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Small-molecule inhibitors and the salivary gland epithelium in Sjögren’s syndrome

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ABSTRACT

Introduction: The salivary gland (SG) in primary Sjögren’s syndrome (pSS) is characterized by its lack of function (hyposalivation) and lymphocytic invasion. Small-molecule inhibitors (SMIs) are a new class of drugs, whose diminutive size permits diffusion into cells. SMIs targeting components of the immune system are eagerly being trialed for their potential therapeutic utility in pSS. Neglected until now, however, is a discussion of the potential effects of SMIs on the SG epithelium.

Areas covered: We begin by reminding the reader of the SG epithelial compartment, its complicity in inflammatory milieu formation in pSS, and categories of SMIs which merit attention. We discuss each SMI category, including pre-clinical data concerning pSS and likely consequences of their application on the SG epithelium.

Expert opinion: Recovery of saliva production in pSS requires restoring the function of the SG epithelium, not solely on inflammation resolution. Many SMIs, for example, those blocking JAK-STAT signaling, interfere with critical epithelial cell pathways, most notably EGF signaling. If the effect of SMIs on SG epithelium is ignored, recovery of SG function will be challenging. We predict that NFκB signaling blockade will impart the least SG epithelium damage whilst reducing inflammation and facilitating recovery from hyposalivation in pSS.

1. Introduction: small-molecule inhibitor use in Sjögren’s syndrome

Primary Sjögren’s syndrome (pSS) is a systemic autoimmune disease, frequently typified, amongst many other symptoms by loss of function of the exocrine glands, autoantibody production and chronic fatigue [1]. The immune landscape of pSS patients is complex and has been described as conforming to a ‘type I interferon signature’, in addition to active BAFF (also induced by interferon), IL-6, IL-7, and IL-21 pathways [2,3]. The interferon signature refers to the enhanced level of expression of interferon genes themselves (α, β, γ), and their downstream targets, such as IFIT1, IFIT3, IFI44L, IFI35, IFI44, MX1, OAS1, and SIGLEC [4]. Induction of the interferon signature genes is physiologically indicative of a viral infection, lending weight to the theory that pSS may originally, or at least partially, be caused by a latent/misprocessed viral infection [5]. The BAFF/IL-6 and IL-21 pathways are likely to be complicit in B-cell hyperactivity, as frequently observed in pSS [6].

In pSS, for as yet unclear reasons, salivary glands (SGs) become infiltrated with lymphocytes. In fully developed pSS, the infiltrate is predominantly composed of T- and B-cells and coincides with dysfunction of the SG (hyposalivation). Although lymphocytic infiltration was originally presumed to be causative of SG dysfunction, this has recently been disputed, largely due to the poor correlation between the amount of infiltration and saliva production [7–9]. Some studies suggest indeed that defects in calcium signaling, and specifically the Orai1, STIM1 and TRPC1 molecules involved in store-operated calcium entry (SOCE), may underlie reduced saliva production prior to extensive lymphocytic infiltration [10,11]. This ongoing dispute fosters the continuing search for new agents to ameliorate hyposalivation in pSS patients, as research efforts continue to pinpoint why saliva production diminishes. Small-molecule inhibitors (SMIs) represent a relatively new class of chemicals, with weights of under 900 Daltons. Their diminutive size permits diffusion across cell membranes, facilitating transport to their intracellular sites of action. Several publications have extensively reviewed the use of SMIs in rheumatic diseases, including pSS [12–15]. These reviews focus mostly on the effect of various SMIs on the immune cells commonly associated with pSS. Here we take an alternative approach. We aim to discuss possible uses and effects of SMIs on the SG epithelium in pSS, and their eventual application to facilitate regeneration of the damaged SG in pSS. We conclude by suggesting that recovery of the function of the SG in pSS will not be achieved without considering the effects of chosen drugs on the SG epithelium.

2. The salivary gland epithelium in Sjögren’s syndrome

In order to provide a full dissection of the potential effect of SMIs on the SG epithelium, we will begin by outlining the respective members in this cell compartment, the evidence of their involvement in the immune response in pSS, and which
SMIs in our opinion must be examined in terms of their effects on the epithelium.

2.1. Types of salivary gland epithelial cells

The SG epithelium is comprised of several cell types (Figure 1). Beginning at the foundations of saliva formation, acinar cells produce and secrete either watery or mucous-rich saliva, from serous and mucous acinar cells, respectively. In both cell types, M3 muscarinic and α1 adrenergic neurotransmitter signaling initiates the IP3R signaling pathway, releasing calcium via the inositol triphosphate receptor (IP3R) from the endoplasmic reticulum into the cytoplasm. This increased intracellular calcium concentration triggers the action of the store-operated calcium entry (SOCE) system. The concerted action of the Orai1, TRPC1, and STIM1/2 trio of SOCE members in the plasma membrane drives the movement of more calcium into the cell [16]. The activation of water, potassium, and chloride channels in response to this calcium deluge results in water, and hence saliva, secretion out of the apical pole of the acinar cell, via the aquaporin-5 channels [16]. Secretion is facilitated by myoepithelial cells, which envelope the acinar cell clusters and contract in response to cholinergic stimulation to expel saliva. Secreted saliva is channeled through small intercalated ducts into striated ducts, comprised of basal and luminal cell types, and finally through the larger excretory ducts into the mouth. Complementing these cell types and critical for the sustained SG function are its resident stem/progenitor cell populations. Stem/progenitor cells, from here on termed SG progenitor cells (SGPCs) proliferate and differentiate into fresh acinar cells, to replenish acinar cells reaching the end of their lifespan. Human SGPCs are proposed to reside in the intercalated and striated ductal compartments, with some SGPC populations also being present in acini [17,18]. Although all of the above named cell types are epithelial in nature, the term epithelium in pSS studies is usually used to refer to the striated ducts. For the purpose of this review and in keeping with the field, we will therefore also use the term ‘epithelial cells’ to refer to the striated ducts. Confusingly, ‘salivary gland epithelial cell (SGEC) cultures’, often used to model the SG epithelium, may also contain intercalated duct cell, acinar cells, and fibroblasts in addition to striated duct cells. SGEC cultures are generated by mincing SG tissue,

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**Figure 1. Epithelial cell types in the adult human parotid salivary gland.** (a) Schematic representation of the five different types of epithelial cells in the adult human salivary gland. The parotid gland contains serous acinar cells as secretory component, whereas the submandibular gland contains a mixture of serous and mucous acinar cells. (b–e) Histological images showing a global view of a section of parotid gland tissue (b), and high-resolution images of striated ducts (c), an intercalated duct (d), and the acinar cell/myoepithelial cell secretory unit (e). Hematoxylin-eosin staining.
typically human minor (lip) biopsies, and depositing them into tissue culture plates. A proportion of cells will adhere to the plastic and proliferate to cover the surface of the dish, and are used to study the SG epithelium [19]. SGECs according to Dimitrou et al. are keratin 8, 18, and 19 positive, all of which are broad SG epithelial markers, including acinar cells, and not specific for the striated ducts [13]. Caution is recommended when extrapolating properties of SGECs to striated duct epithelial cells, and vice versa. Conversely, the recent increase in comprehension of the nature of adult stem/progenitor cells throughout the human body, and what they require to grow, has facilitated their isolation from the SG, and indeed from patients with pSS, in the form of organoids cultures (Figure 2) [20]. Organoids, defined as ‘3D structure(s) grown from stem cells and consisting of organ-specific cell types that self-organizes through cell sorting and spatially restricted lineage commitment’ represent miniature versions of their respective organs of isolation, and contain not only progenitor cells but also terminally differentiated functional cells such as acinar cells [21]. As such organoids may be considered a superior model for probing the mechanisms underlying hyposalivation in pSS, and will presumably be employed more frequently in the future to understand the SG in pSS.

2.2. Salivary gland epithelium as immune conductor

The SG epithelium as a whole was initially considered targeting the process of SG damage in pSS, and at the receiving end of immune signals. SGECs from pSS patients, for example, appear to be more sensitive to apoptosis, undergoing more apoptosis when stimulated with TNFa and IFNα. They are also likely to be an important source of autoantigens, to undergo more detachment-induced apoptosis (anolysis) by toll-like-receptor 3 ligation than healthy controls, and to be susceptible to apoptosis induced by pSS-associated B-cells [22–24]. Indeed, TLR3 stimulated SGECs also demonstrated an enhanced mRNA level expression of the Ro60, Ro52 and SSB autoantigens on their surfaces as a consequence of apoptosis facilitating initiation of an immune response by immune cells [25–27]. In addition to the increased apoptosis of epithelial cells, the SG epithelium has also been demonstrated to contain autophagy machinery that is less active [25,26,28,29]. Using SG organoid cultures, we have recently demonstrated that the proinflammatory cytokines IFNα, TNFα, and IL-6 induce proliferation of SG ductal cells, appearing to promote their premature transition into replicative senescence [20]. We propose that this, in combination with SGPC apoptosis, may underpin persistent hyposalivation state observed in pSS, as the cell pool normally responsible for production of fresh acinar cells is exhausted [20].

Recently, various studies, focusing mostly on immunohistochemical stainings has suggested that the epithelium actively participates in the development of the immune milieu in pSS. SGECs are capable of antigen presentation to the immune system, by means of expression of MHC Class II, CD80, and CD86 stimulatory/co-stimulatory molecules, all signals necessary for T-cell activation [30,31]. SGECs also bind a variety of pathogen-associated molecular patterns (PAMPs), as inferred by expression of toll-like receptors (TLRs) 1, 2, 3, 4, and 7, and express receptors for CXCL10, CXCL12, CXCL13, IFNα, IFNβ, TNFα, in addition to the B-cell activator CD40 and it is receptor CD40L [20,31–39]. They also produce a wide array of cytokines/chemokines including IL-1β, IL-21, IL-1, IL-6, TNFα, BAFF, CXCL-10, CXCL-12, CXCL-13, and CCL-21 under various stimulation conditions [19,32–44]. Taken altogether, it is therefore fair to assume that SMIs targeted at blocking general immune system signaling pathways such as the NFκB, TLR and JAK-STAT pathways may also exert an effect on the epithelium, its progenitor cell population, and effect the subsequent regenerative abilities of the SG. In support of the feasibility of rescuing the SG epithelium in pSS, patients treated with a biological DMARD rituximab demonstrated reversion of lymphoepithelial lesions, whereby striated ducts become invaded by B-cells and proliferate, to normal ductal architecture [45].

2.3. Relevant SMIs for the epithelium in pSS

According to the clinicaltrials.gov website at the time of writing and when searched under the disease type ‘Sjögren’s syndrome’ (last accessed February 2019), 17 of the 166 clinical pSS trials registered are investigating the use of small-molecule inhibitors for treatment of pSS [46]. The cellular pathways inhibited by these agents include those blocking elements of the innate immune response (TLR, NFκB, JAK-STAT and cathepsin S signaling), the adaptive immune response (BTK and calcineurin signaling) and the autophagy pathway (PI3K signaling, mTOR signaling). Considering the presence of TLRs and cytokine receptors on SG epithelial cells, and the implied activity of the NFκB pathway downstream of TLR signaling, examination of the mechanism of action of these SMIs is crucial. Inhibition of B-cells via Btk signaling blockade or calcineurin signaling, or antigen presentation by cathepsin S blockade conversely, will not be considered here. Undoubtedly attenuation of B-cell proliferation, T-cell activation and antigen presentation in the SG epithelium will have a knock-on effect on the epithelium, which remains to be investigated. In the interest of completeness, we will consider the effect so of manipulation of the autophagy, apoptosis and Rho kinase pathways, three general cellular pathways for which no clinical trials are as yet running for pSS, but for which attention may very likely

Figure 2. Representative phase contrast microscopy of salivary gland organoid cultures from the human parotid gland.
turn to in the coming years. Available drugs targeting the pathways discussed are listed in Table 1.

### 3. Inhibitors of major immune signaling pathways

The fastest and first response of the body to attack by a (perceived) foreign body is the triggering of the innate immune system. Many immune responses being with TLR signaling, whereby pathogen-specific molecular signals bind, and activate the NFκB pathway, amongst other pathways. Activated NFκB signaling then proceeds to initiate transcription of proinflammatory genes such as cytokines. Secreted cytokines are capable of activating neighboring cells and transmitting signals through the JAK-STAT pathway. SMIs targeting these crucial inflammatory signaling pathways are commercially available and in clinical trials for use in pSS in all of these signaling pathways. Each pathway will be considered with respect to its blockade in pSS and the potential effect on the SG epithelium.

3.1. **Inhibitors of toll-like receptor signaling**

Pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), such as viral RNA, and include the TLRs [49]. In humans, so far up to 10 different TLRs have been identified. Endosomally located TLRs 3, 7, and 9 recognize viral RNA/DNA or CpG nucleotides, and their activation has traditionally been most commonly associated with pSS. The activation of TLRs stimulates innate immune cells such as macrophages and dendritic cells to produce inflammatory cytokines, induce dendritic cell functional maturation and antigen presentation to naive T-cells [50]. Two cascades are involved in TLR signal transduction process, involving either Myd88 signaling (used by all TLRs except TLR3) and TRIF signaling (used by all TLRs). These pathways are reviewed in Takeda et al. [49] and culminate broadly speaking in NFκB pathway activation. In pSS PBMCs, TLR7 and TLR9 mRNA levels are upregulated, compared with healthy controls, and altered TLR expression phenotype was found in pSS PBMCs [51]. At present, no TLRs inhibitors are being evaluated in clinical trials for pSS. A group of antimalarial drugs, however, namely hydroxychloroquine sulfate (HCQ), chloroquine (CQ), and quinacrine, have been applied to treat arthritis and SLE patients [52]. These SMIs work by preventing endosome acidification and TLR 7, 8 and 9 activation. CpG–52,364 is a quinacrine derivative. A phase I trial of the use of CpG–52,364 for SLE treatment has been completed, and results are likely to be published in the near future. Another SMI of TLRs, ST2825, which exerts its effects by inhibiting MyD88, has been found to suppress TLR9 induced B-cell proliferation, differentiation, and autoantibody production, showing potential therapeutic promise for pSS [53]. Although appearing promising for treatment regimes in pSS, first studies into their effects on pSS patients were disappointing, and did not improve symptoms, including dryness [54].

Focusing now on the SG epithelium, SGECS have been well established to express not only virally associated TLRs (3 and 7) but also those associated with bacterial infections (TLRs 1,2,4) [37]. pSS SGECS and labial SGs displayed higher constitutive expression of TLR1, TLR2, TLR3, TLR4, and MyD88 mRNA compared with healthy control SGECS. In the labial SG of pSS patients, the acinar and ductal epithelial cells strongly expressed TLR2, TLR3, TLR4, and MyD88. Ductal and acinar epithelial cells in pSS parotid glands expressed TLR7 and TLR9 but were found to be limited to the ductal epithelial cells only in the controls [55]. When SGECS were treated with the TLR2, 3 and 4 ligands, dose-dependent upregulation of ICAM-1, CD40, and MHC-I expression was observed; moreover, compared to control SGECS, SGECS from pSS patients displayed significant and constitutive higher gene expression of TLR1, 2 and 4 [39]. TLR signaling in the SG epithelium is therefore active and its blockade is likely to also affect the SG epithelium. The SG epithelium relies on many signaling pathways for its homeostasis. One such pathway is epithelial growth factor (EGF) signaling. In relation to TLR signaling, EGF receptor (EGFR) activation has also been demonstrated to be necessary for TLR3 signaling in an epithelial cell line, and for the TLR-4-mediated activation of the downstream NF-κB pathway, albeit in a cancer cell line model system [56,57]. Vice versa, TLR4 signaling also activates EGFR signaling, activates proliferation in intestinal epithelial cells and regulates expression of EGFR ligands [58]. In terms of rescuing SG parenchymal balance in pSS, we would suggest that TLR

<table>
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<th>Drug</th>
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<th>Registered clinical trial? (stage)</th>
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<td></td>
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<td></td>
<td>GS-9876</td>
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<tr>
<td></td>
<td>JAK-STAT (3.3)</td>
<td>Filgotinib</td>
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<td>LY294002</td>
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<td>Wortmannin</td>
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blockade is not the foremost candidate for therapeutic use based on its cross-talk with the EGF pathway.

### 3.2. Inhibitors of NFκB signaling

The nuclear factor kappa B (NFκB) family is a group of transcription factors, capable of activating an array of inflammatory downstream targets, when permitted to translocate to the nucleus. Their activation can be achieved through both a canonical or non-canonical pathway. The five members of the NFκB family (RelA, RelB, c-Rel, NFκB1, and NFκB2) form homo- or heterodimers with each other, together with the inhibitory molecule IkB. The canonical NFκB pathway is activated by proinflammatory cytokines such as IL-1β and TNFα, that bind to their respective receptors and also by binding of (PAMPs) to the TLRs. Engagement of both types of receptors triggers activity of the IKK (IkB kinase) complex, culminating in phosphorylation IkB proteins in the inhibitory NFκB complex. Phosphorylated IkB is then degraded, and released NIK/K translocate to the nucleus and activate target gene transcription. The non-canonical pathway operates via a slightly different mechanism, and given the expression of CD40 on ductal epithelial cells, is also likely to be operational in pSS. For the purpose of this review however and considering which drugs are currently in clinical trials for pSS treatment, we will focus only on the canonical pathway.

In pSS, overall phosphorylated IKKe, responsible for degrada-
tion of IkB proteins, and NFκB levels were positively and significant-
antly correlated with biopsy focus score, infiltration grade and overall disease activity measured [59]. The levels of BAFF and a large group of proinflammatory cytokines, which are all regu-
lated by NFκB signaling, are elevated in pSS [60,61]. Single nucleotide polymorphisms in NFκB pathway genes have been asso-
ciated with pSS [62], and a specific mutation in the promoter for a member of the inhibitory IkB complex, IkBa-826T, was associated with susceptibility for pSS [63]. Numerous SMIs of the NFκB signaling pathways are currently commercially available for use. Currently, two drugs acting as NFκB modulators are in clinical trial phases for treatment of pSS. Iguratimod is a new synthetic DMARD show to have anti-inflammatory effects in animal models of arthritis or other autoimmune diseases, and preventing activation of the NFκB signaling pathway through as yet unclear mechanisms [64]. A phase 2 trial using the Syk-inhibitor GS-9876 is also currently in progress. Syk-signaling lies upstream of IKK activation, and its blockade results in amelio-
rating NFκB release from its inhibitory complex.

Numerous preclinical studies have examined the role of the NFκB pathway in the SG epithelium in pSS. The NFκB pathway has been soundly demonstrated to be active in pSS SGECs. Phosphorylated IKKe (pIKKe), pIkBa, and pNFκB were all highly expressed in ductal epithelium in minor SGs from pSS patients [65]. Expression of the NFκB inhibitor IkBa, which inhibits NFκB activity by masking its nuclear localization signals, was significant-
ly lower in SGECs from pSS biopsies than in healthy controls [65,66]. Stimulating the TLR2 receptor in SGECs induced IL-2 production via the NFκB pathway in pSS SGECs [67,68]. Treating SGECs with pSS anti-Ro(SSA) autoantibodies resulted in a progressive increase in binding of NFκB to DNA, and transfection of SGECs with IkB-α in anti-Ro(SSA)-treated SGECs lead to a marked reduction of proinflammatory cyto-
kines gene expression and an increase in apoptosis [26,69]. In a human SG cell line, upregulation of IL-6 was regulated by a group of pathways including NFκB [70]. Our recent study demonstrated that knock-out of the natural NFκB inhibitor A20 in K14+ epithelial cells (and thus constitutive pathway activation) was sufficient to trigger the initial phases of pSS, including reduced saliva production, and lymphocyte invasion of the SGs [38]. Although detailed studies focusing on the SG are lacking, there may also be a degree of interplay between the NFκB pathway and acinar cell calcium signaling, whereby calcium signaling is essential for NFκB pathway activity [71]. In pSS patients with reduced saliva flow but minimal lymphocytic infiltration, acinar cell calcium signaling was found to be reduced, and in a separate study employing neuronal cells, blockade of NFκB signaling interfered with function of the membrane calcium channel proteins Orai1/STIM1/2 [10]. If these data can be extrapolated to SG acinar cells, the effect of NFκB blockade via SMIs on acinar cell function may further exacerbate problems with saliva secretion, by disruption of calcium signaling. These studies aside, no studies directly checking the effect of NFκB SMIs on SGECs have been per-
fomed. Cross talk between the NFκB and EGF signaling pathways is mediated via TLR signaling, as discussed in section 3.1. Theoretically, SMIs targeted against NFκB signaling should exert minimal collateral damage on EGFR signaling, which remains a problem with TLR blockade, and should also dam-
pen the inflammatory process.

### 3.3. Inhibition of JAK-STAT signaling

JAK and STAT intracellular messengers relay a membrane-bound signal to the nucleus, through means of dimerization in various combinations of JAK (four members: JAK1,2,3 and TYK2) and transcription factor STAT (seven members: STAT1, 2, 3, 4, 5A, 5B, and 6) family constituents. JAK-STAT signaling is initiated by binding of cytokines to their respective receptors on the cell surface and is utilized by more than 50 cytokines. Cytokine binding induces receptor multimerization, which permits cross-phosphorylation of cytoplasmic JAK kinase. JAKs then activate members of the STAT family by phosphorylation, facilitating their migration to the nucleus and activation of downstream targets. STAT proteins tend to activate defined gene sets. These sets can be broadly grouped into four types of effector responses, but which can vary depending on cell type and tissue origin of the signal transducing cell. In lymphocytes, activation of STAT1 and STAT4 drives production of an anti-viral Th1 response, STAT6 a Th2 response, STAT3 a Th17 response, and STAT5 an anti-
inflammatory Treg response. A defined role for STAT2 remains unclear. Considering the established type-1 interferon signature, one can hazard a guess that blockade of JAK-STAT signaling may be considered of therapeutic applicability in pSS. A common upstream denominator of cytokine receptors associated with these response profiles appears to be JAK1, narrowing down the potential choice of JAK inhibitors in pSS [72]. A clinical trial with filgotinib, a selective JAK1 inhibitor, is currently in progress in pSS, the outcomes of which will be eagerly awaited. Two other clinically approved JAK1 inhibitors, tofacitinib (blocking JAK1 and
JAK3) and ruxolitinib (blocking JAK1 and JAK2) remain to be extensively tested in pSS. Focusing on peripheral blood, Vartoukian et al. [73] demonstrated the potential utility of targeting JAK-STAT, when reporting that SOCS3, a negative regulator of the JAK-STAT pathway, was itself dysregulated in peripheral blood of pSS patients [73]. Filgotinib treatment also reduced IFNo-induced IFIT1 and IFIT2 gene expression, and membrane-bound BAFF expression in SGECs from lip biopsies [74,75]. We have also demonstrated recently that SGPCs react to proinflammatory cytokines by proliferation and apparent cell death, presumably by signaling through the JAK-STAT pathway, implying that inhibitors of JAK-STAT signaling may also interfere with the homeostasis of the SG epithelium [20].

The SG epithelium, including its progenitor cells, is characterized by its reliance on EGF and Wnt signaling for homeostasis, amongst others [18,76,77]. EGF signaling is mediated by several mechanisms including by JAK-independent activation of the STAT3 [78–80]. The influence of JAK inhibitors such as filgotinib on EGFR signaling via STAT3 is currently unclear, and will likely be crucial to the stability of the epithelium following JAK1 inhibition. Another pathway salient to the function of many epithelial cell populations is the TGF/SMAD system. Expression of TβRβ family members is associated with differentiation of SGPCs into acinar cells [81]. Activation of the JAK-STAT pathway may result in enhanced activity of the TGF signaling messenger SMAD7, promoting thus SGPC differentiation. Theoretically, JAK-STAT blockade may prevent acinar cell formation, and eventual saliva production [82]. Interestingly, SMAD7 activation also enables STAT3 activation.

A third pathway salient to epithelial cell survival and of relevance to JAK-STAT inhibition is the Wnt signaling pathway. Wnt signaling has been demonstrated to be crucial to numerous adult stem cell populations, and supplementation of cell culture medium with members of the Wnt family permits generation of mini-SGs in the laboratory [20,76]. The intracellular intricacies of the Wnt pathway reveal a degree of cross-talk with the JAK-STAT pathway. Binding of the Wnt3a ligand to its extracellular receptor in a retinal epithelial cell line has been shown to activate STAT3 [83]. STAT3 has additionally been implicated in the nuclear translocation of β-catenin, a process crucial to Wnt-pathway activation [84]. Vice versa, T-cell factor (TCF), a binding element for a member of the Wnt pathway, was discovered in the STAT3 promoter [85]. β-catenin also induced STAT3 transcription and mRNA/proteins levels, providing robust evidence of the interplay between the two pathways [85]. Knockout of adenomatous polyposis coli (APC), a negative regulator of the Wnt pathway, resulted in activation of the JAK-STAT signaling pathway, albeit in a fly model [86].

All in all, these data imply that despite the likely efficacy of JAK-STAT blockade in terms of resolving inflammation, there are likely to be numerous detrimental effects on the homeostasis of the SG epithelium. JAK-STAT signaling inhibition will affect diverse cellular processes in the SG parenchyme, including epithelial (SGPC) proliferation and differentiation, as a curious interactive web exists between JAK STAT signaling, and at least three important epithelial cell/epithelial stem cell signaling pathways [87].

4. Manipulation of epithelial cell survival mechanisms

The parenchyme of the SG is maintained by proliferation and differentiation of progenitors cells, which replace exhausted or damaged functional cells. These damaged cells presumably undergo apoptosis and are cleared from the tissue. SGECs from pSS biopsies are more sensitive for apoptosis, which can reportedly be induced by anti Ro and La autoantibodies [26,27,42,88,89]. Apoptosis represents therefore an interesting therapeutic modality.

A second homeostatic pathway that should be considered in terms of potential therapeutic utility is the autophagy pathway. Recent studies have also demonstrated the salience of the autophagy system, a cellular resources recycling system, for maintenance of tissue parenchyma in general [90]. During stress situations, the autophagic machinery recycles cellular resources to enable their applications where most needed. Autophagy has been demonstrated to be less active in pSS, pointing to a need to normalize this imbalance in order to restore saliva production [28,29].

A third category of SMIs not generally associated with the immune system but of potential importance to the SG epithelium are the small Rho GTPases. Inhibition of downstream targets of Rho GTPases tends to promote proliferation in epithelial cells [91]. The striated duct epithelial cells in pSS, in addition to becoming apoptotic, are widely proliferative. These proliferative events have been linked, in combination with concurrent B-cell proliferation and differentiation, to the development of MALT lymphomas in pSS patients, and replication-induced senescence of the progenitor cell compartment [20]. Here we consider SMIs inhibiting these three pathways, in relation to their potential application in pSS.

SMIs aimed therefore at restoring SG epithelial cell homeostasis by blocking apoptosis, promoting autophagy or modulating epithelial cell proliferation are therefore also worthy of consideration in the context of therapeutic angles aimed at restoring saliva production.

4.1. Inhibitors of apoptosis

Apoptosis is a form of programmed cell death. SGECs from pSS patients have been well characterized to be more sensitive to apoptosis [42,88]. Curiously, basal cells of the SG striated ducts in healthy and pSS tissue were found to express the anti-apoptotic protein Bcl2, and thus be relatively resistant to apoptosis [92]. Acinar cells, of both controls and patients conversely, did not express Bcl2. How this relates to the enhanced apoptosis in SGECs from pSS patients in the pSS epithelium is not clear, and reiterates the doubts about which cells exactly are found in SGEC cultures.

In terms of aiming to restore homeostasis of the SG in pSS, normalization of the levels of apoptosis would seem to represent a sensible goal. To date, no studies employing SMIs targeted against the apoptotic machinery in pSS have been reported, whilst tens of these drugs exist. Targeting specifically the hyperproliferative epithelium will also be challenging. Apoptosis is also part of the regular turnover of the SG, when exhausted saliva-producing acinar cells die, and are
replaced with new cells generated from progenitor cells. Enhanced EGF signaling, critical to epithelial cell and SGPC homeostasis, and also involved with STAT3-based cytokine signaling, can also result in apoptosis [93]. This provides an additional explanation for enhanced apoptotic levels observed in the epithelium of pSS patients, and also further confirms the complicity of STAT3 in a multitude of salient pathways. Use of SMIs targeting the JAK-STAT pathways may therefore also reduce EGFR-mediated epithelial cell apoptosis, although this has never been tested. The prevention of epithelial cell apoptosis under broad anti-apoptotic SMI application may have undesirable consequences for the SG epithelium as a whole and immune system in general but in lieu of experimental data, we can only speculate how this may manifest.

4.2. Inhibitors of the mTOR pathway

Classical literature states that autophagy is a survival mechanism for cells under stress conditions. It can be viewed as a mechanism for degrading and recycling cellular resources, and broadly speaking antagonizes apoptosis, although this is still fiercely under debate [90]. If not correctly recycled, these cellular components have been suggested to contribute to autoimmune phenotypes, including pSS [94]. The autophagy pathway can be broadly subdivided into that mediated by mTOR1 or mTOR2 subcellular signaling units, with mTOR1 being both the most well characterized, and likely responsible for protein clearance and autophagy. An active mTOR1 pathway inhibits autophagy. Interestingly, TLR stimulation has been shown to initiate autophagy, termed then ‘immunological autophagy’ [95,96], and vice versa that autophagy can negatively regulate TLR signaling (reviewed in [97]). mTOR inhibitors, such as the mTOR1-specific compound rapamycin, promote an active autophagy system and protein clearance. When salivary gland lymphocytes from of pSS patients were examined, numbers of T-, B-, and plasma cells with mTOR activity were elevated compared to controls, and were further associated with local and systemic B-cell hyperactivity [28]. Most strikingly, proliferation of B-cells, γT-cells, Tc cells, CCR9+ Th cells, and IgG production halted in vitro with mTOR inhibitors [28]. Studies relating specifically to the SG parenchyme and autophagy are fairly preliminary. In a comparison of minor SGs from patients with pSS scleroderma and systemic sclerosis, elevated activity of the mTOR pathway (and thus inhibited autophagy) was observed in the epithelium as a whole, although the lack of a healthy control comparison makes the results of this study challenging to interpret [29]. Proctor et al. showed that application of rapamycin, and thus increased autophagy, in a duct ligation injury model transiently stabilized the atrophic murine SG, although this effect was relatively short-lived [98]. Confirming the interaction of the autophagy pathway with the innate immune system, TLR9 stimulation in immortalized human SG cells has recently been demonstrated to increase autophagy [99].

The autophagic pathway of epithelial cells is intricate and intertwined with many cellular processes in addition to apoptosis and TLR signaling. For example, EGFR signaling leads to active PI3K signaling, downstream increased mTOR activity, and hence decreased autophagy. Targeting of PI3K kinases with SMIs, for example, LY294002 and wortmannin, may remove this blockade of autophagy, and improve saliva production according to a recent mouse study, but at the expense of EGFR signaling critical to SG epithelial cell survival [100]. As discussed in section 3.1, EGFR signaling itself functions mechanistically in a similar manner to cytokines, and is likely to be elevated in pSS, and therefore potentially also contributing to the reduced activity of the autophagy machinery in pSS. Stabilization of autophagy in the SG epithelium in pSS would seem a logical, but knock-on effects of tampering with the autophagy machinery on all these additional pathways may make it a less desirable option.

4.3. Inhibitors of small Rho GTPases

Small Rho kinases mediate a host of signals emanating from surface receptors, the extracellular matrix, and mechanical stresses. Their activation culminates in inhibition of cell cycle progression, amongst other outcomes [101]. Rho kinase signaling plays a critical role in epithelial cell biology and Rho kinase inhibitors, blocking the action of Rho-ROCK downstream of Rho kinase, promote human epithelial cell proliferation. Proliferation of limbal epithelial cells was promoted dose-dependently in an in vitro culture system and epithelial wound closure rate was shown to be faster with application of Y-27,632, a Rho-ROCK inhibitor [102]. Treating epithelial cells from various tissues with Y-27,632 increased the total number of epithelial cells harvested, by promoting their proliferation [91].

In SGs, the effect of Rho kinase has been extensively studied from SG development and epithelium polarization to epithelium apoptosis and does not appear to be so clear cut. Firstly, Rho kinase is essential for the development of SGs. A study from William’s group showed that Y-27,632 and H-1152 Rho-ROCK inhibitors stalled submandibular gland branching morphogenesis at the cleft initiation stage [103]. Small Rho GTPases are also important for acinar formation from human SG cell lines [104,105]. Secondly, Rho kinase also exerts a strong effect on epithelial polarization. Li et al. reported the inhibition of Rho kinase abolished correct distribution of tight junction proteins, potentially influencing saliva secretion given the role of tight junctions in paracellular transport of fluid [106]. The activated Rho kinase signaling pathway is also involved in the apoptosis of SG epithelial cells, and when in vitro cultured mouse SG stem cells were incubated with Y-27,632, expression of senescence-related proteins p16 and p21 was downregulated, and cellular proliferation was enhanced [107,108]. Another study using Y-27,632 to treat mouse SG in vitro also suggested increased cell adhesion, viability, migration, and proliferation [109].

Inhibition of the Rho kinase pathway using agents such as Y-27,632 appears may therefore be both beneficial and detrimental to the SG epithelium, dependent on the cell type in question, and process the cell is undergoing at the time of application. If proliferation of epithelial cells is observed with Rho-ROCK application, extended application of Rho kinase inhibitors on pSS patients might however not be advisable, with respect to undesirable SGPC exhaustion induced by
replicative senescence. Transient exposure at the appropriate timing therefore may be a potentially useful therapeutic option in order in combination with stronger anti-inflammatory SMIs, to help rescue SG function in pSS.

5. Conclusion

Small-molecule inhibitor use in treatment of autoimmune diseases is likely to increase dramatically in the coming years, given their potential to inhibit specific signaling pathways. We have considered here the possible effects of SMIs likely to be employed to treat pSS, on the salivary gland epithelium. We would like to propose a two-cell model, to explain these possible effects (Figure 3). We predict that the ‘first’ epithelial cell transmitting a TLR or NFκB signal represents a less complicated target for SMI applications. Specifically, NFκB inhibition does not appear to interfere directly with EGFR signaling, according to current knowledge, or other salient epithelial pathways. More studies will of course be necessary to confirm this. SMI application to block JAK-STAT signaling in a cytokine signal–receiving epithelial cell (the ‘second’ cell) is likely to interfere with a barrage of signaling pathways critical to epithelial cell homeostasis (Figure 2). SMIs targeted against the autophagy, apoptosis and small GTPases also represent modulation of complicated cellular machinery, which also interact with both immune signaling pathways and other important epithelial cell systems (Figure 2). The role of STAT3 as a junction between many signaling pathways is also apparent, when pathways affected by the SMIs examined are ‘combined’ into one cell. Based on this cross talk and its potential effects on the EGF, Wnt, and TGF signaling pathways, we would propose that SMIs that tamper with STAT3 homeostasis should be avoided, when attempting to both resolve inflammation and rescue the function of the SG in pSS. Figure 3 shows a grossly oversimplified two-cell model of potential SMI interference points. In reality, both processes of the first and second epithelial cells may also take place via autocrine signaling in the same epithelial cell. In this scenario, with no divisible up- or – down-stream components to target, a choice of SMI becomes even more challenging. If further research concludes indeed that all SMIs will detrimentally effect the SG epithelium, an alternative approach may be required.

Figure 3. Summary of documented cross-talk between cellular pathways in salivary gland epithelial cells that are potentially affected by small-molecule inhibitor use. Red highlighted boxes indicate likely critical mediators of the effect of inflammation in the salivary gland in primary Sjögren’s syndrome. For the sake of illustration, toll-like receptors are represented in the membrane of the cell, and not additionally in the endosomes.
Modern technology using patient-specific induced pluripotent cells (iPSCs), capable of making all cell types in the body, could be employed to generate new SG progenitor cells. Further genetic modification of these fresh SGPCs may additionally prevent them becoming activated by immune signals, and their transplantation into the patients SG could in principle rescue the damaged SG.

6. Expert opinion

Primary SS is an autoimmune inflammatory disease, often depicted by loss of exocrine gland function and lymphocytic infiltration of the exocrine glands, in addition to chronic fatigue and autoantibody production. In the case of the salivary gland, hyposalivation is commonly observed in the clinic, and carries with it an obligation to use lubricating aids in order to speak, eat, sleep and chew properly, and continuing dental problems. Hyposalivation confers a dramatic decrease in quality of life for the patient, and its resolution represents an unmet clinical need.

The small molecular inhibitors are a new class of drugs with a small weight that allows their movement into cells, and interference with very specific members of many different signaling pathways. These pathways include the NFκB and JAK-STAT pathways belonging to the immune system, and the autophagy, apoptosis and Rho GTPase pathways grouped here under the heading of epithelial cell survival mechanisms. The small molecular inhibitors discussed in this review are either already in use for treatment of pSS (alone or in combination with DMARDs), or likely to be trialed in the coming years. Their effect of the SG epithelium, the tissue type that must recover in order for hyposalivation to be alleviated in pSS, is paramount in choice of the SMI used, and should not be ignored. Having carefully reviewed available literature and knowledge regarding epithelial cell biology, we would like to first emphasize the importance of the epithelium in the choice of SMI made. If inflammation is resolved using a particular SMI, but the homeostasis of the SG is destroyed in the process, its recovery in order for salivation to be re-established. In our opinion and based on current data, SMIs targeting the NFκB receptors may represent the optimum choice with the least collateral damage to the epithelium, although this remains to be fully tested. Any potential future applications are limited at present by the scarcity of exhaustive studies examining the effect of the full spectrum of SMIs on the SG epithelium. Ideally, these studies should include a treatment of SMI effect on SG epithelial cell proliferation, differentiation, and apoptosis, in combination with effective amelioration of inflammation. We also suggest that a more widespread employment of SGPC cultures in organoid format is necessary, where the full repertoire of parenchymal cells in the SG can be examined, and will be key to unraveling the effects of SMIs on the SG epithelium in the coming years. We predict furthermore the emergence of the field of immune–parenchyma interactions in general as one of the most important and potentially influential fields in medicine in the current scientific climate, as we acknowledge the capability of tissues such as the SG epithelium to actively participate in disease pathology.

With every new wave of drugs discovered comes the hurry to apply them to relevant patient groups. In the case of pSS and small molecular inhibitors, this brings with it not inconsiderable risks for the parenchyme of the SG in pSS. Broadening the scope, we would suggest that the core message of our review also applies to other autoimmune diseases with an epithelial cell component, for example, Crohn’s disease, where the same mechanisms and pathway cross-talk are likely to exist.

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Declaration of interest

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References

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.


2. Newly defined criteria for pSS patient classification


... First demonstration of therapeutic potential of stem cells isolated from the human salivary gland.
... Our study suggesting that stem cells of the salivary gland may have reached replicative senescence due to the proliferative effects of proinflammatory cytokines.
... Establishment of the SGEC culture model, which has been employed in many studies to probe the involvement of epithelial cells in pSS.
... Important study demonstrating in the SGEC model that the epithelium of pSS patients may be more sensitive to apoptosis than healthy controls.

• Study demonstrating the expression of TLRs by the 5G epithe-
lium, and therefore their potential to contribute to the immune response.