal SD cells, implying again either switching of luminal cells from striated duct to intercalated duct phenotype (plasticity), or unusual differentiation of the BSD progenitor cells into intercalated duct cell types (Fig. 2F). Again, multiplex immunostaining would be valuable in teasing apart if ectopic expression of LCN2 coincides in the same cell with that of AQP5, KRT14 and KRT7.

In summary, considering the presence of AQP5$^+$ and LCN2$^+$ cells in the striated ductal cells of LELs, it would appear feasible that differentiation from KRT14$^+$ basal SD progenitor cells into luminal duct intercalated cells may occur in LELs, and perhaps even the initial phases of their transition into (AQP5$^+$) acinar cells, although these observations remain speculative, pending further validation.

Reduced sodium channel expression by luminal SD cells is likely mediated by soluble factors produced by periductal B cells

Acting as a vessel for the transport of saliva from acinar cells to the mouth, and housing progenitor cells, are not the only functions of the striated ducts. Through the action of transporter channels, the luminal cells of the striated ducts also modify the ionic content of saliva, critical for maintaining the correct calcium ion concentration needed for continuous mineralisation of the enamel of the teeth (63). Several studies have attempted to use ion content in saliva as biomarkers for pSS with varying degrees of success. Increases in sodium content between saliva from pSS patients compared to various control groups were the most robust compared to other ions (64-69). Translation of these correlations into sodium levels as

a clinical readout for pSS however has been slow, presumably due to the lack of specificity for sodium ion concentration alterations for pSS. Patients with aspecific sialadenitis may also demonstrate increased sodium levels, for example. Parotid gland specific salivary sodium levels in pSS significantly correlated with the degree of parotid gland CD45+ cell infiltration, in particular with the number of B cells in the infiltrate, and with the degree of LEL severity (63). Downregulation of sodium transporting machinery was observed in both normal (appearing) SDs close to immune foci and in LELs, implying that LELs serve in the context of sodium dysregulation as a proxy for degree of lymphocytic infiltration, and are not necessary per se for sodium transport disruption (63, 70-73).

Expression of senescence markers in LELs

Senescence is a state of permanent cell cycle arrest, where cells both lose their ability to proliferate, but are also not apoptotic. Senescent cells furthermore also secrete a panel of proinflammatory factors, together termed the senescence-associated secretory proteome (SASP), capable of spreading the senescent state to neighbouring cells. Senescence can be induced by DNA damage (for example radiation), and also by extensive replication. In the case of the latter, progressive shortening of telomeres with every division eventually leads to a critical telomere length which triggers cell cycle arrest to avoid chromosomal DNA damage. If a stem cell population becomes senescent, losing its ability to proliferate, it cannot perform its function in maintenance of tissue homeostasis. p16 is a cell cycle inhibitor and is commonly employed as a marker of senescence. Our previous work showed that BSD cells in pSS are likely to have reached replicative senescence, due to the pro-mitotic influence of pSS-associated pro-inflammatory cytokines such as IFN-α, IL-6 and TNF-α (64). p16 expression by BSD cells correlated negatively with the amount of produced unstimulated whole saliva, stimulated whole saliva and stimulated parotid gland saliva (65). The degree of BSD cell p16 expression was positively correlated with the extent of CD45+ cell infiltration in the gland (74). In relation to LELs, the number of p16+ BSD cells was larger (p=0.056) in patients demonstrating presence of LELs, compared to those without (74), although we did not distinguish between what proportion of those cells were physically situated in LELs or in normal (appearing) striated ducts. We now also appreciate that p16 is also expressed by non-epithelial (likely B or T cells) in LELs, denoted by lack of expression of the epithelial-broad marker EGFR (Fig. 2G, H). Attainment of a state of senescence by B cells in LELs, namely a lack of apoptosis and expression of SASP proteins, may also augment the inflammatory environment, and encourage further proliferation of (non-senescent) B cells and the epithelium, and the potential development of MALT lymphomas.

Model of LEL formation and concluding remarks

To summarise, a perfect storm involving cytokines, B cells and ductal epithelial cells may develop in pSS patients' salivary glands, ultimately leading to LELs. In Figure 3, we propose a stepwise model for LEL formation. The first step is probably inflammatory pathway activation in the ductal epithelium and consequent secretion of pro-inflammatory cytokines and chemokines, including CXCL10, BAFF and type-I IFN. CXCR3+ B cells are then attracted and migrate into the epithelium, potentially aided by basement membrane disruption. Within the epithelium, B cells acquire and/or maintain an activated and more 'innate' phenotype characterised by expression of surface receptors FcRL4 and TACI, among others, and NF-kB pathway activation, resulting in *e.g.* IL-6 secretion. Intra-epithelial B cells stimulate BSD cell proliferation, leading to the characteristic epithelial hyperplasia typical for LELs and aberrant expression of AQP5 and LCN2. In more severe LEL stages, numbers of intra-epithelial B cells increase, most likely stimulated by chronic antigen exposure and presence of immune complexes. These severe LEL stages are characterised by complete disruption of epithelial structure, which will compromise the function of ducts, *i.e.* saliva transport to the mouth and ion resorption. Ultimately, uncontrolled proliferation of B cells within LELs may lead to MALT lymphoma development.

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