Review

One year in review 2020: pathogenesis of primary Sjögren's syndrome

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ABSTRACT

The pathogenesis of primary Sjögren's syndrome (pSS) remains poorly understood. However, important efforts have been made during the last few months. In this review, following the others of this series we will summarise the most recent literature on pSS pathogenesis focusing in particular on new insights into pSS animal models, genetics and epigenetics, innate and adaptive immune system abnormalities and tertiary lymphoid structures. Hopefully, novel insights into pSS pathogenesis will pave the way to new therapeutic approaches to the disease, improving patients' management and prognosis.

New insights into pSS pathogenesis

Primary Sjögren's syndrome (pSS) is a chronic slowly progressing autoimmune disease, characterised by dysfunction and destruction of salivary and lacrimal glands associated with a wide spectrum of systemic manifestations (1-5). The pathogenesis of pSS is a result of a complex interplay between the activated immune system and the epithelial cells, targets and players of the autoimmune response (6). Environmental factors may act in several susceptibility genes and perpetuate aberrant cellular and humoral immune responses that, in essence, are the effector mechanisms mediating tissue damage and the generation of the clinical manifestations of the disease (7). In this review, following the others of this series (8-12), we will summarise the most recent literature on this topic.

Findings from animal models

Over the past years a variety of murine models, have been introduced in an effort to elucidate pSS pathogenetic mechanisms and to identify novel therapeutic targets (13). The first and most popular are non-obese diabetic (NOD) mice, that exhibit lymphocytic infiltration of the exocrine glands. Allushi *et al*. (14) using NOD mice disclosed that the salivary gland dysfunction was strongly related with the onset of hyperglycemia and pro-inflammatory cytokines and confirmed the previously reported dissociation between the grade of sialadenitis and the severity of salivary gland dysfunction.

Klinngam *et al*. (15) proposed the inhibition of Cathepsin S (CTSS) in male NOD mice as a potential therapeutic approach of pSS ocular manifestations. Their study revealed that systemic intraperitoneal administration of the peptide-based inhibitor, Z-FL-COCHO (Z-FL) significantly reduced CTSS activity in tears, lacrimal glands and spleen, as well as the total number of lymphocytes invading the lacrimal glands resulting in increased stimulated tear secretion.

Limaye *et al*. (16) developed an aquaporin 5 (AQP5) cAMP-responsive element (Cre) mouse which was bred with TNF-α^{glo}. This mouse model with salivary gland-specific overexpression of TNF-α, displayed severe inflammation through the AQP5-Cre only in the salivary glands, with acinar cell atrophy, fibrosis, and dilation of the ducts, accounting for significant hyposalivation and masticatory dysfunction. Apostolou *et al*. (17) created and studied an ERdj5$^{-/-}$ mouse as a potent murine model for pSS. The chaperone protein ERdj5 is an ER-resident disulfide reductase required for the translocation of misfolded proteins during ER-associated protein degradation and is considered as a significant molecule to maintain the integrity of the salivary glands. This study disclosed that the mice exhibit SG inflammatory infiltrates, more prevalent in female than in males, high levels of anti-Ro/SSA and anti-La/SSB autoanti-

bodies, reduced saliva secretion, excessive cell death through apoptosis, and deregulated cytokine levels within the SG tissue, similar to those described in human disease. These results support the amount of evidences indicating salivary gland epithelial cells (SGEC) endoplasmic reticulum (ER) stress as a key player in the generation and perpetuation of glandular autoimmune inflammatory responses (18). Finally, Choi SS *et al*. (19) used a SKG strain of mice which is a BALB/c congenic line bearing a W163C mutation of Zap70, and proposed that Th17-mediated increased serum levels of anti-M3R IgG might be able to block the M3R-mediated Ca2+ signalling, leading to hypofunction of the salivary glands.

Take home message

NOD mice and a variety of other murine models, have been introduced to investigate novel key players in pSS pathogenesis such as cathepsin S, aquaporin5 and salivary gland epithelial cells endoplasmic reticulum stress.

Genetics in pSS

Attempts at understanding the genetic component of pSS are still in their infancy. However, recent studies have begun to elucidate familial links of disease, identify specific risk alleles and even stratify patients based on global gene expression levels. One such study was able to differentiate patients suffering from extra-glandular manifestations (EGMs) from those with glandular features (GF) and widespread pain (WP) using microarray analysis, based on both number of differentially expressed genes (DEGs) and the characterisation of predominant protein pathways. For example, patients with EGMs had higher expression of genes related to innate (apoptosis, TLR and interferon signalling) and adaptive (T and B cell activation) immune responses which play a fundamental role in pSS, whereas those patients with GF and WP had highest differential gene expression related to sensory perception and pain (20). Min *et al*. (21) sought to categorise pSS patients into two clusters based on the agglomerative hierarchical clustering method. Cluster 1 represented a high-grade inflammatory state of moderate to advanced disease and was enriched in a number of immune cells, especially B cells and Th1 cells. Whereas cluster 2 reflected an early to moderate disease status with low-grade inflammation enriched with epithelial cells and Th17 cells. Unsurprisingly, the expression values of key driver genes were remarkably higher in cluster 1 than cluster 2 and increased in correlation with the histopathological score. Remarkably, the clusters were able to predict response to rituximab therapy; patients in cluster 2 had better outcomes whereas those in cluster 1 did not respond to the therapy.

Genetic techniques have also been used to identify genetic predispositions to pSS and whether the disease also shows familial links with other autoimmune diseases, specifically rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). GWAS has confirmed that there is a strong genetic risk for SS in the major histocompatibility complex, however many other studies have also highlighted other risk alleles (7). Wang *et al*. performed whole exome sequencing on 31 families with at least 2 first degree relatives suffering from pSS, RA or SLE and identified 39 variants in immune related genes across these three diseases (22). These variants predominantly involved genes related to the regulation of T cell activation and T cell receptor signalling including in CD45, LCK, LAT, TEC and NFAT5. This suggests that families suffering from autoimmune diseases may have multiple rare variants that act on common pathways that underlie many autoimmune diseases. The reason why these variants give rise to different clinical manifestations in the autoimmune spectrum remains undefined. Looking at susceptibility genes specifically associated with pSS, several papers have reported particular single nucleotide polymorphisms (SNP) that can be considered risk alleles in pSS. Yao *et al*. have taken a microarray approach to identify key SS related genes and pathways across their cohort of patients and controls using the weighted co-expression network analysis tool (23). 19 hub genes were identified including EIF2AK2, GBP1, PARP12, PARP14, and TDRD7 which were also able to distinguish SS patients from controls, and two (EIF2AK2 and TDRD7) also correlated with inflammatory and interferon response (23). Two particular polymorphisms have also been identified in the TNFAIP3 gene in a cohort of pSS, RA and SLE patients (24). Ciccacci *et al*. described two SNPs rs2230926 and rs6920220 in the TNFAIP3 gene, the first was highly associated with SLE but not RA or pSS, although interestingly this variant confers a higher risk of pSS patients developing lymphoma. The latter SNP was highly associated with SLE, RA and pSS at the genotypic level (24). Additionally, Liu *et al*. (25) described 285 differentially expressed genes between pSS patients and controls. pSS patients had strong expression levels of genes related to interferon signalling including IRF1, CXCL9 and CXCL10, chemokines and their receptors including CCR1, CXCL11, CXCL13 and CXCR4 and immunological synapse formation including CCL19, CCR7, DOCK2 and DOCK8. Furthermore, the expression levels of both CCL19 and CCR7 correlated with increased levels of SSA antibody and serum IgG. In a Mexican SS cohort, risk alleles in immune synapse genes CD28 and CTLA4 were identified; however as participant number was low these results need to be corroborated (26). Variant alleles in the STAT4 gene were demonstrated to be more prevalent in pSS patients than controls and Colafrancesco *et al*. (27) showed that patients carrying the STAT4 rs7574865 SNP had a higher risk of developing leukopenia. Moreover, a particular HCP5 SNP was associated with a higher risk of developing anti-SSA, anti-SSB, RF, hypergammaglobulinaemia, leukopenia and lymphoma whereas two variants of the IL10 gene seemed to give contrasting susceptibility to disease development with one conferring a protective effect and the other giving a higher susceptibility of developing SS.

Take home message

Genetic studies have highlighted many risk alleles for pSS, some overlapping with other autoimmune diseases. The

challenge remains to understand the biological consequences of specific risk alleles and how to use these data to aid in early diagnosis of disease and patient stratification for treatment options.

Epigenetics

In complex diseases such as pSS, switch-on and off signals of gene expression, related to inflammatory pathways are governed by epigenetic mechanisms. Imgenberg-Kreuz *et al*. (28) performed comparative analyses of genome-wide DNA methylation profiles in peripheral blood samples from well characterised cohorts of patients with SLE, pSS and healthy controls, in an attempt to find potential epigenetic sharing between diseases, as well as, disease-specific alterations. Comparison between SLE and pSS revealed decreased methylation in SLE compared to pSS. Interestingly, anti-Ro/SSA and/or anti-La/SSB positive pSS patients exhibited hypomethylation in type I interferon induced genes.

Lopes *et al*. (29) sought to investigate the molecular mechanisms that regulate cDC2 function in pSS, focusing onto the miRNA profile of purified circulating cDC2s. They found that the expression of both miR-708 and miR-130a was consistently decreased in cDC2s of pSS patients, being also downregulated upon stimulation of purified cells with TLR3 and TLR7/8 ligands. Mitogen- and stress activated protein kinase-1 (MSK1) was found to be an endogenous target of miR-130a in primary cDC2s. Activation of MSK1 leads to the stimulation of NF-kB-depended cytokine genes including TNF-a, IL-12, and IL-6. These observations imply that inhibition of MSK1 can be tested as a novel therapeutic approach in pSS.

Hillen *et al*. (30) screened 758 miRNAs by an Open Array quantitative PCR-based technique in two independent groups of pSS patients and identified 10 miRNAs that were differentially expressed in pSS plasmacytoid dendritic cells (pDCs), as compared to healthy controls. Most of the dysregulated miRNAs were involved in phosphoinositide 3-kinase-Ak strain transforming and mammalian target of rapamycin signalling, as well as the regulation of cell death. In addition, they detected a set of novel protein targets of miR-29a and miR-29c, including five molecules that were regulated by both miRNAs and were involved in cell death. Downregulation of miR-29a and miR-29c was associated with a prolonged survival of pDCs in pSS patients. The authors suggested that the major effects of pDCs type I interferon production and cell death, might be controlled via miRNAs. Su *et al*. (31) were the first to evaluate the expression profile of circRNA in PBMCs of pSS patients. They identified 234 differentially expressed circRNAs with hsa_circRNA_001264, hsa_circRNA_104121 and hsa_circRNA_045355 being strongly related to some clinical (*e.g*. renal involvement and arthritis), laboratory parameters (*e.g*. ANA, anti-Ro/SSA, anti-La/SSB positivity and elevated ESR) and disease activity index in pSS patients. Talotta *et al*. (32) analysed both plasma and saliva from 28 pSS patients and observed an altered expression of salivary miR17, 18a and 146b in pSS patients, associated with worse ultrasonography imaging and ESSPRI scores, as well as anti-La/SSB positivity.

Jang *et al*. (33), applying whole transcriptome profiling of minor salivary glands from pSS patients, found miR-1248 to be significantly elevated and correlated with increased expression of IFN signature genes. In this study the authors confirmed two direct targets of miR-1248: DAK, a negative regulator of type I IFN and ITPR3, a key component of calcium signalling in epithelial cells, thus providing for the first time a mechanistic link between these pathogenetic pathways in pSS. Regarding long noncoding RNAs (lncRNAs) Fu *et al*. (34) reported that lncRNA PVT1 was involved in reprogramming of the glycolytic metabolism and proliferation of CD4+ T cells upon activation. lncRNA PVT1, was found to be upregulated in the CD4+Tcells of pSS patients and responded to classical TCR signalling, maintaining the expression of the transcription factor Myc, thus controlling the proliferation and effector functions of CD4+ T cells, through the regulation of glycolysis. The authors suggest that IncRNA PVT1, through this action, might contribute in CD4+ T-cell activation and beyond that in the pathogenesis of pSS. Kim *et al*. (35) compared the miRNAs expression in the tears of pSS patients and healthy controls. The researchers concluded that miR-16-5p, miR-34a-5p, miR-142-3p, and miR-223-3p were significantly upregulated in patients with SS whereas, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-92a-3p, miR-134-5p, miR-137, miR-302d-5p, miR-365b-3p, miR-374c-5p and miR-487b-3p were significantly downregulated.

Take home message

Several epigenetic mechanisms (*i.e*., DNA methylation, miRNAs, lncRNAs) contribute to switch-on and off the expression of genes related to inflammatory pathways and could be targeted to improve pSS therapy.

Dendritic cells, interferons and innate immune response

Plasmacytoid dendritic cells (pDC) are the premier type-I interferon (IFN)-producing cells. Hillen *et al*. identified four signatures in pSS-pDC, including an IFN-induced gene signature and a ribosomal protein gene-signature that indicated pDC activation. A comparison with a dataset derived from *in vitro* activated pDCs, revealed that pSS pDCs produced higher levels of pro-inflammatory cytokines, including type-I IFNs, upon *in vitro* stimulation with endosomal Toll-like receptor ligands. The cytokine production of activated pDCs was associated with expression of hub-genes from the IFN-induced and ribosomal protein gene-signatures, indicating that the transcriptional profile of pSS pDCs underlies their enhanced cytokine production. (36) Upregulation of IFN-stimulated genes (ISGs) has been found in salivary glands, PBMCs, isolated monocytes, pDCs and B cells of pSS patients (37). Davies *et al*. performed an exploratory analysis of MAPK/ERK and JAK/STAT signalling networks in PBMCs from 25 female pSS patients and equally matched healthy controls. The study showed increased signalling potentials in peripheral B cells of pSS patients in response to TLR7 and -9 stimulation through STAT3 S727

and NF-kB that correlate with a type I IFN signature, thus suggesting that patients displaying elevated potentiation of STAT3 S727 and NF-kB signalling could benefit from therapies targeting these pathways (38). In another study, TLR-7 was found to be dominantly expressed in both mononuclear and ductal cells with downstream signals for type I IFNs. The authors suggested that TLR7-dominant innate immunity might be related to the development of sialadenitis in pSS (39).

Immunofluorescent analysis in MSG sections from pSS patients showed that C-X-C motif chemokine 10 (CXCL10) and matrix metalloproteinase 9 (MMP9) were strongly co-expressed in expanded ductal cells and were associated with the presence of infiltrating immune cells around expanded ducts. In contrast, acinar cells did not express CXCL10 and MMP-9. MMP-9 inhibition could suppress the CXCL10 expression in human salivary gland ductal cells via a decrease in STAT1 phosphorylation and therefore, IFN-γ signalling (40). CXCL10 can be increased by the Poly (ADP-Ribose) Polymerase Family Member 9 (PARP9) through upregulating IFIT1 which is mediated by phosphorylation of STAT1, thus suggesting PARP9 as a potential therapeutic target for pSS patients (41). Xin *et al*. (42) demonstrated that myocardial infarction associated transcript 2 (Myrt2) synergistically with miR-377 can block IFN-γ-evoked activation of NF-κB and JAK/STAT signalling pathway, suggesting that Mirt2-miR-377 mediates the inflammatory process in the salivary glands of pSS patients.

In a recent study, Chen *et al*. (43) analysed the cytokine profiles in both tears fluid and saliva of 29 pSS patients. In saliva, pSS patients showed significantly higher level of IP-10 (CXCL10 - interferon gamma-induced protein 10), associated with a higher candida score, and increased level of MIP-1a (CCL3 - macrophage induced protein), associated with lower whole saliva secretion rates. Indeed, the activation of the innate immune system in pSS is triggered by environmental insults. However, the agents implied in the initiation and progression of disease remain largely unclear. From this perspective, the analysis of the microbiome in pSS is an emerging field of research. Particularly, Rustehen *et al*. (44) analysed bacterial profiles in saliva, and showed that *Porphyromonas pasteri* was less represented in pSS samples, whereas Bellocchi *et al*. (45) demonstrated that in the gut microbiome of patients with autoimmune disease, bacteria able to produce tolerogenic substances such as *Lachnoclostridium, Lachnospira* and *Sutterella* were reduced, whereas bacteria with pro-inflammatory features (*i.e*., *Bifidobacterium, Rumniclostridium, Streptococcus*) were increased.

Take home message

Important efforts have been made in clarifying the role of innate immune system in pSS pathogenesis. Of interest, the transcriptional profile of pSS pDCs has been explored and correlated with pDCs enhanced cytokine production. Moreover, several evidences have supported the role of TLR7-dominant innate immunity in the development of sialadenitis in pSS suggesting that patients displaying elevated potentiation of STAT3 S727 and NF-kB signalling could benefit from therapies targeting these pathways.

B and T cells in Sjögren's pathogenesis

Antigen (Ag)-driven T cell-mediated B cell hyperactivity is a hallmark of pSS leading to autoreactive B cell activation, formation of tertiary lymphoid structure with ectopic germinal centre in the SG and B cell lymphomagenesis (5). Interestingly, Skarstein *et al*. reported that autoantigen-specific Ro52 and Ro60 CD27+ B cells accumulate most prominently in areas of fatty infiltration in the SG of SS patients, suggesting a chemotactic gradient favouring B cell recruitment in these areas (46). Among T cell subsets involved in SS pathogenesis (47), Tfh facilitate T cell-dependent B cell responses in GC-like structures, mainly by secretion of IL-21, which drives B cell activation and differentiation towards plasma cells. Tfh and the recently described pathogenic peripheral-helper T-cells (Tph) are the main source of IL-21. Its release is mainly the result of inducible T-cell costimulator (ICOS)-ICOS-L interaction, respectively expressed by T and B cells. Pontarini et al. described both these two IL-21 producing T-helper subsets, invariably expressing ICOS, to be enriched in the SS peripheral compartment, labial SG with ectopic GCs and parotid with MALT lymphoma (MALT-L). Interestingly, ICOS blockade in *ex vivo* SG-organ cultures significantly reduced the production of IL-21 and inflammatory cytokines IL-6, IL-8 and tumour necrosis factor-α (TNF-α) (48). In addition to Tfh cells, their regulatory counterparts, T follicular regulatory cells (Tfr) cells, exert an immunosuppressive effect on Tfh and B cell proliferation and activation in secondary lymphoid tissues. Ivanchenko *et al*. (49) confirmed previous published data by showing an increased frequency of circulating Tfr, identified as Foxp3+CXCR5+CD4+ cells, in SS patients with a particular expansion of Tfr expressing PD-1. Tfr and Tfh cell ratio was increased in SS patients when compared with healthy controls suggesting an imbalance between pro-inflammatory and immunoregulatory pathways in SS. In keeping with this scenario, a subset of CCR7loPD-1hi circulating Tfh cells was selectively increased in SS patients with high SG focus score, and correlated with disease activity scores and circulating plasmablasts (50). Tfh cell response in SS have been nicely described to be restrained by IL-10-producing regulatory B cells both in SS patients and in an experimental SS mouse model induced by sub-cutaneous injection of a SG protein extract (51). Thus, although B cells are generally regarded as central in the pathogenesis of SS, as extensively reviewed in the paper by Mielle *et al*. (52), they can also display a regulatory function via IL-10 secretion suggesting that fine tuning of B cell responses in SS is critical to control autoimmunity and T cell responses. Restriction of T cell mediated B cell hyperactivity might therefore be an important target for the treatment of SS patients. In this regard, several costimulatory pathways involved in B-T cross talk have been considered as potential therapeutic tar-

gets including the CD40–CD40 ligand (CD40L) co-stimulation (53). The expression of CD40, together with other costimulatory molecules part of B7 family, such as CD80, CD86 or ICOSL, PDL1 are enhanced on the surface of SG epithelial cells in SS patients, acting as antigen presenting cells and supporting T cell activation. Now an additional member of B7 family molecules, B7 H3 was found up-regulated on SG epithelial cells in SS patients, with increased level of its soluble form in the saliva. B7-H3 promoted inflammation by activating NF-κB pathway resulting in increased levels of IL6 and TNFa and enhanced apoptosis of SG epithelial cells (54). In line with the attempt of inhibiting B and T cell activation, mammalian/mechanistic target of rapamycin (mTOR) inhibitor, rapamycin (sirolimus), was tested *in vitro* as a proof of concept of its efficacy in inhibiting B and T activation in SS. B cell and T cell proliferation and production of IgG and IFN-γ were inhibited *in vitro* by rapamycin, upon activation of SS peripheral blood mononuclear cells by a combination of superantigen SEB and TLR9-ligand CpG-C to induce mTOR pathway activation (55).

Take home message

The role of B and T cell subsets has been extensively explored: particularly, Tfh and their regularly counterparts Tfr. New data highlighted that Tfr and Tfh cell ratio was increased in SS patients when compared with healthy controls suggesting an imbalance between pro-inflammatory and immunoregulatory pathways in SS.

Tertiary lymphoid structures in pSS

Tertiary or ectopic lymphoid structures (TLS) are lymphoid aggregates of T and B cells forming in non-lymphoid organs in response to chronic inflammation (56). TLS forming in the target organ of autoimmune diseases, including pSS, are generally associated with worse disease progression (56) whereas in solid cancer TLS are mostly associated with good prognosis (57, 58). A progress in our understanding of TLS in pSS was the recent recognition that these structures are formed and maintained by the continuous crosstalk between immune and resident non-immune cells. Nayar *et al*., using an inducible murine model of TLS formation in the salivary glands, showed that lymphocyte-independent IL-13 signalling can induce an early priming in salivary gland immune-fibroblasts that is necessary for the subsequent phases of establishment and expansion of TLS, which they found depend on IL-22 signalling (59). Using the same model, Lucchesi *et al*. showed that the absence of IL-27 signalling (an immunomodulatory cytokine mainly produced by resident myeloid cells) led to an uncontrolled Th17 expansion in the glands which in turn fostered an exaggerated expansion and aberrant activity of salivary glands TLS. In SS, they found that despite pSS patients expressing high local and peripheral levels of IL-27, this cytokine was unable to inhibit Th17 differentiation and conversely induced a strong IFNγ response compared to healthy donors (60).

Interestingly, IL-17 and IL-22 were also recently shown to play a major role in the epithelial-to-mesenchymal transition in healthy human salivary gland epithelial cells (SGEC) *in vitro*, a process that has been associated with salivary gland fibrosis in SS (61). The authors of this work showed how in histological sections from SS salivary glands, epithelial cell markers inversely correlated with inflammatory score and disease severity while a positive correlation was found between salivary gland inflammation and mesenchymal cell markers. The inflammatory milieu that induces and maintains TLS in SS salivary glands might also be involved in a premature aging of salivary gland stem cells (SGSC). Pringle *et al*. showed that organoids generated from SS SGSC have significantly less epithelial cells and an inferior regenerative potential compared to healthy donors SGSC. This was associated with an upregulation of senescence markers in SS SGSC. Interestingly, the authors showed that healthy donors SGEC up-regulated senescence-related genes when exposed to proinflammatory cytokines *in vitro* (62). This growing body of evidence seems to suggest that not only fibroblasts priming is necessary for TLS formation but also that once the inflammatory environment associated with TLS is formed in the salivary glands of SS patients, this might drive the epithelial to mesenchymal transition. Thus, inflammation leads to the accumulation of more fibroblasts, resulting in fibrosis, the loss of SGEC and a decreased regenerative capacity of the gland, that might ultimately contribute to the development of xerostomia. Remarkably, a recent study addressing the role of the lipid-sensing receptor GPR55 in murine and human salivary glands showed that this molecule is important both in replication and proliferation control of ductal epithelial cells and in the induction of salivation, rising the hopes for a pharmacological target capable to restore salivary gland architecture and mitigate hyposalivation (63).

Understanding the nature of the interactions between haematopoietic and non-haematopoietic cells in SS TLS, also helps to gain invaluable insights in other sequelae of the disease, such as the development of MALT lymphoma. Van Ginkel *et al*. recently showed that LEL, hyperplastic ductal basal cells that can form a multi-layered stratified epithelium, are specifically rich in B cells (64) but not in other lymphocytes. Furthermore, they found that B cells localised in highly proliferating areas of LEL and that the number of B cells correlated with LEL severity, concluding that there might be a pathological association between the presence of intraepithelial B lymphocytes and formation of LEL, a pre-lymphomatous lesion marking pSS patients at higher risk of lymphoma.

Take home message

Novel insights regarding TLS formation and maintenance are paving the way to new therapeutic approaches to pSS.

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