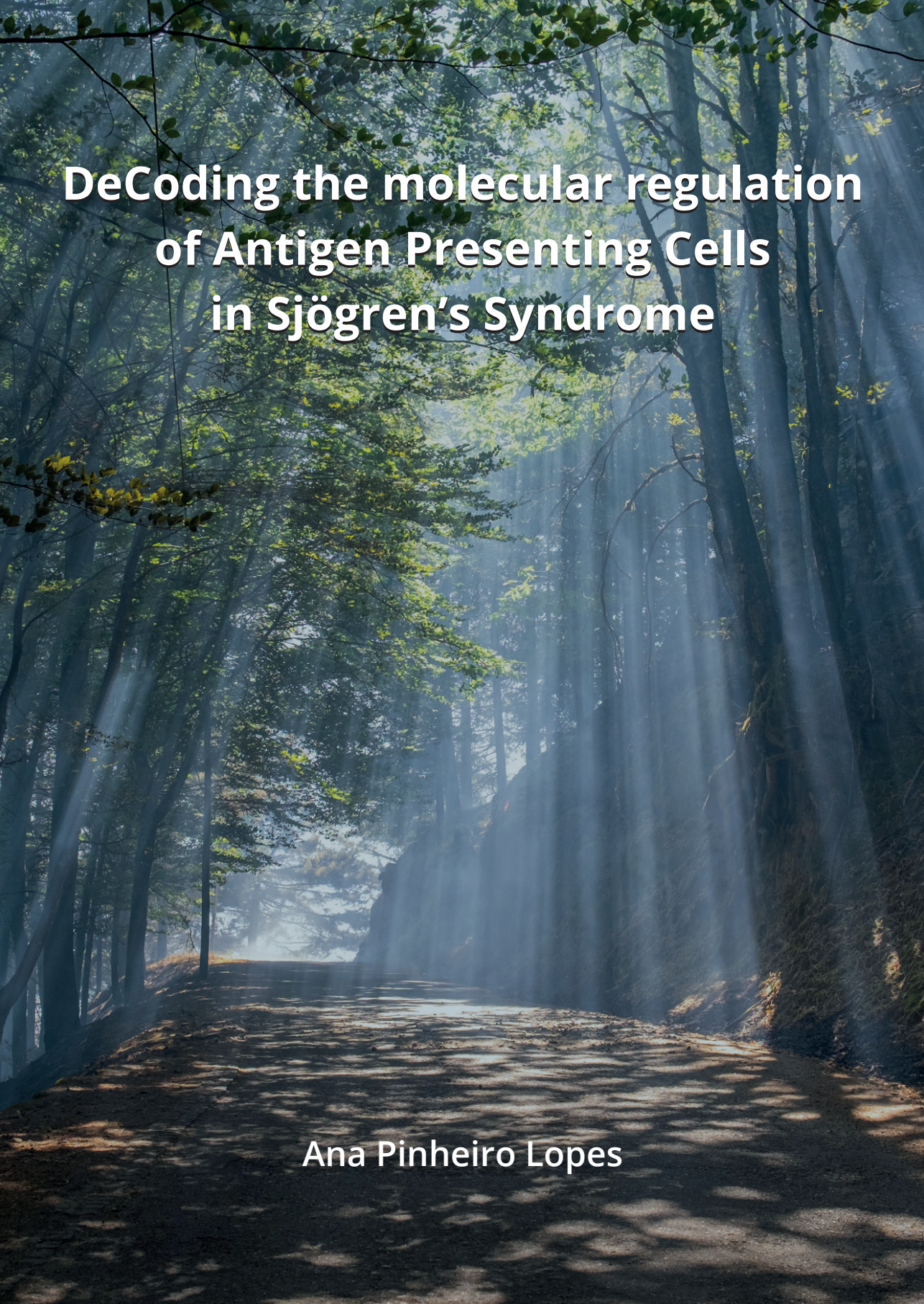

DeCoding the molecular regulation of Antigen Presenting Cells in Sjögren's Syndrome

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DeCoding the molecular regulation of Antigen Presenting Cells in Sjögren's Syndrome

DeCodering van de moleculaire regulatie van antigeen presenterende cellen bij het syndroom van Sjögren

(met een samenvatting in het Nederlands)

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Contents

Chapter 1

General Introduction

Sjögren's syndrome: beyond the dryness and clinical heterogeneity

Sjögren's syndrome (SS) is a chronic, systemic autoimmune disease characterized by immune cell infiltration of exocrine glands, particularly, salivary and lacrimal glands, leading to glandular atrophy and impaired function. The clinical consequences of Sjögren's syndrome are typically keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). In addition, SS also exhibits extraglandular systemic features of other autoimmune diseases, including fatigue, arthritis, pulmonary involvement, interstitial nephritis, peripheral neuropathy, and vasculitis (1-3). Systemic manifestations out of the EULAR Sjögren's syndrome disease activity index (ESSDAI) occur in approximately 30 to 40% of the patients with primary Sjögren's syndrome (4). SS can occur alone, defined as primary Sjögren's syndrome (pSS), or termed secondary Sjögren's syndrome if the disease occurs in association with another systemic autoimmune disease, such as rheumatoid arthritis, systemic lupus erythematosus, scleroderma, or dermatomyositis (1, 5).

pSS affects approximately 0.1–0.4% of the general population with large female predominance with a female-to-male ratio of 9:1 (6). Although pSS can occur at all ages, it is mainly diagnosed between 30 and 50 years of age (3), with a peak incidence at approximately 50 years of age (6). The estimated prevalence is 0.3 to 1 per 1000 persons (5). On the basis of 2016 ACR-EULAR criteria (7), pSS classification requires the presence of immunologic abnormalities (with a total score of ≥4) including the presence of autoantibodies against Sjögren's-syndrome antigen A and/or B (anti-SSA/Ro, anti-SSB/La, respectively), focal lymphocytic sialadenitis with focus score ≥1 on biopsy of labial salivary glands and/or objective ocular or oral dryness.

Although several studies show conflicting evidence regarding the mortality risk in pSS (8-10), a subset of patients with extraglandular manifestation and/or severe disease has a higher mortality risk (9, 11, 12). B-cell lymphoma, severe organ-specific features (principally, interstitial lung disease, renal failure and severe cryoglobulinaemic vasculitis), in addition to infections and cardiovascular disease are commonly associated with increased mortality (13). B-cell lymphoma occurs in about 5-10% of pSS patients (9, 14) and the risk of B-cell lymphoma is 15 to 20 times higher among pSS patients compared to the general population (5, 14), possibly related to chronic B-cell activation. The developed lymphomas are predominantly of the B-cell non-Hodgkin's type, with a predominance of the low grade, marginal-zone histologic type and often develop in organs in which pSS is active, such as the salivary glands, and thus are primarily mucosa-associated lymphoid tissue lymphomas (5, 15).

Lack of effective treatment for Sjögren's syndrome patients

Despite the enormous efforts to successfully treat pSS patients, currently, there is no effective treatment approved for pSS. The therapeutic management of pSS has remained unchanged in the past years (16) and it mainly consists of symptomatic treatment of sicca complaints and systemic features. The treatment algorithm initially focusses on the oral, ocular and systemic manifestations and the therapeutic decisions are dependent on symptoms and disease severity as well as the degree of internal organ involvement (17).

Current treatment is based on local tear and saliva substitutes, immunosuppressants and systemic secretagogues, yet these strategies are frequently ineffective and scarcely tolerated (18). In patients with systemic involvement, treatment strategies are mostly based on expert opinion and the use of pharmacological approaches is supported from the evidence in other autoimmune and inflammatory diseases. In spite of the numerous anti-inflammatory and biologics strategies that have been tried over the past decades, there is a huge unmet need for an effective treatment as many therapeutic options have failed (19). Nevertheless, recently results demonstrated clinical efficacy in systemic complications as measured by ESSDAI upon treatment with ianalumab (anti-B-cell activating factor (BAFF) receptor) (20) and iscalimab (anti-CD40) (21). On the other hand, there is great interest in unravelling the molecular pathways targeted by the use of classical disease-modifying antirheumatic drugs (DMARDs) (22). In fact, combined treatment with leflunomide and hydroxychloroquine was successful in pSS patients with significant reduction of disease activity and unprecedented inhibition of B cell hyperactivity (23).

Pathophysiology of Sjögren's syndrome: a complex interplay of non-immune and immune cells

Sjögren's syndrome is considered as a multifactorial disease with an unknown aetiology despite the widespread molecular, histological and clinical studies. Nevertheless, several factors such as genetic predisposition and environmental factors, such as infections, and hormonal imbalance (24), seem to influence the development of Sjögren's syndrome. Genome-wide association studies (GWAS) have shed light on the mechanisms involved in the pathogenesis of pSS, with implications of both components of the innate and adaptive immune systems. The strongest association with pSS relates with the human leukocyte antigen (*HLA*) genes. Nevertheless, the non-*HLA* genes including interferon regulatory factor 5 (*IRF5*) and signal transducer and activator of transcription 4 (*STAT4*) and

B lymphocyte kinase (*BLK*) also show consistent pSS risk loci (25, 26). Thus, the genetic factors implicated in pSS are associated with well-established genes of key importance for antigen presentation, innate immune responses, and lymphocyte activation and signalling, indicating a clear involvement of the immune system. Interestingly, most of the variants detected in GWAS studies are located in non-coding regions, suggesting that they have a regulatory role that is fairly unexplored. Moreover, the identified pSS-associated genetic variants only explain a small proportion of the heritability observed in pSS, supporting a possible contribution of other factors (27).

A widely accepted model of pSS pathophysiology has been proposed (28-30), implicating the activation of mucosal epithelial cells. It is hypothesized that in genetically predisposed individuals, viral stimulation or abnormal production of endogenous viral elements drives glandular epithelial cell activation and apoptosis, triggering inflammation and autoimmune - mediated tissue injury. This process is anticipated to lead to the activation of the innate and adaptive immune systems, associated with production of type-I and -II interferons (IFN), B cell hyperactivity and production of autoantibodies, such as anti-SSA/SSB (31). The combination of all these events leads to the destruction of the exocrine gland architecture, and development of keratoconjunctivitis sicca and xerostomia. The presence of autoantibodies, an hallmark of pSS, favours immune complex formation, that maintain and amplify the production of IFNs, resulting in an inflammatory loop that leads to tissue damage (5).

The activated immune cells and the inflammatory microenvironment in turn can further activate epithelial cells and regulates their survival, creating a vicious cycle of epithelial cell and immune cell interaction that perpetuates the autoimmune responses observed in pSS (32). Histological studies of labial salivary glands from pSS patients have demonstrated the interaction and contribution of different cell types, including T and B cells, macrophages, dendritic cells, and epithelial cells, to orchestrate a persistent chronic inflammatory response (**Figure 1**).

The triad of immunological hallmarks in pSS includes lymphocytic infiltration of exocrine glands, B cell hyperactivity and IFN-signatures. The lymphocytic recruitment is facilitated by the expression of immune-competent molecules by exocrine glands epithelial cells. These molecules include adhesion molecules, pro-inflammatory cytokines and chemokines and are involved in lymphoid cell recruitment and homing, immune cell activation, differentiation and proliferation, and ultimately in immune cell organization within the tissue lesions.

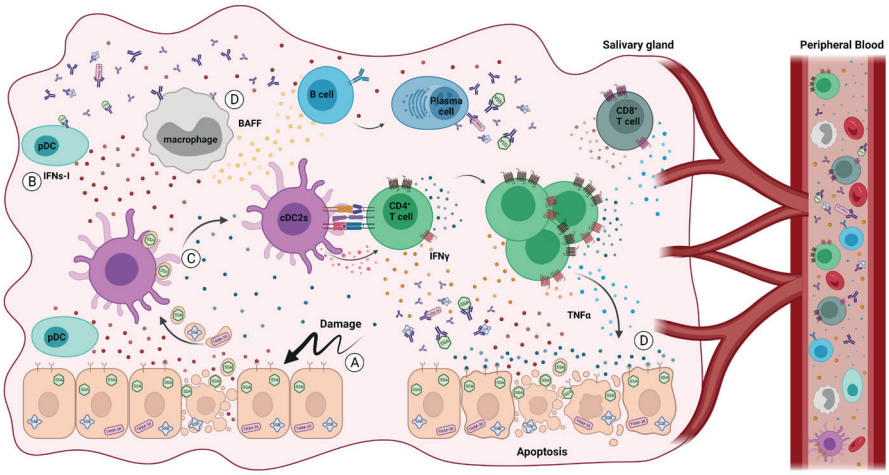

Figure 1. The central role of immune cells in Sjögren's syndrome pathogenesis. In a multi-factorial model, **(A)** combinations of genetic predisposition and danger signals from the environment initiate a cascade of immune responses leading to destruction of salivary gland epithelial cells and accumulation of apoptotic debris. **(B)** Plasmacytoid DC (pDC) produce a large amount of type-I interferon (IFN) which in turn, activates innate immune cells including monocytes, B cell, and type-2 conventional dendritic cells (cDC2s). **(C)** Activated cDC2s uptake self-apoptotic debris and stimulate $CD4^+$ T cell to become mainly T helper type 1 (Th1, IFNγ, tumour necrosis factor alpha (TNFα)) producers, perpetuating salivary gland destruction, and creating a positive feedback loop. **(D)** Macrophage stimulation by IFNs increases BAFF production to sustain B cell activation and plasma cell differentiation. **(E)** The resulting cytokine milieu promotes continuous immune cell infiltration and the development of a persistent chronic inflammatory response.

The microenvironment of inflamed glandular tissue of pSS patients is also rich in B-cell-chemoattracting molecules, such as chemokine CXC-chemokine ligand (CXCL)-12, CXCL13 and cytokines, including interleukin (IL)-21 and BAFF, that promote B-cell trafficking, survival, plasma cell formation and ultimately a humoral immune response (33). B cell hyperactivity is a key hallmark of pSS, manifested by hypergammaglobulinemia, increased levels of serum immunoglobulin (Ig)-G and the presence of autoantibodies, including antinuclear antibodies, anti-SSA/SSB, both antibodies against two ribonucleoprotein complexes. Additional signs of local B cell activation consist of marked increase of IgG-secreting plasma cells in the exocrine glands and the occurrence of germinal centre-like or ectopic lymphoid structures (34). In view of its critical role in the maintenance of ectopic tertiary lymphoid structures, organization of B-cell follicles and the migration of B cells into ectopic germinal centre (35, 36), the CXCL13-CXCR5 axis has been extensively studied in pSS. In fact, increased serum levels of CXCL13 are associated with minor salivary glands

histologic features, non-Hodgkin's lymphoma, and increased risk of lymphoma development (37-40). In the inflamed salivary glands, *CXCL13* expression strongly correlates with the presence of follicular T cells and the lymphocytic focus score (41). The ectopic germinal centre formation and lymphoid neogenesis that takes place in the salivary glands of patients with pSS supports the hypothesis that in pSS patients an antigen-driven immune response occurs (42, 43).

Despite the many mechanisms and mediators described to link activation of the innate and adaptive immune systems in pSS, type-I IFNs (mainly IFNα and IFNβ) have gained widespread attention (44). Besides the anti-viral and anti-tumour activities, type-I IFNs have wide variety of immunomodulatory effects such as induction of BAFF, immunoglobulin switching, increased antigen presentation, T and natural killer cell-mediated cytotoxicity (45). Type-I IFNs response is a rapidly induced, self-amplifying pathway, which generates a feed-forward loop of IFNs production in the targeted and neighbouring cells (29). Moreover, the abundant presence of autoantibodies favours the formation of immune complexes that further activates inflammatory cells through complement and Fc receptors, leading to the production of IFNα by infiltrating plasmacytoid dendritic cells (33). This mechanism is thought to be the main trigger inducing the IFN production and therefore the IFN-signature in pSS patients. The IFN-signature is defined as the increased expression of a set of IFN induced genes (e.g., *MX1, IFIT3, IFI44*), and currently is the most used method to demonstrate an upregulation of IFN activity (45). IFN-stimulated monocytes produce inflammatory mediators that drive B cell survival and maturation, thereby contributing to survival of plasma cells resulting in prolonged autoantibody production. This, further triggers IFN-signalling as well as increased immune complexes deposition in target tissues leading to chronic inflammation, damage and, ultimately, loss of function (46). In fact, despite being associated with systemic disease activity assessed by ESSDAI, autoantibodies and serum IgG (47), reduction of type-I IFN signature and laboratory parameters including erythrocyte sedimentation rate (ESR), serum IgG levels by hydroxychloroquine did not improve clinical response in pSS patients (48). This suggests that in pSS, some immunological findings might be dependent on type-I IFN, however they are not related to the clinical response. As such, the complexity of the cell types involved and the mechanisms that lead to immune cell activation and tissue damage in pSS could explain the challenges and difficulties of developing an efficient therapeutic strategy (49).

Unravelling the cross-talk between the innate and adaptive immunity to identify novel therapeutic strategies in Sjögren's syndrome

The immunological mechanisms behind the self-directed damage of exocrine gland tissue, glandular dysfunction and extraglandular manifestations are still only partly understood, despite decades of research. A better understanding of these mechanisms is crucial to untangle the redundancy and complex interplay of immune cells in the immunopathology of pSS patients.

The role of innate immune response in initiation of pSS, is linked with the activation of the immune cascade and coordination of the adaptive immune response. This mediates the skewing, activation, and differentiation of different cell types in peripheral blood and exocrine tissue and is considered to be indicative of disease onset (50). In this inflammatory environment, the adaptive immune system is activated and T-cells can mediate a direct destruction of glandular tissue and induce B-cell activation, leading to the production of autoantibodies. This process ultimately leads to chronic autoimmune responses resulting from adaptive immune cells.

The composition of the tissue lesions seen in minor salivary glands of pSS patients points towards a crosstalk between the innate, adaptive immunity and the salivary epithelial cells. This combination of different networks and players leads to a complex vicious inflammatory loop culminating in the destruction of the exocrine gland architecture, which perpetuates disease development. Furthermore, comprehending the individual cell type contributions and the relationship between the immune system and the microenvironment holds promise to elucidate pSS pathogenesis and the development of novel effective therapies (49).

Dendritic cells and Monocytes: orchestrators of inflammation in Sjögren's syndrome

Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells that play a crucial role in regulation of immune response by the initiation of immune responses and induction of tolerance. They act as sentinels capturing and processing antigens, migrating in T cell areas to initiate an immune response and reshaping in response to a variety of stimuli such as toll-like receptor (TLR) ligands, cytokines, and immune complexes (51).

Although DCs are relatively rare in human peripheral blood, they can be broadly classified into two major subsets on the basis of different phenotype and function: conventional DCs (cDCs) and plasmacytoid (pDCs). cDCs recognize both extracellular and intracellular pathogens, efficiently process and present exogenous antigens to naive $CD4^+$ and $CD8^+$ T cells and elicit effective adaptive immunity. Conversely, pDCs are highly effective in sensing viral or self -DNA and RNA, mainly via TLR9 and TLR7, respectively and rapidly producing large amounts of type-I IFNs (52-54). Additionally, cDCs can be subdivided into type 1 (cDC1s; CD141-expressing cDCs) with superior antigen cross-presentation to $CD8^+$ T cells, and type 2 (cDC2s; CD1c-expressing cDCs) with a wide spectrum of functions, including antigen presentation and priming of $CD4^+$ T cells. Both subsets arise from distinct committed precursors, and are dependent on a distinct sets of transcription factors (55). Recently, new studies applying high-throughput technologies at a single-cell level highlighted the heterogeneity within the cDC2s, and identified two new subsets, the DC2 ($CD5^+$) and DC3 ($CD5^-CD14^+$) (56-58). Both DC2 and DC3 subsets can activate and differentiate naïve T cells, however DC3 subset is more efficient in inducing a T helper 17 cell response (57) and priming $CD8^+$ T cells with a tissue homing signature (59). As DCs play a central role in the maintenance of tolerance to self-antigens, defects in DC functions and populations might contribute to the aberrant immune activation seen in patients with autoimmune disorders such as pSS (60, 61).

In pSS patients, circulating pDCs are decreased (62, 63) and display an aberrant phenotype (64, 65) leading to their accumulation in the salivary glands. For instance, pDCs from pSS patients, display an upregulation of C-C chemokine receptor type 5 (CCR5), relevant for effective migration of pDCs to inflamed tissues (66). In addition, the increased levels of CCR5 ligands including, C-C chemokine ligand (CCL) type 3 and CCL4, in the saliva of pSS patients sustains pDCs infiltration (67). In the tissues, pDCs can be activated via TLRs by self-antigens and immune complexes, leading to increase production of type-I IFNs (64). IFNs can act in autocrine manner to self-perpetuate IFN production and sustain an inflammatory loop, and in a paracrine manner inducing BAFF production by monocyte and DCs to the activate and differentiate B cells into plasma cells secreting antibodies (68, 69).

The frequency of circulating cDC2s in pSS patients is similar to healthy individuals (62, 63) and up to date scarce research has focus on the functions of these cell in pSS. Recently, transcriptomic analysis of minor salivary glands from pSS patients confirmed the presence of a cDC2 gene signature in the inflamed salivary glands

which was strongly associated with naïve and resting memory CD4$^+$ T cells (70, 71). In addition, due to its capacity to produce BAFF, cDC2s can stimulate humoral responses (72) and play an important role in pSS. In fact, BAFF levels are increased in the circulation of pSS patients and correlate with autoantibody titter (73). Furthermore, immature DCs are decreased in the blood of pSS patients, while mature DCs accumulate in the salivary glands (74, 75), where they can have access to autoantigens derived from apoptotic glandular epithelial cells (76). cDC2s can present these autoantigens to autoreactive T cells (77) and thus perpetuate immune cell infiltration in the salivary gland.

Due to the very low frequency of cDC1s in circulation, on average 0.02% among leucocytes, little is known about their function in pSS. In circulation of pSS patients cDC1s are reduced (63), and negatively correlate with the frequency of tissue-infiltrated DCs suggesting that the decreased circulating levels observed are a result of an increased DC trafficking to the salivary gland (74). In view of its superior ability to produce IFNβ and cross-present necrotic cell antigens upon activation (78), the functional role of cDC1s and their potential contribution to the development of pSS should be considered.

Monocytes

Monocytes are critical innate immune cells that sense environmental changes, migrate to tissues in response to infection and injury and are highly specialized in phagocytosis and antigen presentation. They secrete a large range of different cytokines and chemokines, and once recruited to tissues, monocytes are capable of differentiating into macrophages and dendritic cells and thus further contribute to local inflammation (79).

Circulating blood monocytes are traditionally divided into three major subsets: the classical monocytes (CD14$^+$CD16$^-$), the intermediate monocytes (CD14$^+$CD16$^+$), and the non-classical monocytes (CD14$^-$CD16$^+$), according to morphology and molecular markers detected by flow cytometry (80). Classical monocytes are primed for phagocytosis, and migration, and are known to be important scavenger cells. Intermediate monocytes represent a transitional population between the classical and non-classical monocyte subsets, with the highest capacity to present antigen, and induce CD4$^+$ T cell proliferation. Non-classical monocytes have a pro-inflammatory behaviour, secrete inflammatory cytokines in response to infection, and are involved in antigen presentation and T cell stimulation (80-82). Nevertheless, the precision of this classification is oversimplified and biased by the limited selection of cellular markers and strict gating strategies.

High-throughput technologies based on gene expression, are emerging as powerful tools to identify new monocytes subpopulations, emphasizing the significant intercellular variation existed within the above-mentioned subsets (56, 83). Recently, six distinct monocyte populations were identified in the circulation of healthy individuals with unique gene expression profiles and functional annotations (84). The usage of these techniques could shed light on the current understanding of the functional heterogeneity and plasticity of each monocyte subset and their individual role and contribution to several diseases, including autoimmune diseases.

In pSS, monocytes are relevant not only in the initial immune response but also in the maintenance of chronic inflammation. In the salivary glands of pSS patients, the increased numbers of intermediate monocytes and increase IL-34 expression favours the differentiation and survival of monocytes within the tissues (85). Furthermore, the presence of macrophages in the salivary glands of pSS patients, and their increase expression of protease genes, suggests that these cells could orchestrate tissue destruction and abnormal repair processes (86). Monocytes are also important responders and amplifiers of type-I IFN response, due to the high surface expression of interferon-α/β receptor and the production of cytokines that influence proliferation, differentiation and survival of autoreactive B cells (87). In fact, the presence of infiltrating macrophages in pSS salivary glands was associated with lymphocytic infiltration and lymphoma (88).

Epigenetic regulation in Sjögren's syndrome: other level to control immune cell activation

Epigenetic modifications are gene regulatory mechanisms that controls gene expression, thereby altering cellular function, through alterations in DNA accessibility and/or translation without changing the underlying DNA sequence (89). Epigenetic mechanisms can be influenced by several environmental factors (90), and although they are stable over time, to maintain cell identity, they are also dynamic enough to change in response to external factors (91). This reversible nature, results in a variable expression of identical genetic information, based on the surrounding conditions, resulting in enhanced or silencing gene expression. The epigenetic modifications represent a novel challenging avenue to understand the underlying mechanisms of auto-immune diseases and to the development of innovative therapeutic interventions (92). Epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNA (ncRNAs) and despite DNA methylation being the most widely studied epigenetic mechanism, recently new insight into the study of ncRNAs have highlighted their importance in pSS (93).

While protein coding genes account for less than 2% of the human genome, transcription of ncRNAs from intronic or intergenic genomic regions constitute an important regulatory mechanism with implications in tissue differentiation, development, and proliferation (94). ncRNAs comprise a large family of different RNAs, including microRNA (miRNAs), short interfering RNA, and long non-coding RNA (lncRNAs), with miRNAs being the most extensively studied. The ultimate goal for the epigenetic studies in pSS, would be the understanding of disease pathogenesis, through the fine regulation of gene expression to improve patient classification and enhance treatment outcome as result of specialized and targeted therapeutic approaches.

micro-RNAs involvement in Sjögren's syndrome

Micro-RNAs are small single-stranded RNA molecules with a length of 19 - 25 nucleotides which regulate gene expression at the post-transcriptional level. Interaction with mRNA mainly at the 3' untranslated region (3'UTR) leads to cleavage of their target gene, preventing protein translation (95, 96). miRNAs have multiple targets and functions and are suggested to be involved in the regulation of up to 80% of human genes. This versatile capacity seems to be associated with the capacity of a single miRNA to target multiple genes and a single target gene be regulated by multiple miRNAs (97). Dysregulation of miRNA expression is found to be associated with the onset and progression of inflammatory autoimmune diseases including pSS. In pSS patients several dysregulated miRNAs were identified and associated with the presence of autoantibodies, local inflammation, and the production of pro-inflammatory cytokines (98). Furthermore, an increasing number of studies show that the expression of miRNAs is altered in circulation (peripheral blood mononuclear cells (PBMCs) and sera), in inflamed tissues (minor salivary gland and salivary gland epithelial cells) and in saliva of pSS patients (25, 92, 99).

Among others, miR-146a is up-regulated in PBMCs of pSS patients (100-102), is induced by NF-kB activation, and up-regulates phagocytosis and can act as a negative regulator of TLR/IFN signalling. As such, this miRNA has been implicated in early phase of pSS (103). Among other functions, miRNAs can also regulate apoptosis of epithelial cells in the salivary glands, contributing to autoantigens exposure and self-directed immune response in salivary glands (76, 104). For instance, miR-1207-5p and miR-4695-3p, both anti-apoptotic miRNAs, are able to repress pro-apoptotic gene expression and are downregulated in the salivary glands of pSS patients. Moreover, these miRNAs can also regulate the expression of tripartite motif containing 21 (*TRIM21*), one component of SSA autoantigen,

known to be upregulated in the salivary glands of pSS patients (105). Furthermore, differential miRNA expression in salivary epithelial cells has been reported (98, 106) with implication for tissue function. For instance, miR-1248 is upregulated in the salivary epithelial cells and regulates IFNβ production and calcium signalling through the down-regulation of its targets (*DAK* and *ITPR3*) (107). By modulating both IFN-signature, a hallmark of pSS, and the expression of *ITPR3*, miR-1248 has a functional mechanism which underlies loss of salivary fluid secretion in pSS patients (108). As such, the expression of this miRNA might contribute to pSS immunopathology by affecting two important functions of salivary gland epithelial cells.

Moreover, miRNAs with key regulator function of B cell activation and differentiation are dysregulated in B cells from pSS patients (109). Inhibition of miR-30b expression in B cells, mimics the dysregulation found in pSS patients and increased the expression of BAFF (109), which is associated with disruption of B-cell tolerance and increase autoantibody production (110). Moreover, the fact that several miRNAs correlate and target SSA or SSB autoantigens expression suggests that they can regulate the expression of autoantigen in pSS (111), and at last, the production of autoantibodies and lymphomagenesis in patients with pSS (92). Actually, the level of dysregulated miRNAs, in B cells, from pSS patients with anti-SSA antibodies is 3.5 times higher than in anti-SSA negative pSS patients. Despite the complexity of the miRNA network pathways, their capacity to regulate multiple targets at a variety of expression sites highlights the potential role of miRNA in pSS pathophysiology.

Long non-coding RNAs expression in Sjögren's syndrome

LncRNA are defined as RNAs longer than 200 nucleotides that are not translated into functional proteins. Depending on their position regarding the protein coding genes, lncRNA can be categorized as: sense or antisense, bidirectional, intronic or intergenic (112, 113). LncRNAs regulate gene expression at multiple levels by transcriptional, post-transcriptional or epigenetic regulation. Despite controlling gene expression in nearby proximity, lncRNAs can also affect DNA replication or the response to DNA damage and repair. In addition, lncRNAs can function apart from their target DNA sequences, impacting structural and/or regulatory functions in the different stages of mRNA life, including splicing, turnover and translation. Thus, lncRNAs can affect several important cellular functions, including haematopoiesis and immune activation, and alteration of their expression is present in numerous diseases (113).

Although the exact functional roles and mechanisms of most lncRNAs remains largely unknown, emerging evidences reveals that their functional versatility can contribute to the pathogenesis of autoimmune diseases, including pSS (114, 115). However, to date, the majority of the functional studies of lncRNAs in pSS focus on their role in circulating immune cells possibly due to the complexity and diversity of the inflamed tissues.

The expression of several lncRNAs, such as *NRIR*, *BISPR*, *LINC00426*, *CYTOR* and *TPTEP1* is dysregulated in PBMCs of pSS patients. These lncRNAs are involved in numerous inflammatory pathways including, NF-kB signalling, MAPK signalling and JAK-STAT signalling. As both *NRIR* and *BISPR* lncRNAs regulate important genes involved in type-I interferon signalling pathway (116), their dysregulation in pSS could contribute to perpetuate the presence of IFN-signature observed in pSS patients. Furthermore, dysregulation of *CTD-2020 K17.1*, *LINC00657*, *RP11-169 K16.9*, *LINC00511*, *RP11-372 K14.2* and *RP11-214O1.2* lncRNAs in PBMCs of pSS patients are relevant as their target genes are implicated in epithelial cell damage, autoimmunity and B cell hyperactivation (117). Moreover, *PSMA3-AS1* and *GABPB1-AS1* were significantly upregulated in PBMCs of pSS patients, and *GABPB1-AS1* expression positively correlated with the percentage of B cells and IgG levels (118).

Additionally, changes in the expression of lncRNAs were also found in B cell of pSS patients. Among others, the up-regulation of *LINC00487* in all B cell subsets of pSS patient seems to be associated with higher levels of type-I IFNs. Their expression is induced by IFNα stimulation, and IFNα itself is the upstream regulator of this lncRNA (119). Thus, this dysregulation might induce a self-perpetuating inflammatory loop, sustaining B cell activation.

In circulating $CD4^+$ T cells from pSS patients, the expression of the lncRNA *TMEVPG1* is increased and positively correlates with the presence of anti-SSA antibodies, ESR, total IgG levels and the frequency of Th1 cells. LncRNA *TMEVPG1* functions as transcriptional enhancer, promoting the transcription of IFNγ through a mechanism involving *T-bet* expression. In view of its positive regulation of IFNγ production, it is suggested that this lncRNA contributes to initiate and/or accelerate $CD4^+$ T cell activation in pSS (98). Additionally, *NEAT1* expression is upregulated in circulating $CD4^+$ T cells from pSS patients, and its expression is induced upon T cell stimulation (120). *NEAT1* silencing affects T cell differentiation by inhibiting Th17 polarization through modulating of STAT3 ubiquitination (121). Furthermore, *NEAT1* upregulation promotes the expression of CXCL8 and

tumour necrosis factor (TNF)α and activation of MAPK signalling pathway upon stimulation (120). By acting as a positive feedback molecule in RIG-I pathway, *NEAT1* leads to the production of IFNβ (122), contributing in this way to perpetuate the inflammatory loop present in the inflamed exocrine tissues of pSS patients.

Moreover, the expression of the lncRNA *PTV1*, is increased in lesioned samples and in circulating $CD4^+$ T cells of pSS patients. The expression of this lncRNA is induced upon cell activation by antigen simulation and is involved in glycolytic metabolism and T cell proliferation (123). As $CD4^+$ T cells respond to external signalling and rapidly infiltrate the lesioned salivary glands, representing the major lymphocytic subset, it is possible that the increased expression of *PTV1* in the tissue is mainly due to the presence of $CD4^+$ T cells (124). Interestingly, a GWAS study revealed an association with the occurrence of non-Hodgkin B cell lymphoma and the expression of this lncRNA (125). As such, the upregulation of *PVT1* might be sustained by a continuous activation and proliferation of $CD4^+$ T cells, which can lead to B hyperactivation, and explained the link found with the occurrence of non-Hodgkin B cell lymphoma.

In line with the evidences pointing to the altered lncRNA expression in circulating immune cells, in labial salivary glands from pSS patients the expression of these epigenetic regulators is also dysregulated. Out of the several dysregulated lncRNAs found in the salivary glands of pSS patients, Shi, N. et al. validated the increased expression of eight lncRNA including *LINC00426-003*, *AC017002.1-001* and *RP11-81H14.2-006*. The expression of the validated lncRNA correlates with important immunological and clinical features including circulatory levels of IgA, IgM, β2 microglobulin, ESR, and local visual analogue scale of parotid swelling and dry eyes (126). These correlations with circulating inflammatory mediators and exocrine gland function suggest that lncRNAs are involved in the pathophysiology of pSS, nevertheless additional functional studies are needed to shed some light into their regulatory mechanisms.

Aims and thesis outline

Primary Sjögren's Syndrome (pSS) is a systemic multifactorial disease, characterized by prominent lymphocytic infiltration of the exocrine glands, T and B cell hyperactivation and exocrine dysfunction. cDC2s and monocytes are key orchestrators of inflammatory responses, linking innate and adaptative immunity and its altered function is linked to autoimmune diseases. In this

thesis we set out to investigate the epigenetic, transcriptional and functional mechanisms underlying cDC2s and monocytes function in pSS.

In **Chapter 2**, we assessed the expression of circulating small non-coding RNAs (ncRNAs), including microRNAs (miRNAs), in light of their important role in the regulation of immune functions. To gain further insight into the relation between the expression of sncRNA and the disease parameters, pSS patients were stratified based on sncRNA profile.

In **Chapter 3**, we evaluated the miRNome of cDC2s from pSS patients and identify the triggers by which miRNAs where dysregulated in pSS. Furthermore, we exploit the functional consequences of this dysregulation in cDC2s function by identifying the affected miRNA targets and their contribution to pSS immunopathology. We extended the study of epigenetic regulation on cDC2s cell activation in autoimmunity to long non-coding RNAs (lncRNAs). In **Chapter 4**, we unravelled the role of lncRNA HLA Complex P5 (*HCP5*) in regulating crucial cDC2s functions including regulation of type-I IFN signalling, cell recruitment and interaction with B and T cells. In addition, we screened and validated increased *HCP5* expression in circulating cDC2s and in the salivary glands of pSS patients.

In view of the potential involvement of cDC2s in pSS, in **Chapter 5**, we investigated their transcriptomic profile of in pSS, non-Sjögren's sicca patients (nSS) and healthy individual in order to understand the mechanisms underlying cDC2 dysregulation in pSS. Furthermore, we identified key functional pathways dysregulated in pSS patients, in relation to IFN signature and relevant for antigen clearance and T cell activation.

To enlighten the contribution of monocytes in pSS immunopathology, in **Chapter 6**, we exploited RNA-sequencing to investigate the transcriptional profile of circulating monocytes of pSS and nSS patients and identified dysregulated processes related to translation, IFN-signalling, and TLR signalling.

In **Chapter 7**, we conclude with the overall discussion of the findings presented in this thesis, aiming to enlighten the complex molecular mechanisms trough which immune dysregulation occurs in pSS patients. The potential implications of the results presented here for the development of novel therapeutic avenues for treatment of pSS patients is also addressed.

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Chapter 2

Circulating small non-coding RNAs reflect IFN status and B cell hyperactivity in patients with primary Sjögren's syndrome

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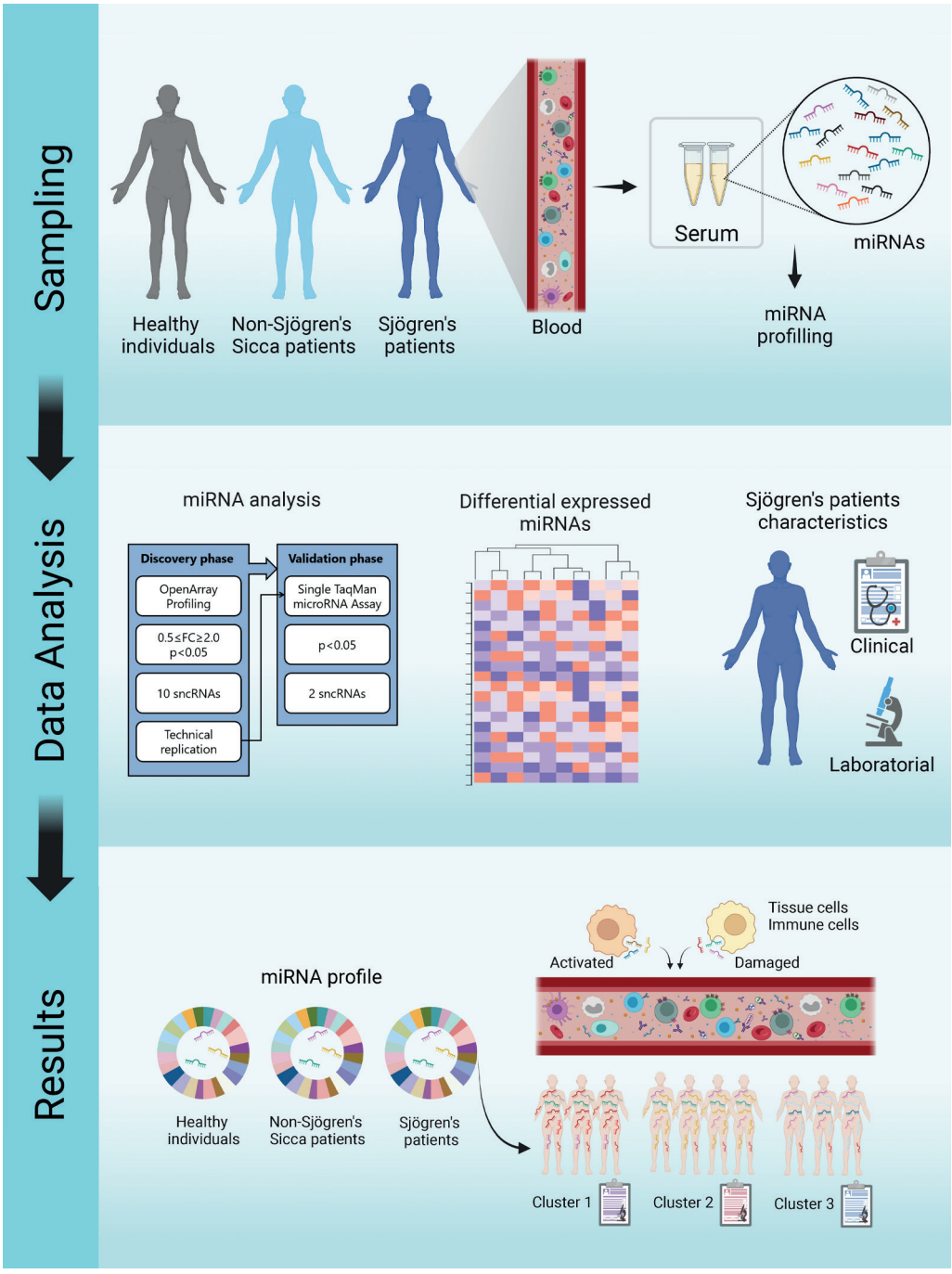
Background Considering the important role of miRNAs in the regulation of post–transcriptional expression of target genes, we investigated circulating small non-coding RNAs (snc)RNA levels in patients with primary Sjögren's syndrome (pSS). In addition, we assessed if serum sncRNA levels can be used to differentiate patients with specific disease features.

Methods Serum RNA was isolated from 37 pSS patients as well as 21 patients with incomplete Sjögren's Syndrome (iSS) and 17 healthy controls (HC) allocated to two independent cohorts: discovery and validation. OpenArray profiling of 758 sncRNAs was performed in the discovery cohort. Selected sncRNAs were measured in the validation cohort using single-assay RT-qPCR. In addition, unsupervised hierarchical clustering was performed within the pSS group.

Results Ten sncRNAs were differentially expressed between the groups in the array. In the validation cohort, we confirmed the increased expression of U6-snRNA and miR-661 in the iSS group as compared to HC. We were unable to validate differential expression of any miRNAs in the pSS group. However, within this group several miRNAs correlated with laboratory parameters. Unsupervised clustering distinguished three clusters of pSS patients. Patients in one cluster showed significantly higher serum IgG, prevalence of anti-SSB autoantibodies, IFN-score, and decreased leukocyte counts compared to the two other clusters.

Conclusion We were unable to identify any serum sncRNAs with differential expression in pSS patients. However, we show that circulating miRNA levels are associated with disease parameters in pSS patients and can be used to distinguish pSS patients with more severe B cell hyperactivity. As several of these miRNAs are implicated in the regulation of B cells, they may play a role in the perpetuation of the disease.

Graphical abstract



Introduction

Primary Sjögren's syndrome (pSS) is a systemic chronic autoimmune disease characterized by lymphocytic infiltration of salivary and lacrimal glands, associated with dryness of mouth (xerostomia) and eyes (keratoconjunctivitis sicca). pSS patients may present with extra-glandular manifestations such as renal, pulmonary or neurologic involvement and around 5% of the patients develop lymphoma, primarily of the mucosa-associated lymphoid tissue (MALT) [1, 2]. B cell hyperactivity is one of the hallmarks of pSS, demonstrated by the presence of hypergammaglobulinemia and autoantibodies against intracellular autoantigens Ro/Sjögren's syndrome associated autoantigen (SS)A and La/SSB, which are expressed by almost all cell types. The immune complexes formed by autoantibodies lead to innate immune activation and type I interferon (IFN) production, contributing to the chronicity of the disease. Although the pathogenesis of pSS is still unknown, a complex interplay of several factors has been implicated including genetic predisposition, environmental factors, and epigenetic factors [3, 4].

MicroRNAs (miRNAs) are single-stranded, small non-coding (snc)RNAs of 19–25 nucleotides in length that regulate gene expression at the post-transcriptional level [5]. Numerous studies have demonstrated that miRNAs are expressed in different tissues, cell types, and are also present in various biological fluids such as saliva, serum and plasma. Circulating miRNAs can be found in combination with specific carrier proteins or enclosed in different types of vesicles, including exosomes [6, 7]. miRNAs account for 1–5% of the human genome and can negatively regulate expression of at least 30% of protein-coding genes at the post-transcriptional level [8]. A single miRNA can influence many different mRNA targets and conversely, several different miRNAs can bind to a single mRNA target. This regulation can occur at different levels, by mediating mRNA cleavage, repressing mRNA translation or causing mRNA destabilization [9, 10].

miRNAs are involved in the control of immunologic processes such as cell differentiation, proliferation, and apoptosis [11]. As such, miRNAs are thought to play a critical role in autoimmunity and in numerous autoimmune diseases [12]. Recently, several studies in pSS patients demonstrated the dysregulation of specific miRNAs in salivary glands or PBMCs from pSS patients [13, 14]. The expression of miR-768-3p and miR-574 in the salivary glands of patients with pSS is different from those with non-Sjögren's syndrome and can distinguish subsets of pSS patients with low or high grade salivary gland inflammation [15].

Furthermore, significantly lower expression of miR200b-5p in salivary gland tissue was described in pSS patients with MALT lymphoma compared to pSS patients without history of lymphoma [16].

sncRNAs, including miRNAs, are present in serum and circulating miRNA levels are associated with a range of diseases, including nervous system disorders, metabolic and autoimmune diseases [7, 12, 17]. As serum is easily accessible and collection is relatively easy to standardize, we investigated whether there are differences in the serum levels of 758 sncRNAs between pSS patients and incomplete Sjögren's Syndrome (iSS) patients or healthy controls (HC). In addition, we assessed if serum sncRNA levels can be used to differentiate patients with specific disease features.

Materials and methods

Patients and controls

Two independent cohorts of patients followed up in the department of rheumatology & clinical immunology at the University Medical Center Utrecht and controls were established: a discovery cohort (14 pSS, 8 iSS, 8 HC) was used to screen the serum abundance of a large panel of 758 sncRNAs, while a validation cohort (23 pSS, 13 iSS, 9 HC) was used to test the reproducibility of the results (**Figure 1**). Donors were allocated to each cohort random. The patients with pSS were classified according to the AECG criteria [18]. The iSS patients presented with dryness complaints without a known cause, were not clinically considered to have any generalized autoimmune disease including pSS, and did not fulfil the classification criteria for pSS. The study was approved by the ethics committee of the University Medical Center Utrecht. All patients gave their written informed consent in accordance with the declaration of Helsinki. The characteristics of the individuals included in the study are depicted in **Table 1**.

Serum RNA preparation

Fresh blood samples were collected in Vacutainer SSTII Advance tubes (BD Biosciences, Franklin Lakes, NJ, USA). Serum was collected as per manufacturer's instructions, snap frozen in liquid nitrogen and stored at -80°C until further use. RNA was extracted from 240uL of serum using the miRcury RNA isolation kit for biofluids (Exiqon, Vedbaek, Denmark). At the first step of extraction, 300pg of a synthetic miRNA (Arabidopsis thaliana ath-miR-159a) was added to each sample as a spike-in.

Table 1. Characteristics of the patients and controls enrolled in the study.

	Discovery Cohort (n=30)			
	HC	**iSS**	**pSS**	**p-value**
N (M/F)	8 [0/8]	8 [0/8]	14 [3/11]	*0.149*
Age (yr.)	56 [51-67]	42 [25-68]	54 [29-70]	*0.229*
LFS (foci/4 mm^2)	-	0.0 [0.0-1.0]	1.9 [1.0-4.0]	***<0.001***
ESSDAI	-	-	2.0 [0.0-19]	-
ESSPRI	-	-	3.7 [2.0-8.8]	-
Schirmer (mm/5 min)	-	3.0 [0.0-24]	5.0 [0.5-25]	*0.620*
ANA (no. positive [%])	-	0 [0%]	10 [71%]	***0.002***
SSA (no. positive [%])	-	3 [38%]	8 [57%]	*0.659*
SSB (no. positive [%])	-	0 [%]	4 [29%]	*0.254*
RF (no. positive [%])	-	0 [%]	5 [42%]	*0.114*
IFN-score	2.94 [-2.0-7.0]	-	8.84 [-5.0-14.9]	*0.124*
Serum IgG (g/L)	-	10 [6.8-17]	14 [8.3-30]	*0.070*
ESR (mm/hour)	-	12 [4.0-17]	11 [5.0-36]	*0.645*
CRP (mg/L)	-	1.9 [0.0-4.0]	1.0 [0.0-8.0]	*0.244*
C3 (g/L)	-	1.1 [0.6-1.7]	1.1 [0.7-1.3]	*0.578*
C4 (g/L)	-	0.3 [0.2-0.4]	0.3 [0.0-0.3]	*0.425*
Not treated (no. [%])	-	7 [88%]	11 [79%]	*>0.999*
Only HCQ (no. [%])	-	1 [12%]	1 [7%]	*>0.999*
Other (no. [%])	-	0 [0%]	2 [14%]	*0.515*

Values are Median [Range] unless stated otherwise. Groups were compared per cohort using Kruskall Wallis test, Fisher's exact test or Mann-Whitney U test where appropriate. Significant differences ($p<0.05$) are depicted in bold. HC: Healthy control; iSS: incomplete Sjögren's syndrome; pSS: primary Sjögren's syndrome; LFS: Lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: Anti-nuclear antibodies; SSA: Anti-SSA/Ro; SSB: Anti-SSB/La; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein, HCQ: Hydroxychloroquine. Other treatment group includes Azathioprine, alone or in combination with Prednisone (n=5); Mesalazine (n=1); HCQ in combination with Prednisone (n=1); Prednisone (n=1).

Validation Cohort (n=45)			
HC	**iSS**	**pSS**	**p-value**
9 [1/8]	13 [0/13]	23 [1/22]	*0.461*
45 [29-55]	47 [24-71]	55 [26-77]	*0.324*
-	0.0 [0.0-0.7]	2.0 [1.0-5.0]	***<0.001***
-	-	5.0 [0.0-13]	-
-	-	5.3 [1.0-8.0]	-
-	5.8 [1.5-32]	5.5 [0.0-30]	*0.483*
-	7 [54%]	19 [86%]	***0.050***
-	4 [31%]	18 [78%]	***0.011***
-	0 [0%]	12 [52%]	***0.002***
-	1 [10%]	9 [47%]	*0.098*
-1.59 [-3.5-4.7]	-	10.7 [-5.1-17.8]	***0.048***
-	13 [6.5-15]	15 [5.6-33]	*0.061*
-	13 [2.0-29]	14 [3.0-63]	*0.384*
-	1.0 [0.0-9.3]	1.2 [0.0-13]	*0.834*
-	1.2 [0.8-1.5]	1.0 [0.8-1.4]	***0.029***
-	0.3 [0.2-0.4]	0.2 [0.1-0.4]	*0.075*
-	10 [77%]	15 [65%]	*0.708*
-	2 [15%]	3 [13%]	*>0.999*
-	1 [8%]	5 [22%]	*0.385*

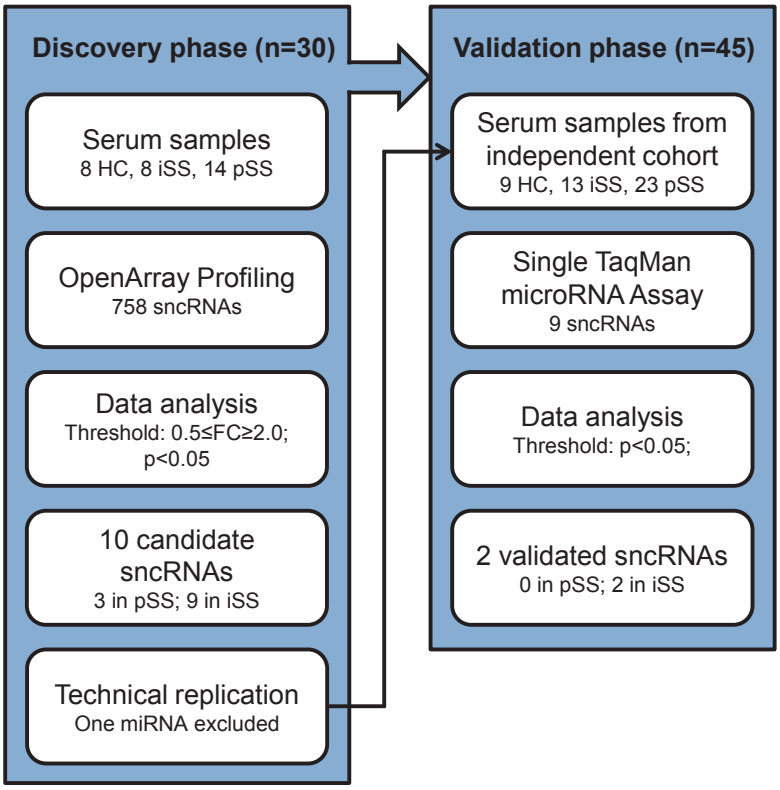


Figure 1. Workflow of discovery and validation approach. sncRNAs were considered to be validated in the validation phase when they reached the threshold of p<0.05 with a difference in the same direction (ie. up/down regulated) as was observed in the discovery phase. HC: healthy control; iSS: incomplete Sjögren's Syndrome; pSS: primary Sjögren's syndrome; sncRNA: small non-coding RNA.

sncRNA profiling array

sncRNA profiling in the discovery cohort was performed on the OpenArray platform (Life Technologies, Carlsbad, CA, USA). Profiling was performed as previously described [19]. Data were analyzed using ExpressionSuite software (Life Technologies), using the relative threshold cycle (Crt) and the comparative threshold cycle method. Data were normalized using both the global mean normalization approach [20] and normalization by ath-miR-159a spike-in [21]. Low expressed sncRNAs (Crt higher than 27) were set at 27; samples with an amplification score lower than 1.24 were excluded from all analyses. Relative expression was calculated by dividing the Crt of each sample by that of a random sample in the healthy control group, which was set at 1. Differences in sncRNA

expression between the groups in the discovery cohort using global mean normalization with a FC difference of ≤0.5 or ≥2.0 at an uncorrected p-value of p<0.05 between any of the groups were selected for validation analysis.

sncRNA validation

For biological validation, miRNA-specific TaqMan RT-qPCR was performed on the samples from the validation cohort. In the same experiment, all samples from the discovery cohort were re-measured for technical replication and to allow the merging of the data for studying associations with clinical parameters and clustering analysis. To this end, the following sncRNA assays were ordered from Life Technologies: U6-snRNA (ID 001973), hsa-miR-23a-3p (ID 000399), hsa-miR-223-5p (ID 002098), hsa-miR-661 (ID 001606), hsa-miR-143-3p (ID 002249), hsa-miR-342-3p (ID 002260), hsa-miR-150-5p (ID000473), hsa-miR-140-5p (ID 001187), hsa-miR-29c-3p (ID 000587), hsa-miR-212-3p (ID 000515) and for the exogenous control ath-miR-159a (ID 000338). From 2.5 uL of serum RNA, cDNA was synthesized by using the individual miRNA-specific RT primers contained in the TaqMan miRNA assays in the presence of 3.3 U/uL MultiScribe RT enzyme (Life Technologies), by using the following thermal cycler conditions: 10 min at 4°C, 30 min at 16°C, 30 min at 42°C, 5 min at 85°C. miRNA levels were quantified in duplicate from 3uL of cDNA using TaqMan fast advance master mix and miRNA-specific primers from the TaqMan miRNA assays, using these amplification conditions on the Quantstudio 12k Real-Time PCR system (Life Technologies): 2 min at 50°C, 20 sec at 95°C, followed by 40 cycles of 1 sec at 95°C, 20 sec at 60°C. sncRNA expression was calculated after normalization by ath-miR-159a spike-in (ΔCt = Ct mean target–Ct mean miR-159a). The relative fold change (FC) of each sample was calculated in comparison with the ΔCt mean of the HC group (reference) according to the formula FC = 2-ΔΔCt, where ΔΔCt = ΔCt sample—ΔCt reference. Technical replication was considered to be successful if there was a robust correlation between the Ct in the discovery array (Crt) and the Ct in the single-assay RT-qPCR (r>0.5 and p<0.05). Validation was considered successful if the direction of the difference (ie. up/downregulation) was identical to what was observed in the discovery cohort and the difference was significant at an uncorrected p-value of p<0.05.

Hierarchical clustering

For unsupervised hierarchical clustering, Euclidian distance with complete linkage was used on the FC of sncRNA levels to divide the pSS patients into clusters using the Multi Experiment Viewer online software (http://mev.tm4.org).

Interferon signature quantification

At the time of blood drawing for serum collection, additional blood was drawn from 13 of the healthy controls and 25 of the pSS patients (randomly selected) to determine the IFN signature. To this end, mononuclear cells were isolated from heparinized peripheral blood by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). CD14+ monocytes were isolated by magnetic-activated cell sorting using CD14+ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. To confirm consistent purity of isolated monocytes, cells were stained with the following monoclonal antibody combination: anti-CD45 Peridinin chlorophyll (clone: HI30; Sony Biotechnology, San Jose, California, USA), anti-CD16 Phycoerythrin (clone: DJ130C; Agilent, Santa Clara, California, USA) and anti-CD14 Fluorescein isothiocyanate (clone: TÜK4; Miltenyi Biotec) and the proportion of CD14+ cells within the isolated fraction was measured using Fluorescence associated cell sorting (FACS) and a FACSCanto II flow cytometer (BD Bioscience, San Jose, USA). The purity of the monocyte samples was (median [range]) 98% [90–99%], there were no significant differences in cell purity between the groups. Cells were lysed in RLTPlus buffer (Qiagen, Venlo, Netherlands) supplemented with 1% of Beta-mercaptoethanol. Total RNA was purified using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was assessed with Qubit RNA Kit (Life Technologies). To determine the IFN-score, the relative expression of 5 Interferon-induced genes (*IFI44L*, *IFI44*, *IFIT3*, *LY6E* and *MX1*) was assessed as previously described [22] relative to the expression in the healthy control group, using the Quantstudio system (Life Technologies).

Statistics

Statistical analyses were performed using GraphPad Prism software version 6.02 (GraphPad, Lo Jolla, CA, USA) and IBM SPSS version 21 (IBM Corp, Armonk, NY. USA). Mann-Whitney U-test was used to compare groups in the discovery and validation analyses, without correction for multiple testing. Kruskal-Wallis H test with post-hoc Dunn's test of multiple comparisons was used to compare clusters. For correlations, Spearman's rho was used and p-values were corrected for multiple testing using B&H FDR. Fisher's exact test was used to compare categorical variables. Two-sided testing was performed for all analyses. Differences and correlations were considered statistically significant at $p<0.05$.

Results

Discovery of sncRNAs using OpenArray-based miRNA profiling

OpenArray-based analysis of 758 sncRNAs was performed in the serum of pSS patients, iSS patients, and healthy controls (HC) from the discovery cohort (n = 30). All differences in sncRNA abundance between the groups that were significant when using spike-in normalization were also significant when using global mean normalization (**Supplementary Table 1**). We based our further analysis on the data from the global mean normalization to be as inclusive as possible and because this methodology is considered to be the gold standard [23, 24]. When global mean normalization was used, the levels of three sncRNAs were significantly different in pSS patients and nine sncRNAs were significantly different in patients with iSS as compared to HC. Two of these sncRNAs (U6-snRNA and miR-29c-3p) were different in both patient groups compared to HC. There were no differences in sncRNA abundance between pSS and iSS patients that met the set thresholds (**Table 2**).

Table 2. Results from discovery and validation cohort analyses.

	Discovery Cohort (n=30)		**Validation Cohort (n=45)**	
	iSS vs HC	pSS vs HC	iSS vs HC	pSS vs HC
miR-29c-3p	**5.308 (0.004)**	**5.659 (<0.0001)**	1.070 (0.744)	1.009 (0.910)
U6-snRNA	**4.280 (0.003)**	**2.461 (0.016)**	**2.388 (0.020)**	1.962 (0.490)
miR-23a-3p	**2.718 (0.007)**	2.124 (0.123)	0.706 (0.009)	0.881 (0.305)
miR-661	**2.609 (0.021)**	1.978 (0.019)	**1.743 (0.007)**	1.861 (0.312)
miR-150-5p	**2.168 (0.028)**	1.265 (0.353)	1.247 (0.558)	1.162 (0.405)
miR-143-3p	**2.088 (0.007)**	1.692 (0.016)	0.903 (0.595)	1.080 (0.993)
miR-140-5p	**2.062 (0.021)**	1.364 (0.182)	0.980 (0.632)	0.900 (0.300)
miR-223-5p	**2.001 (0.049)**	1.141 (0.642)	1.123 (0.618)	1.355 (0.790)
miR-342-3p	**2.384 (0.001)**	1.496 (0.309)	1.081 (0.683)	1.013 (0.516)
miR-212-3p	0.417 (0.232)	**0.129 (0.002)**	-	-

Results are expressed as mean FC (p-value). Differences between groups that met the threshold for the corresponding analysis (for discovery: FC difference of ≤0.5 or ≥2.0 at p-value of p<0.05; for validation: FC difference in same direction as seen in discovery at p<0.05) are indicated in bold. Mann–Whitney U test was used to test all comparisons. No differences that met the set thresholds were observed between pSS and iSS in the discovery cohort. miR-212-3p was not technically replicated and therefore was not included in the validation cohort analysis.

For technical replication, we measured the ten differentially expressed sncRNAs in all of the donors included in the discovery cohort using single-assay RT-qPCR. Nine out of these ten sncRNAs (all but miR-212-3p) showed a robust correlation between the relative expression measured in the OpenArray and in the single-assay RT-qPCR, and were therefore included in the validation phase (**Supplementary Table 1**).

Validation of the selected sncRNAs in an independent cohort

The nine sncRNAs that were technically replicated were measured in an independent validation cohort (n = 45) using single-assay RT-qPCR. None of the sncRNAs that were identified as differentially expressed in the pSS group in the discovery cohort were validated. Of the sncRNAs that were differentially expressed in the iSS group compared to HC in the discovery cohort, two were validated: U6-snRNA and miRNA-661 (**Table 2**).

Serum sncRNA expression is associated with laboratory disease parameters in pSS patients

Although the sncRNAs included in the validation phase were not differentially expressed in pSS patients within the validation cohort, we observed a large spread in expression for all nine of the sncRNAs within this group (**Figure 2**). This observation prompted us to investigate whether sncRNA abundance was related to clinical or laboratory parameters within the pSS patients. For this, we used the single-assay RT-qPCR data from both cohorts. None of the sncRNAs showed a significant association with demographic data (sex, age) or clinical features (ESSDAI, ESSPRI, Schirmer), yet several of them showed correlations with laboratory parameters including LFS (**Table 3**). Interestingly, many of the parameters known to be associated with high disease activity (i.e. low C3/C4, decreased leukocyte count, high lymphocytic focus score) were negatively correlated with the abundance of the sncRNAs investigated (**Table 3**). In addition, pSS patients who are positive for anti-Ro (SSA) and/or anti-La (SSB) showed decreased expression of several of the sncRNAs when compared to the antibody-negative pSS patients (**Supplementary Table 3**). Thus, the spread in sncRNA expression in the pSS group is related to their heterogeneity in disease parameters.

Hierarchical clustering

Since each sncRNA was associated with a distinct set of laboratory parameters, we next investigated whether specific patterns of sncRNA expression could distinguish subsets of patients with a certain disease phenotype within the pSS

group. To this end, unsupervised hierarchical clustering was used to group the most similar pSS patients on the basis of their expression of each of the nine sncRNAs measured by single-assay RT-qPCR. This allowed the identification of three distinct clusters of patients based on different sncRNA patterns (**Figure 3A**). Clustering analysis showed that one group of patients (cluster 3) had an overall decreased expression of all nine sncRNAs in their serum, while two groups (clusters 1 and 2) had higher serum levels for at least one of the measured miRNAs. Comparison of clinical parameters between clusters showed that patients in cluster 3 presented with higher serum IgG and IFN-score, as well as decreased leukocyte counts compared to cluster 1. In addition, an increased frequency of patients in this cluster was positive for anti-La (SSB) (**Figure 3B**).

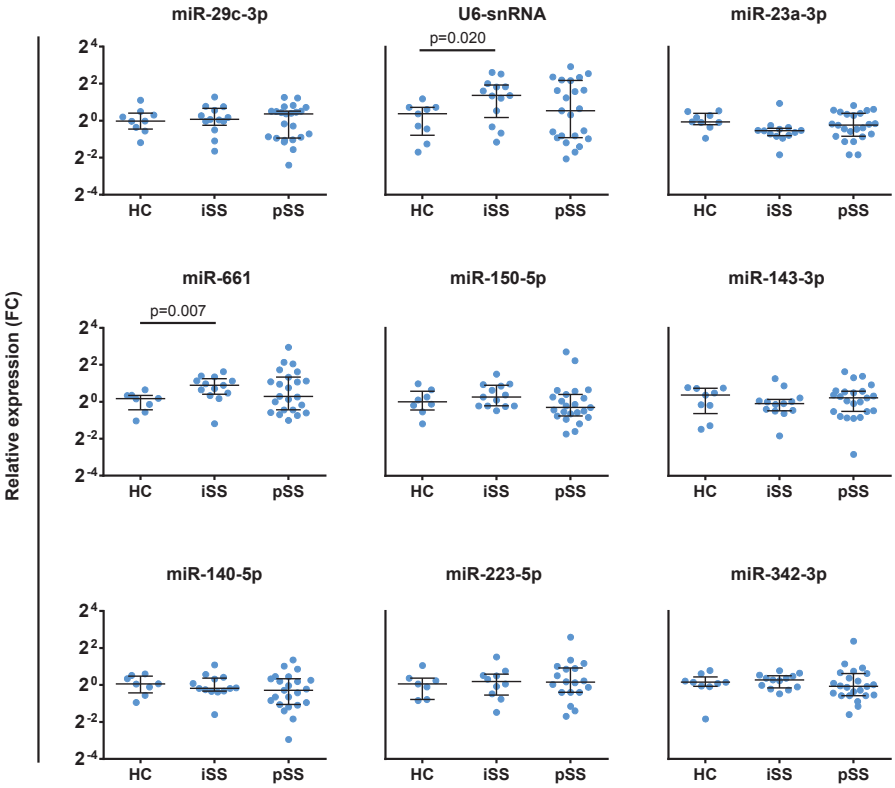


Figure 2. RT-qPCR data of all nine sncRNAs included in the validation phase. Serum sncRNAs were measured using single Taqman qRT-PCR in the validation cohort (n = 45). ΔCt per sample was calculated using the expression of an exogenous spiked-in Arabidopsis thaliana miRNA to correct for technical variation. The relative expression of each sample was calculated as fold change (FC) in comparison with the ΔCt mean of the HC group in the respective cohort. Medians ± IQR are shown.

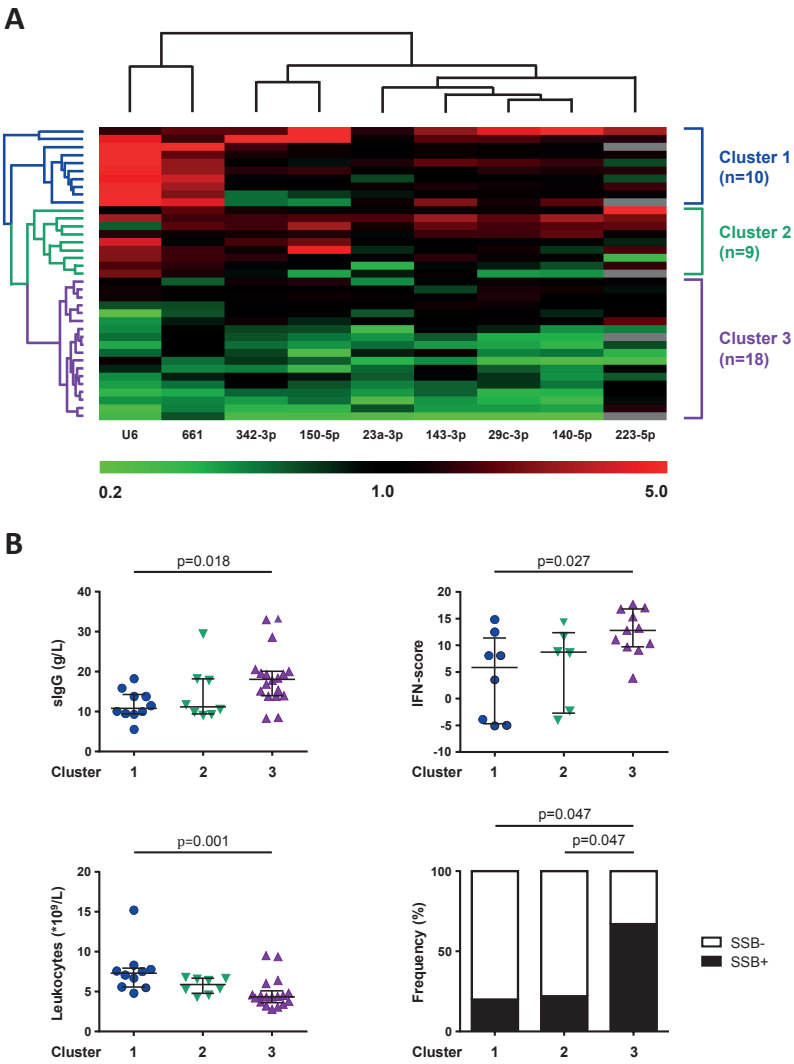


Figure 3. pSS patients with increased B cell hyperactivity can be identified using hierarchical clustering of serum sncRNA expression levels. Nine sncRNAs were selected based on their differential expression in the discovery array and subsequent technical replication. These sncRNAs were measured using single-assay RT-qPCR in samples from both cohorts (n = 75). Unsupervised hierarchical clustering was performed on the expression of the nine selected sncRNAs in all 37 pSS patients. Grey fields depict unavailable data points (**A**). Clinical parameters and frequency of positivity for anti-La (SSB) autoantibodies were compared between the three clusters (**B**). The patients in each cluster were compared using Kruskal-Wallis H test with post-hoc Dunn's test of multiple comparisons and Fisher's exact test. For dot plots, medians ± IQR are shown.

Table 3. Correlations between serum sncRNA levels and disease parameters in pSS patients.

		C3 (g/L)	C4 (g/L)	sIgG (g/L)	Leuko-cytes (*10⁹/L)	Lympho-cytes (*10⁹/L)	LFS (foci/ 4mm²)	IFN score
miR-29c-3p	ρ	0.372	**0.415**	**-0.429**	**0.520**	0.183	-0.282	**-0.502**
	p	0.050	**0.042**	**0.038**	**0.009**	0.387	0.147	**0.039**
U6-snRNA	ρ	0.381	**0.400**	**-0.432**	**0.697**	0.226	-0.208	**-0.545**
	p	0.050	**0.047**	**0.038**	**0.000**	0.285	0.269	**0.029**
miR-23a-3p	ρ	**0.463**	0.268	**-0.401**	**0.382**	0.111	**-0.393**	-0.442
	p	**0.029**	0.171	**0.046**	**0.049**	0.602	**0.049**	0.050
miR-661	ρ	0.374	0.236	**-0.520**	**0.605**	0.112	-0.245	**-0.675**
	p	0.050	0.218	**0.009**	**0.003**	0.602	0.204	**0.004**
miR-150-5p	ρ	0.253	0.534	-0.291	**0.434**	**0.483**	**-0.387**	-0.250
	p	0.190	0.009	0.131	**0.038**	**0.038**	**0.049**	0.269
miR-143-3p	ρ	0.378	0.229	**-0.491**	**0.527**	0.070	-0.372	**-0.491**
	p	0.050	0.229	**0.017**	**0.009**	0.731	0.050	**0.042**
miR-140-5p	ρ	**0.403**	0.346	**-0.422**	**0.543**	0.103	-0.264	**-0.468**
	p	**0.047**	0.071	**0.039**	**0.009**	0.621	0.176	**0.048**
miR-223-5p	ρ	0.275	0.327	-0.343	0.207	0.163	-0.410	-0.302
	p	0.190	0.122	0.095	0.296	0.498	0.050	0.207
miR-342-3p	ρ	0.292	**0.386**	-0.222	0.332	0.406	**-0.421**	-0.072
	p	0.133	**0.049**	0.233	0.079	0.056	**0.041**	0.731

Spearman's correlation coefficients (ρ) and B&H FDR-corrected p-values are shown. sIgG: serum immunoglobulin G; LFS: lymphocytic focus score. Correlations that are significant at $p<0.05$ are depicted in bold.

Discussion

In the present study, we investigated whether serum sncRNAs can be used to distinguish pSS patients from iSS patients and HC. Using two independent cohorts with patients and controls, we were unable to identify any sncRNAs that reproducibly differ between pSS patients and patients with iSS or HC. However, we did show that circulating sncRNA levels reflect disease parameters in pSS patients and can be used to distinguish pSS patients with higher markers of B cell hyperactivity.

We chose to measure circulating miRNAs in serum, as it is easily accessible and its preparation is well standardized. Measurements of miRNAs in plasma may yield different results, as a recent study showed that around 6% of studied miRNAs show differences in expression between the 2 fluids [25]. Future studies need to be conducted to investigate whether more clear differences in miRNA levels can be found in plasma from pSS patients. However, levels of eight of the nine sncRNAs included in our validation phase (all but miR-661) were previously compared between serum and plasma and none of them exhibited significant differences [25]. As such, these miRNAs should be similarly expressed in plasma measurements.

Our discovery-validation approach allowed us to measure a large number of sncRNAs in the discovery cohort and follow nine of these up in the validation cohort. Technical replication showed that the differences found in the discovery cohort were not artifacts of the array. In addition, we compared two methods of data normalization for the discovery cohort to ensure optimal data analysis. We chose to use global mean normalization as it was the most inclusive and appropriately corrects for limitations intrinsic to the qPCR methodology [20, 23]. As such, any serum sncRNAs that were included in the array and are robustly dysregulated in pSS patients compared to iSS or HC should have been identified here. Using this approach, we validated that two sncRNAs, U6-snRNA and miR-661, are increased in iSS patients compared to HC. To our knowledge, these sncRNAs have not previously been described in any autoimmune disease and future studies on the function of these sncRNAs should clarify what their role is in the disease. Although the iSS patients who were studied presented with mild local and systemic parameters of inflammation, these features may explain the increased sncRNA levels. In-line with this hypothesis, data showed an association between increased circulating U6-sncRNA and markers of inflammation in a range of inflammatory conditions [26].

However, the lack of differences between pSS patients and iSS patients or HC in sncRNAs can be largely attributed to the heterogeneity of this group. The expression of the nine sncRNAs measured in the validation phase was strongly overlapping between pSS patients, iSS patients and HC. As our analyses show an association between serum sncRNA levels and several biological disease parameters, the large variation in the pSS group seems to be related to differences in markers of inflammation. In particular, autoantibody presence was an important parameter in this regard, as a range of sncRNAs showed significant differences between autoantibody positive and negative pSS patients. As such, the increased prevalence

of SSA and SSB positivity in the validation cohort compared to the discovery cohort, although not statistically significant, may have contributed to the lack of validated targets. However, both cohorts presented with an autoantibody presence that is within acceptable range to those reported in very large cohorts of pSS patients [27].

Within the nine sncRNAs measured in the validation phase, we observed an overall trend of increased expression in the pSS patients from clusters 1 and 2 compared to the patients in cluster 3. The pSS patients in cluster 3 showed an overall decrease in serum levels of the measured sncRNAs and presented with more pronounced autoimmune activity, including increased B cell hyperactivity, as measured by sIgG and autoantibody positivity, and a higher IFN-score. The IFN-score was previously shown to correlate with the disease activity and autoantibody presence in pSS patients [22], which is in-line with the increased serum IgG and SSB-positivity we observed in the patients in this cluster. The association of lower sncRNA levels with higher parameters of B cell hyperactivity may be explained by a change in the composition of circulating B cell pool. In line with this hypothesis, three of miRNAs analyzed in the validation phase (miR-150-5p, miR-223-5p, and miR-342-3p) are highly expressed by naïve B cells while their expression is down-regulated upon B cell activation. In addition, these miRNAs were implicated in the regulation of B cell differentiation [28]. As such, this set of miRNAs may be involved in the increased B cell hyperactivity observed in the patients of cluster 3. Alternatively, the lower sncRNA levels observed in cluster 3 may be a reflection of changes in the composition of circulating cells, as these patients also have a decreased leukocyte count. Possibly, the decreased levels of sncRNAs can be explained by migration of the leukocytes responsible for the production of these sncRNAs to sites of inflammation. This is supported by the correlation of leukocyte counts with the expression of the majority of the sncRNAs measured in the validation phase in the pSS group as a whole. In line with this, levels of circulating miRNAs that are expressed by leukocyte subsets correlate with the presence of these cells in the blood [29].

Conclusions

In conclusion, we validated increased expression of two serum sncRNAs in iSS patients compared with HC, U6-snRNA and miR-661, but did not find any differences in serum sncRNA levels between pSS patients and iSS patients or HC. Furthermore, we show that the heterogeneity in sncRNA expression within the pSS patients is associated with differences in clinical and laboratory parameters.

Moreover, pSS patients with a higher IFN score and signs of increased B cell hyperactivity can be distinguished on the basis of overall lower expression levels of the sncRNAs studied. These lower serum sncRNA levels may be related to migration of the miRNA-producing cells from the circulation towards the site of inflammation. In addition, as several of these miRNAs are implicated in B cell activation and differentiation, they may play a role in the more pronounced B cell hyperactivity observed in these patients.

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Supplementary material

Supplementary Table 1. Comparison between global mean and spike-in normalization in the discovery cohort.

	Global mean normalization		Spike-in normalization	
	iSS vs HC	pSS vs HC	iSS vs HC	pSS vs HC
miR-29c-3p	**5.308 (0.004)**	**5.659 (<0.0001)**	**9.686 (0.003)**	**7.531 (0.0001)**
U6-snRNA	**4.280 (0.003)**	**2.461 (0.016)**	**3.848 (0.003)**	2.634 (0.106)
miR-23a-3p	**2.718 (0.007)**	2.124 (0.123)	**2.284 (0.007)**	2.079 (0.110)
miR-661	**2.609 (0.021)**	1.978 (0.019)	2.146 (0.161)	2.000 (0.165)
miR-150-5p	**2.168 (0.028)**	1.265 (0.353)	1.936 (0.161)	1.356 (0.441)
miR-143-3p	**2.088 (0.007)**	1.692 (0.016)	1.698 (0.015)	1.635 (0.095)
miR-140-5p	**2.062 (0.021)**	1.364 (0.182)	1.696 (0.159)	1.356 (0.330)
miR-223-5p	**2.001 (0.049)**	1.141 (0.642)	1.648 (0.336)	1.144 (0.971)
miR-342-3p	**2.384 (0.001)**	1.496 (0.309)	1.736 (0.123)	1.235 (0.616)
miR-212-3p	0.417 (0.232)	**0.129 (0.002)**	0.342 (0.195)	**0.135 (0.002)**

Results are expressed as mean FC. Differences between groups that met the threshold for the corresponding analysis (FC difference of ≤0.5 or ≥2.0 at p-value of p<0.05) are indicated in bold. Mann–Whitney U test was used to test all comparisons.

Supplementary Table 2. Correlation between array and single RT-qPCR results in the discovery cohort.

	Correlation	
	ρ	p
miR-29c-3p	**0.9035**	**<0.0001**
U6-snRNA	**0.8545**	**<0.0001**
miR-23a-3p	**0.7044**	**<0.0001**
miR-661	**0.5704**	**0.0012**
miR-150-5p	**0.9547**	**<0.0001**
miR-143-3p	**0.8645**	**<0.0001**
miR-140-5p	**0.6089**	**0.0005**
miR-223-5p	**0.8976**	**<0.0001**
miR-342-3p	**0.7783**	**<0.0001**
miR-212-3p	0.0	>0.999

Correlation between Crt in profiling array and CT measured with single-assay Taqman RT-qPCR in patients and controls from the discovery cohort (n=30). Spearman's correlation coefficients (ρ) and p-values are shown. Correlations that are significant at p<0.05 are depicted in bold

Supplementary Table 3. Differences between SSA/SSB positive and negative pSS patients in circulating sncRNA abundance.

	SSA		SSB	
	negative	positive	negative	positive
miR-29c-3p	**1.5 [0.5-4.5]**	**1.3* [0.2-2.4]**	1.4 [0.5-4.5]	1.1 [0.2-2.4]
U6-snRNA	1.3 [0.7-5.9]	1.6 [0.2-7.6]	**2.4 [0.6-6.6]**	**0.6* [0.2-7.6]**
miR-23a-3p	1.2 [0.6-2.4]	0.9 [0.3-1.5]	0.9 [0.3-2.4]	0.7 [0.3-1.5]
miR-661	2.2 [0.6-7.8]	1.2 [0.5-4.4]	**2.2 [0.6-7.8]**	**0.9** [0.5-4.4]**
miR-150-5p	**1.6 [0.4-6.4]**	**0.9* [0.3-6.5]**	1.3 [0.3-6.4]	0.8 [0.3-6.5]
miR-143-3p	**1.5 [0.7-3.5]**	**1.0* [0.1-3.1]**	**1.5 [0.6-3.5]**	**1.0* [0.1-3.1]**
miR-140-5p	1.4 [0.5-5.4]	0.9 [0.1-2.7]	1.3 [0.4-5.4]	0.8 [0.1-2.6]
miR-223-5p	**2.3 [0.8-6.0]**	**1.1* [0.3-2.5]**	**1.5 [0.4-6.0]**	**1.0* [0.3-2.5]**
miR-342-3p	1.6 [0.6-2.5]	1.0 [0.3-5.2]	1.3 [0.5-2.5]	1.0 [0.3-5.2]

sncRNAs were measured using RT-qPCR in all pSS patients from the discovery and validation cohort (n=37). Fold changes (FC) were calculated as compared to the mean of the healthy control group in the corresponding cohort. Results are expressed in FC as median [range]. Statistically significant differences (Mann–Whitney U test) between autoantibody positive and negative pSS patients are indicated in bold. SSA: anti-Ro/Sjögren's syndrome antigen A; SSB: anti-La/Sjögren's syndrome antigen B. * and ** depict significant differences at $p<0.05$ and $p<0.01$ respectively.

Chapter 3

MicroRNA-130a contributes to type-2 classical DC-activation in Sjögren's syndrome by targeting mitogen- and stress-activated protein kinase-1

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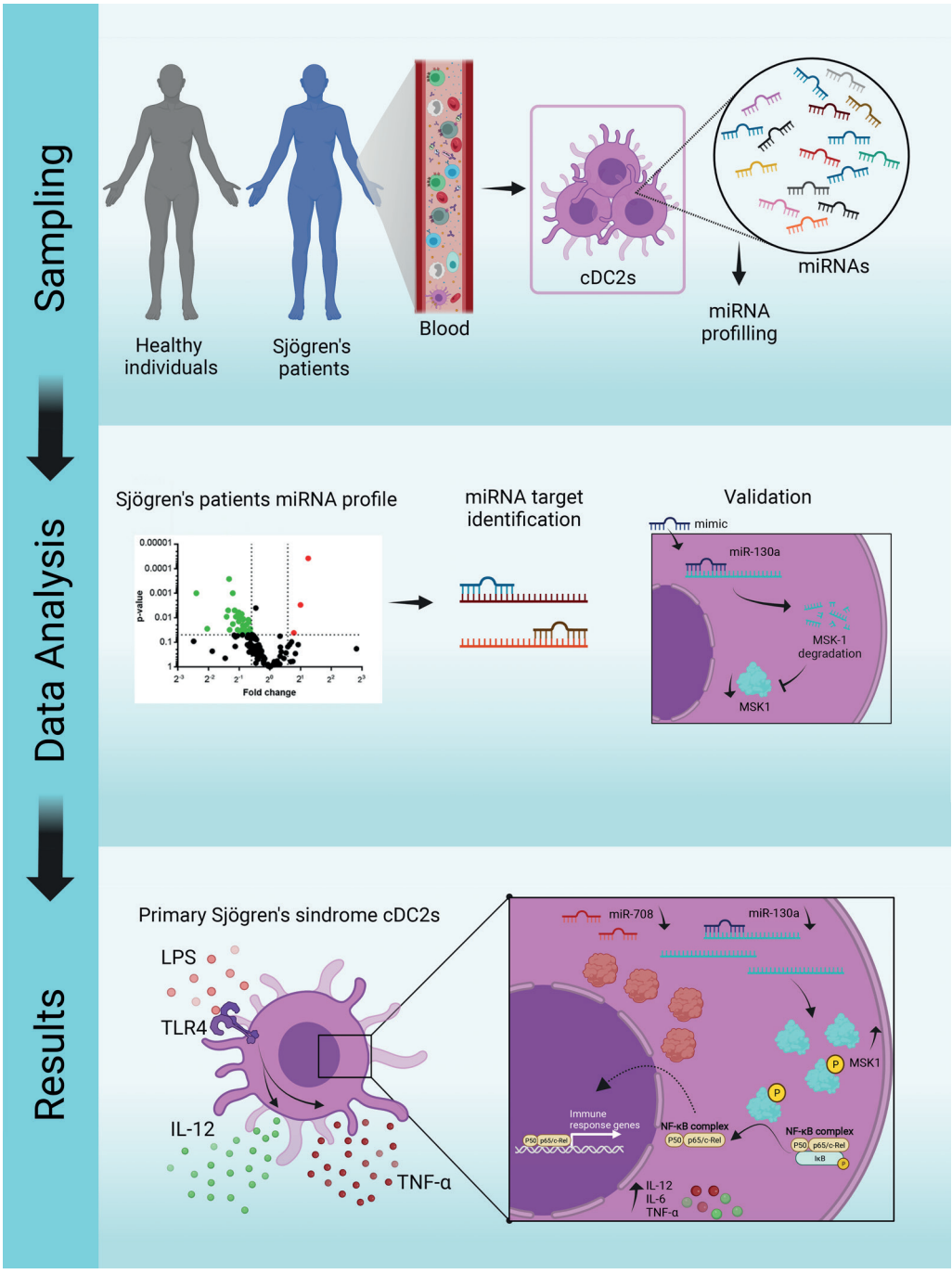
Objectives Considering the critical role of microRNAs (miRNAs) in regulation of cell activation, we investigated their role in circulating type-2 conventional dendritic cells (cDC2s) of patients with primary Sjögren's syndrome (pSS) compared to healthy controls (HC).

Methods CD1c-expressing cDC2s were isolated from peripheral blood. A discovery cohort (15 pSS, 6 HC) was used to screen the expression of 758 miRNAs and a replication cohort (15 pSS, 11 HC) was used to confirm differential expression of 18 identified targets. Novel targets for two replicated miRNAs were identified by SILAC in HEK-293T cells and validated in primary cDC2s. Differences in cytokine production between pSS and HC cDC2s were evaluated by intracellular flow-cytometry. cDC2s were cultured in the presence of MSK1-inhibitors to investigate their effect on cytokine production.

Results Expression of miR-130a and miR-708 was significantly decreased in cDC2s from pSS patients compared to HC in both cohorts, and both miRNAs were downregulated upon stimulation via endosomal TLRs. Upstream mediator of cytokine production MSK1 was identified as a novel target of miR-130a and overexpression of miR-130a reduced MSK1 expression in cDC2s. pSS cDC2s showed higher MSK1 expression and an increased fraction of IL-12 and TNF-α-producing cells. MSK1-inhibition reduced cDC2 activation and production of IL-12, TNF-α, and IL-6.

Conclusions The decreased expression of miR-130a and miR-708 in pSS cDC2s seems to reflect cell activation. miR-130a targets MSK1, which regulates pro-inflammatory cytokine production, and we provide proof-of-concept for MSK1-inhibition as a therapeutic avenue to impede cDC2 activity in pSS.

Graphical abstract



Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by keratoconjunctivitis sicca, xerostomia, and lymphocytic infiltration of salivary and lacrimal glands (1, 2). pSS is associated with multiple factors such as genetic predisposition and environmental factors including viral infection (3). Although the cause of pSS remains poorly understood, the contribution of the immune system is evident. Activated autoreactive B cells and T cells as well as increased levels of pro-inflammatory cytokines drive chronic inflammation of the exocrine glands, associated with loss of function (4).

Conventional dendritic cells (cDCs) are potent antigen-presenting cells with an important role in the initiation and control of immune responses, mainly due to their superior ability to take up and present antigens to T cells. cDCs can be divided into two phenotypically and functionally distinct subsets defined by their expression of CD141 (cDC1s) and CD1c (cDC2s). cDC2s are the most predominant in human blood, tissues and lymphoid organs (5). They produce a variety of cytokines (e.g., IL-12, IL-6, and TNF-α) and chemokines (e.g., CXCL8, CCL3, CCL4, CCL5, and CXCL10) (6) and present antigen to potently activate T cells (7, 8). The primary target cells of cDC2s, CD4 T cells, are thought to play a crucial role in pSS immunopathology (2, 4) and increased numbers of DCs are present in salivary glands of pSS patients (9, 10). As such, cDC2s are suspected to play an important role in driving salivary gland inflammation (11, 12), however their molecular regulation has not yet been studied in pSS.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by inducing cleavage of their target mRNA or preventing translation into protein (13). miRNAs are critical regulators of numerous biological processes, such as cell proliferation and differentiation, metabolism, and cell activation (14, 15). In pSS, several studies showed that miRNA expression is dysregulated in peripheral blood mononuclear cells (PBMCs), purified immune cells, and salivary gland tissue (16–18). Changes in miRNA expression were associated with the presence of autoantibodies, local inflammation, and the production of pro-inflammatory cytokines (19–22). To unravel the molecular mechanisms that regulate cDC2 function in patients with pSS, we investigated the miRNA profile of purified circulating cDC2s from patients with pSS and identified two miRNAs that are expressed at a lower level in pSS in two independent cohorts. In addition, we establish mitogen- and

stress-activated protein kinase-1 (MSK1) as a novel endogenous target of miR-130a in primary cDC2s and provide proof-of-concept for targeting this kinase to inhibit the production of pro-inflammatory cytokines by cDC2s.

Materials and methods

Patients and controls

All pSS patients met the 2002 AECG classification criteria (23). A group of healthy controls (HC) was included as a control group. For identification of differentially expressed miRNAs, two independent cohorts of patients and controls (discovery and replication) were established. An independent set of HC and pSS donors was included for follow-up experiments (**Table 1**). The study was approved by the medical ethics committee of the University Medical Center Utrecht (METC no. 13-697). All patients gave their written informed consent in accordance with the declaration of Helsinki.

cDC2 Isolation

cDC2s were isolated from peripheral blood or buffy coats (Sanquin) by magnetic-activated cell sorting using CD1c (BDCA-1)+ Dendritic Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. To confirm consistent purity of isolated cDC2s, the proportion of CD19-BDCA1+ cells within the isolated fraction was measured using fluorescence activated cell sorting (FACS). The purity of the isolated samples was (median [interquartile range]) 94% (88–97%), and there were no significant differences in cell purity between any of the groups.

RNA Isolation

For mRNA and miRNA studies, cells were lysed in RLTPlus buffer (Qiagen) and total RNA was purified using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was assessed with Qubit RNA Kit (Thermo Fisher Scientific).

miRNA Quantification in Discovery and Replication Cohorts

Identification of differentially expressed miRNAs was carried out in two independent phases. First, expression of 758 miRNAs was screened in MACS-isolated CD1c-expressing cDC2s from the donors included in the discovery cohort using the OpenArray platform as previously described (24, 25) (discovery phase; **Supplementary Figure 1**).

Table 1. Characteristics of the patients and controls enrolled in the study.

	miRNA profiling			
	Discovery phase (n=21)		Replication phase (n=26)	
	HC	pSS	HC	pSS
N (M/F)	6 [0/6]	15 [3/12]	11 [1/10]	15 [1/14]
Age (yr.)	56 [54-67]	53 [29-77]	50 [26-55]	55 [26-69]
LFS (foci/4 mm^2)	-	1.7 [1.0-4.0]	-	2.0 [1.0-4.0]
ESSDAI	-	2.0 [0.0-19]	-	5.0 [0.0-13]
ESSPRI	-	3.3 [1.8-8.8]	-	5.3 [1.0-8.0]
Schirmer (mm/5 min)	-	4.5 [0.5-25]	-	15 [0.5-30]
ANA (no. positive [%])	-	10 [67%]	-	13 [93%]
SSA (no. positive [%])	-	8 [53%]	-	12 [80%]
SSB (no. positive [%])	-	3 [20%]	-	9 [60%]
RF (no. positive [%])	-	4 [31%]	-	7 [64%]
Serum IgG (g/L)	-	14 [8.3-30]	-	18 [9.3-33]
ESR (mm/hour)	-	9 [5.0-30]	-	17 [4.0-77]
CRP (mg/L)	-	1.0 [0.0-8.0]	-	1.0 [0.0-49]
C3 (g/L)	-	1.1 [0.8-1.3]	-	1.0 [0.5-1.3]
C4 (g/L)	-	0.3 [0.1-0.3]	-	0.2 [0.1-0.3]
Not treated (no. [%])	-	12 [80%]	-	9 [60%]
Only HCQ (no. [%])	-	1 [7%]	-	2 [13%]
Other (no. [%])	-	2 [13%]	-	4 [27%]

HC: healthy control; pSS: primary Sjögren's syndrome; LFS: lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: anti-nuclear antibodies; SSA: anti-SSA/Ro; SSB: anti-SSB/La; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein, HCQ: hydroxychloroquine. Other treatment group includes azathioprine, alone (n=2) or in combination with prednisone (n=2); mesalazine (n=2); prednisone, alone (n=4) or in combination with HCQ (2); methotrexate (n=1). Values are median [range] unless stated otherwise.

MSK1 analyses		Intracellular staining	
(n=41)		(n=25)	
HC	pSS	HC	pSS
16 [0/16]	25 [3/22]	12 [0/12]	13 [0/13]
57 [45-67]	58 [34-77]	58 [26-63]	61 [22-81]
-	2.0 [1.0-5.0]	-	3.3 [1.0-6.4]
-	3.0 [0.0-19]	-	5.0 [0.0-13]
-	4.9 [1.3-8.8]	-	6.0 [2.0-9.0]
-	5.5 [0.0-28]	-	5.0 [0.0-24]
-	18 [75%]	-	13 [100%]
-	17 [68%]	-	13 [100%]
-	11 [44%]	-	11 [85%]
-	9 [43%]	-	7 [78%]
-	14 [5.6-42]	-	12 [8.4-22]
-	14 [4.0-43]	-	14 [2.0-27]
-	2.0 [0.0-13]	-	1.7 [0.6-22]
-	1.1 [0.8-1.6]	-	0.9 [0.9-1.1]
-	0.3 [0.1-0.4]	-	0.2 [0.1-0.3]
-	19 [76%]	-	5 [38%]
-	2 [8%]	-	5 [38%]
-	4 [16%]	-	3 [23%]

miRNAs with a poor amplification score (<1.24) were excluded from the analysis and low expressed miRNAs (CT>27) were set at 27. Data were normalized using the global mean normalization approach. miRNAs with a fold change (FC) difference of >1.5 at a nominal p-value < 0.05 between the groups were considered to be differentially expressed. Selection of miRNAs to be measured in the replication cohort was based on the expression level and quality of the amplification (**Supplementary Figure 1**). Consistent differential expression was confirmed for 18 selected miRNAs in the replication cohort using a custom array (Thermo Fisher Scientific). The array included the 18 selected miRNAs and 4 reference small non-coding RNAs (sncRNAs) (miR-17, miR-191, RNU48, U6-snRNA) that all showed good abundance and stable expression between patients and controls (replication phase; **Supplementary Figure 1**). Data were analyzed according to the comparative threshold cycle (CT) method and the expression of each sample was normalized by the mean expression of the four reference sncRNAs. Relative expression was depicted as FC compared to one HC sample, which was set at 1. Replication was considered successful if the direction of the difference (i.e., up/downregulation) was identical to what was observed in the discovery cohort and the difference was significant at a nominal p-value < 0.05.

cDC2 Stimulation

cDC2s were cultured in RPMI glutamax (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biowest Riverside) and 1% penicillin/streptomycin (Thermo Fisher Scientific). cDC2s were cultured at a concentration of 0.5×10^6 cells/mL in a 96-well round-bottom plate. Cells were left unstimulated or were stimulated for 24 h either with 25 µg/mL of toll-like receptor (TLR) 3 ligand Poly(I:C) or with 1 µg/mL of TLR7/8 ligand R848 (both InvivoGen).

miRNA Quantification in Cultured Cells

Analysis of miRNA expression in cultured cells was performed using TaqMan miRNA Human Assays hsa-miR-130a-3p (ID 000454), hsa-miR-708-5p (ID 002341) and RNU44 (ID 001094) (Thermo Fisher Scientific). cDNA was prepared by using the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's instructions (Thermo Fisher Scientific). miRNA levels were quantified using TaqMan fast advance master mix and miRNA-specific primers from the TaqMan miRNA assays on the Quantstudio 12k Real-Time PCR system (Thermo Fisher Scientific). Relative miRNA expression was calculated after normalization by RNU44, which was stably expressed across groups and conditions, using the comparative CT method. The relative fold change (FC) of each sample was calculated in comparison with the unstimulated or the non-targeting miRNA control (SCR) transfected condition where appropriate.

miRNA Transfection of HEK-293T Cells and Primary cDC2s

HEK-293T cells were cultured in DMEM (Thermo Fisher Scientific) with 10% FCS (Biowest) and 1% penicillin/streptomycin (Thermo Fisher Scientific). 24 h prior to transfection, HEK-293T cells were plated in a 6-well plate at a concentration of 0.5×10^5 cells/mL. On the day of transfection, the medium was replaced and cells were transfected with miR-130a mimic or with non-targeting miRNA control (SCR) (Thermo Fisher Scientific) at a final concentration of 30 nM, together with lipofectamine RNAiMAX and Opti-MEM (both Thermo Fisher Scientific) for 48 h.

cDC2s were isolated from buffy coats and plated at a density of 1.0×10^6/mL in a 12-well plate and rested for 6 h. Then, cells were transfected with miR-130a mimic or SCR at a final concentration of 30 nM together with lipofectamine 2,000, Opti-MEM, and PLUS Reagent (all from Thermo Fisher Scientific). 18 h post transfection, cells were washed and seeded at the same cell density and kept in culture for an additional 24 h.

Transfection efficiency was confirmed in HEK-293T by transfection of cells with a fluorescently labeled-SCR (Thermo Fisher Scientific) as described above (**Supplementary Figure 2**). 48 h after transfection, cells were washed and the percentage of positive cells was assessed by FACS. Additionally, the expression of miR-130a was measured in both SCR and miRNA-transfected conditions for both cell types to confirm miRNA overexpression.

Protein Extraction and Immunoblotting

Cells were lysed in Laemmli's buffer and protein content was quantified with a BCA Protein Assay Kit (Pierce). Proteins were separated on 4–12% Bis-Tris SDS NuPAGE gels (Thermo Fisher Scientific) and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in 5% non-fat milk (Bio-Rad) in TBST and probed overnight at 4°C with antibodies recognizing mitogen- and stress-activated protein kinase-1 (MSK1) (#3489, rabbit anti-human, Cell Signaling Technology) and histone 3 (H3) (#9715, rabbit anti-human, Cell Signaling Technology). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated swine anti-rabbit antibody (Agilent Technologies), and protein visualization was performed using a ChemiDoc MP system (Bio-Rad).

Intracellular Cytokine Measurement in cDC2

To evaluate differences in cytokine production between patients and controls after in vitro TLR stimulation, whole blood was diluted 1:1 in RPMI-1640 medium (Thermo Fisher Scientific) with 1% L-glutamine (Thermo Fisher Scientific) and stimulated with

TLR4 ligand LPS (25 µg/mL, Sigma). 1 h after stimulation, 10 µg/mL of Brefeldin A (Sigma) was added and incubated for 5 h. Cells were then stained with anti-BDCA-1 APC (L161, Thermo Fisher Scientific), anti-CD19 BV510 (HIB19, BioLegend), anti-HLA-DR BV605 (G46-6, BD Biosciences) and anti-CD14 BV785 (M5E2, BioLegend). After washing, fixation and permeabilization with FIX&PERM (Thermo Fisher Scientific) according to manufacturer's instructions, cells were stained with anti-IL-6 AF700 (MQ-13A5, Thermo Fisher Scientific), anti-IL-12 FITC (C11.5, BD Biosciences), anti-IL-8 PerCP-Cy5.5 (BH0814, Sony Biotechnology) and anti-TNF-α BV421 (MAb11, BioLegend). Data acquisition was performed using a BD LSRFortessa (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

cDC2 Stimulation and Exposure to MSK1 Inhibitors

cDC2s isolated from buffy coats were plated at a density of 0.5×10^6/mL in a 96-well round-bottom plate. Cells were left unstimulated or were treated with MSK1 inhibitors [H89, 10 µM (Bio-Techne); SB 747651A, 10 µM (Bio-Techne); or Ro 31-8220, 5 µM (Sigma)] for 1 h. Then, cells were stimulated with TLR4L at a final concentration of 100 ng/ml. After 6 h, supernatants were stored and cells were lysed for RNA extraction or processed for flow cytometry.

After harvesting, cells were washed in Annexin V Binding Buffer and stained with Annexin V–APC, 7-AAD–PerCP (all from BD Biosciences), anti-CD80–PE (L307.4, BD Biosciences), anti-CD83–FITC (HB15a, Beckman Coulter) and anti-CD86–PB (IT2.2, Sony Biotechnology). Data acquisition was performed using a FACSCanto II flow cytometer (BD Bioscience) and data were analyzed using FlowJo software (Tree Star). The percentage of viable cells after stimulation was measured as the proportion of Annexin V/7AAD double negative cells (**Supplementary Figure 3**). The expression of co-stimulatory molecules given by the mean fluorescent intensity was evaluated within the viable cells.

Statistics

Differences in miRNA expression between pSS patients and HCs in the discovery cohort were analyzed using Thermofisher Cloud software. For analysis of the replication cohort data, differences in miRNA expression between pSS and HC were assessed using the Mann-Whitney U test (two-sided). For unsupervised hierarchical clustering, Euclidean distance and Ward's linkage method were used on the miRNA FC using MetaboAnalyst online software (https://www.metaboanalyst.ca/). Wilcoxon signed-rank test was used for paired comparisons in in vitro cultures. Statistical analyses were performed using SPSS v20 (IBM) and Graphpad Prism (GraphPad Software). Differences were considered to be statistically significant at $p < 0.05$.

Detailed descriptions of stable isotope labeling of amino acids in cell culture (SILAC), selection of in silico predicted miRNA targets, quantitative real-time PCR and cytokine analysis are provided in the Online Supplementary Methods.

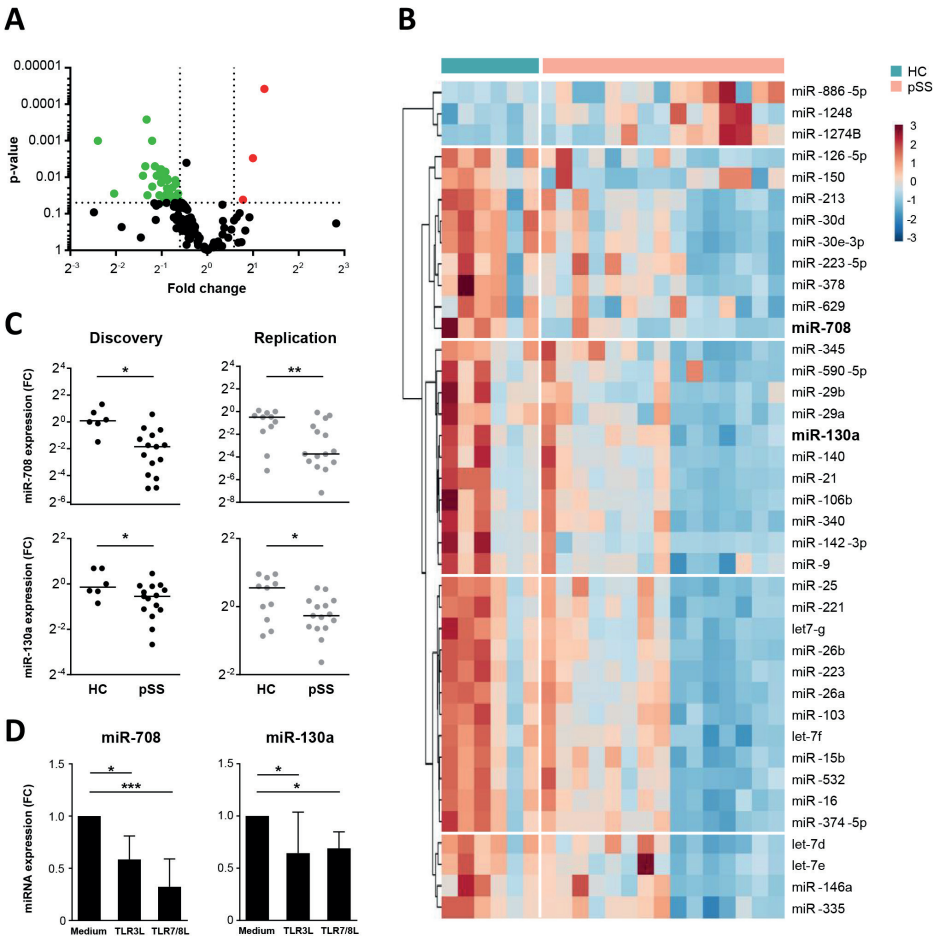


Figure 1. Expression of miR-708 and miR-130a is decreased in pSS patients. Volcano plot displays the relationship between fold change (x-axis) and the statistical significance (y-axis) for the compared groups (pSS patients vs. HC) (**A**). Hierarchical clustering of the 39 differentially-expressed miRNAs between pSS patients and HC using Euclidean distance and Ward's method (**B**). miR-708 and miR-130a expression was consistently downregulated in pSS patients compared to HC in both cohorts (**C**). cDC2s stimulated with TLR3 (25 µg/mL) and TLR7/8 (1 µg/mL) ligands for 24 h showed a reduced expression of miR-708 and miR-130a measured by qPCR compared to medium control (**D**). Medians ± IQR are shown *p < 0.05, **p < 0.01, and ***p < 0.001, respectively.

Results

Expression of miR-130a and miR-708 Is Consistently Decreased in cDC2s From pSS Patients, Associated With Cell Activation

Using two independent cohorts of patients and controls (**Table 1**) we identified differentially expressed miRNAs in cDC2s from pSS patients compared to HC. In the discovery phase, we screened the expression of 758 miRNAs, of which 143 were expressed in cDC2s (**Supplementary Figure 1**). Of these, 39 were differentially expressed in pSS patients compared to HC ($0.67 < FC > 1.5$; $p < 0.05$) (**Figures 1A,B and Supplementary Table 1**). Of the 39 identified miRNAs, we selected 18 miRNAs for quantification in the replication cohort based on their expression level and the quality of amplification in the array (**Supplementary Figure 1**). The expression of miR-708 and miR-130a was consistently decreased in pSS patients compared to HC in two independent cohorts (biological replication) (**Figure 1C**).

Immune cells are known to modulate their miRNA expression upon activation (26). To investigate whether cDC2 activation is associated with downregulation of miR-708 and miR-130a in pSS, cDC2s from HCs were stimulated with ligands for TLR3 and TLR7/8, which are endosomal nucleic acid receptors that are relevant in pSS pathophysiology (27, 28). We observed that upon cell activation with either TLR ligand the expression of both miR-708 and miR-130a was decreased when compared with the unstimulated condition (**Figure 1D**). This supports the notion that the decreased expression of these miRNAs in pSS reflects cDC2 activation.

MSK1 Is a Novel Target of miR-130a

To investigate the regulatory effect of the replicated miRNAs on gene expression at the protein level, we used stable isotope labeling of amino acids in cell culture (SILAC). This proteomic approach allows the identification of miRNA targets by comparing the protein production in cells transfected with a miRNA mimic and non-targeting control (**Figure 2A**). For miR-708, overexpression resulted in downregulation of 23 proteins (**Figure 2B**), of which 2 represented in silico-predicted targets: inosine-5'-monophosphate dehydrogenase 1 (IMPDH1) and prolyl 4-hydroxylase subunit alpha 1 (P4HA1) (**Figure 2C and Supplementary Table 2**). Upon overexpression of miR-130a, the expression of 40 proteins was downregulated (**Figure 2D**). 7 of these proteins represented in silico-predicted targets (**Figure 2E and Supplementary Table 2**). One of the identified miR-130a targets, MSK1, is an important mediator upstream of NF-κB that controls

the production of pro-inflammatory cytokines by cDC2s (29, 30). As such, we selected MSK1 as a target to investigate in follow-up experiments. For this, we first sought to confirm MSK1 regulation by miR-130a at the protein level in HEK-293T cells using western blot. Indeed, in three independent experiments MSK1 was downregulated upon miR-130a overexpression (**Figures 2F,G**).

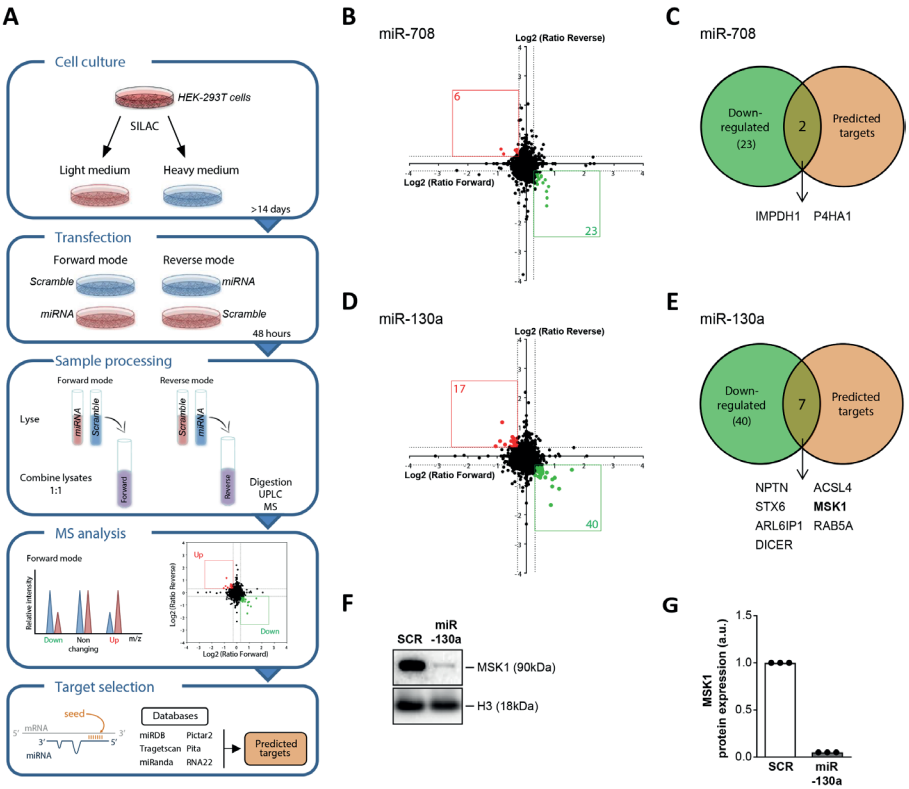


Figure 2. SILAC-based proteomics approach identifies MSK1 as novel target of miR-130a. HEK-293T cells were cultured in light medium or in heavy medium and transfected with either miRNA or with non-targeting miRNA control (SCR) for 48 h. After transfection, light and heavy medium-cultured cells were lysed and combined for mass spectrometry analysis. The intensity of the peak ratios between heavy and light peptides reflects changes in protein production. In silico predicted and experimentally validated targets of the miRNAs were retrieved from six publicly available databases (**A**). Proteins that were downregulated after miRNA-708 or miR-130a overexpression, depicted in green (**B,D**), were selected and compared to the selected targets for each miRNA (**C,E**). Of the 7 proteins that were both downregulated upon miRNA-130a overexpression and contained a seed-region, MSK1 was selected for further analysis. The downregulation of MSK1 in HEK-293T cells upon miRNA-130a overexpression was confirmed in 3 independent experiments by western blot (**F**) (representative of 3 independent experiments) and protein amount was quantified by densitometry in relation to the paired sample transfected with the SCR control, which was normalized to 1 (**G**).

MSK1 Is an Endogenous Target of miR-130a in Primary cDC2 and MSK1 Expression Is Increased in pSS Patients

To determine whether miRNA-130a also regulated MSK1 protein expression in cDC2s, miR-130a was overexpressed in purified cDC2s from HCs. In-line with the data from HEK-293T cells, miR-130a overexpression resulted in significantly decreased expression of MSK1 (**Figures 3A-B**). Following our observation that miRNA-130a was decreased in cDC2s from pSS patients, we investigated whether MSK1 expression was also dysregulated in these cells. Further corroborating the regulation of MSK1 by miR-130a, the expression of MSK1 was increased in pSS cDC2s compared to HC cDC2s (**Figure 3C**). Thus, our data suggest that due to their decreased expression of miR-130a, pSS cDC2s have increased expression of MSK1.

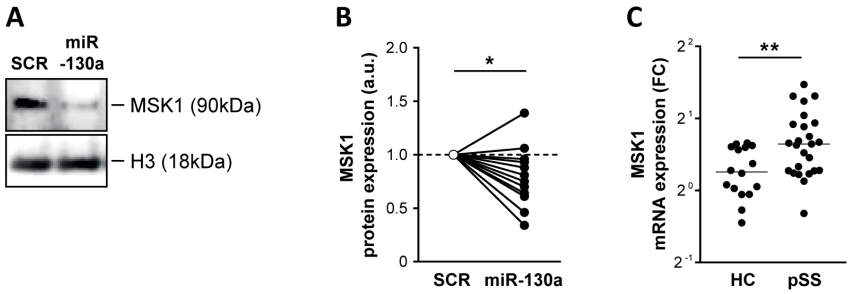


Figure 3. miR-130a regulates MSK1 in cDC2s and MSK1 expression is increased in pSS cDC2s. Downregulation of MSK1 upon miR-130a overexpression in primary cDC2s was confirmed at the protein level by western blot (**A**) (representative of 13 independent experiments). Protein amount was quantified by densitometry in relation to the paired sample transfected with the SCR control, which was normalized to 1 (**B**). MSK1 mRNA expression was measured by qRT-PCR and calculated as fold change (FC) in comparison with the ΔCt mean of the HC group (**C**). Medians are shown. *$p < 0.05$ and **$p < 0.01$, respectively.

Increased Fraction of IL-12 and TNF-α-producing cDC2s in pSS

As MSK1 regulates cytokine production and its expression was increased in pSS cDC2s, we next assessed whether cDC2s from pSS patients produce enhanced levels of pro-inflammatory cytokines downstream from MSK1. As activation via TLR4 efficiently induces MSK1 activation (31), this trigger was used as a model to study MSK1 induction in the context of cDC2 activation. Whole-blood from pSS patients and HC was stimulated with TLR4 ligand LPS for 6 h and the production of cytokines by cDC2s was evaluated by flow cytometry. We observed a significantly increased fraction of cDC2s producing IL-12 and TNF-α in pSS patients compared to HC while no significant differences were found for IL-6 or IL-8 (**Figures 4A-B**).

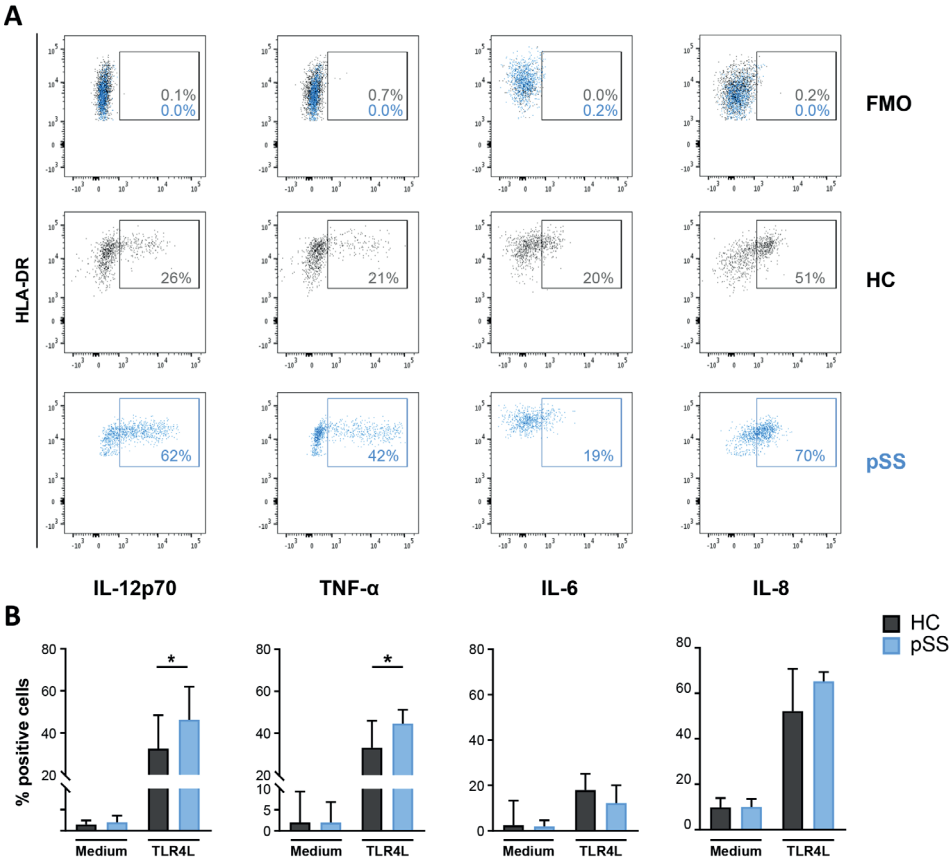


Figure 4. Fraction of cDC2s that produce IL-12 and TNF-α is increased in pSS patients. Intracellular cytokine production was assessed in cDC2s after whole blood stimulation by FACS. Representative flow cytometry dot plots of IL-12, TNF-α, IL-6, and IL-8 expression by cDC2s in HCs (black) and pSS patients (blue) as well as fluorescence minus one (FMO) control upon TLR4L stimulation (25µg/mL) for 6 h are depicted (**A**). Percentages of cDC2s producing cytokines in resting conditions (medium) or after stimulation (TLR4L) are shown for HC and pSS patients (HC n = 12, pSS n = 13) (B). Results are represented as median ± IQR. *p < 0.05.

MSK1 Blockade Inhibits Pro-inflammatory Cytokine Production by cDC2

As MSK1 expression is associated with production of pro-inflammatory cytokines (29, 30), we investigated whether MSK1 inhibition can be used to impede cytokine production in cDC2s. For this, we used 3 different inhibitors previously shown to block MSK1 activation (H89, SB 747651A, and Ro 31-8220) (32). The inhibitor Ro 31-8220 was excluded from further analyses due to its significant negative impact on cell viability (**Figure 5A**). As expected, TLR4 triggering resulted in upregulation of the co-stimulatory molecules CD80

and CD83. Exposure to MSK1 inhibitor SB 747651A lead to clearly reduced expression of both CD80 and CD83, H89 did not affect expression of these molecules (**Figure 5B**). In addition, we investigated the effects of MSK1 inhibition on the production and secretion of MSK1-dependent cytokines. TLR4 triggering induced expression of IL-6, TNF-α, IL-10, IL-12, and IL-8 on mRNA level. Inhibition of MSK1 signaling with either H89 or SB 747651A before stimulation resulted in a significant reduction of IL-6, TNF-α, and IL-10 mRNA (**Figure 5C**). Furthermore, the mRNA expression of both IL-12 subunits was decreased upon exposure to SB 747651A, while no effects were observed on IL-8 (**Figure 5C**). In concordance with the mRNA data, we observed significant inhibition of IL-6, TNF-α, and IL-10 production upon exposure to H89 and SB 747651A at the protein level (**Figure 5D**); IL-12p70 protein production was not detected (not shown). These data support the targeting of MSK1 to inhibit cDC2s activation and subsequent pro-inflammatory cytokine production.

Discussion

Using two independent cohorts of patients and controls, we show that the expression of miR-708 and miR-130a is consistently decreased in cDC2s of pSS patients. In addition, these miRNAs are downregulated upon stimulation via TLR3 and TLR7/8. These TLRs have been implicated in the pathogenesis of pSS (27, 28), as they can be triggered by endogenous RNA molecules such as those contained in immune-complexes and are upregulated in salivary gland epithelial cells and PBMCs from pSS patients (33–35). As activation of cDC2s via TLR3 and TLR7/8 leads to downregulation of miR-130a and miR-708 and literature shows that both these miRNAs negatively impact pro-inflammatory cytokine production via regulation of NF-κB signaling (36, 37), these miRNAs seem to have an immune-inhibitory role. In concordance with this, we show for the first time that MSK1 is an endogenous target of miR-130a in primary cDC2s, and that this regulatory axis is altered in cDC2s from pSS patients. Our data indicate that MSK1 is a link between miR-130a and cytokine production, as MSK1 activation leads to the stimulation of NF-κB-driven genes including TNF-α, IL-12, and IL-6 (38, 39).

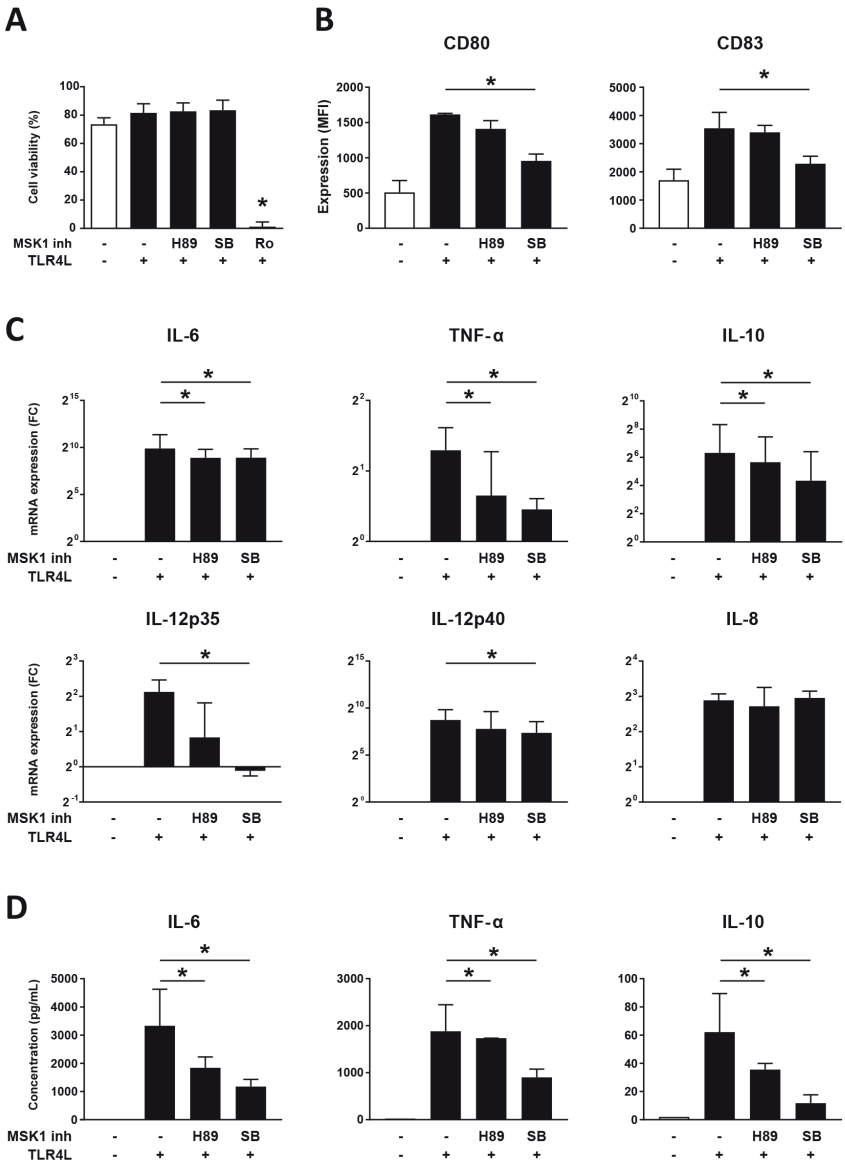


Figure 5. MSK1 inhibition reduces cDC2 activation and inhibits the production of pro-inflammatory cytokines. Isolated cDC2s from buffy coats were treated with H89 (10 μM), SB 747651A (SB; 10 μM), Ro 31-8220 (Ro; 5 μM), or left untreated for 1 h. Then, except for medium control, cells were stimulated with TLR4L (100 ng/mL) for 6 h. FACS was used to assess cell-viability (**A**) and the expression of CD80 and CD83 shown as median fluorescence intensity (MFI) (**B**). Cytokine production upon TLR4L stimulation in the presence or absence of MSK1 inhibitors was measured by qRT-PCR (**C**) and ELISA (**D**). Results are represented as median ± IQR. *$p < 0.05$.

Circulating cDC2s from pSS patients have increased MSK1 expression, supporting the concept that the observed decrease in miR-130a leads to MSK1 upregulation. Using TLR4 triggering as a model for MSK1-driven cDC2 activation, pSS cDC2s produced higher levels of IL-12 and TNF-α, suggesting that the increased expression of MSK1 contributes to their enhanced cytokine production. In addition, MSK1 activation is downstream of several TLRs (31, 40), in-line with the downregulation of miR-130a upon TLR triggering. Thus, pSS cDC2s have decreased expression of miR-130a and increased expression of MSK1, associated with increased production of TNF-α and IL-12. Both of these cytokines are increased in salivary glands and serum of pSS patients (41, 42). High levels of IL-12 promote immune cell infiltration into the salivary glands, which is associated with the presence of autoantibodies in pSS (43). In addition, IL-12 strongly induces T cell proliferation and Th1 polarization, as well as IFN-γ secretion by effector T cells and NK cells (44). TNF-α is a potent pro-inflammatory cytokine and has been implicated in apoptosis of salivary gland epithelial cells, with subsequent release of nuclear autoantigens that contribute to the production of pSS-specific autoantibodies (41, 45). Thus, the demonstrated enhanced activation of pSS cDC2s supports a crucial role for these cells in local immunopathology.

One limitation of this study concerns the differences in clinical and laboratory parameters between the patient groups included. In this regard, the most relevant differences are those in prevalence of auto-antibodies in the two cohorts used for miRNA profiling, as these may have had effect on miRNA replication, preventing the identification of additional dysregulated miRNAs. Still, no statistically significant differences exist between these two cohorts, and all cohorts included largely represent the average pSS patient population as reported in large patient cohorts (46). MSK1 inhibitor SB 747651A prevented the upregulation of co-stimulatory molecules upon TLR4 triggering. In addition, SB 747651A inhibited the production of pro-inflammatory cytokines IL-6, TNF-α, and IL-12 upon TLR4 stimulation. Dissecting the exact contribution of MSK1 inhibition to reduced cytokine production is hampered by a lack of specific inhibitors without off-target effects. Of the available MSK1 inhibitors, SB 747651A is the most selective and only has minor off-target activity against PKA, RSK, PKB, and S6K (32). Though we cannot rule out off-target effects of SB 747651A, the effects we observe seem to be largely MSK1-specific. First, inhibition with H89, which more strongly inhibits activity of PKA rather than MSK1, showed less inhibition of cytokine production compared to SB 747651A, suggesting that PKA targeting would not lead to the observed effects. Second, specific inhibition of S6K and PKB

activity in DCs was previously shown to result in increased IL-12 production and no changes in TNF-α and IL-6 production, which contradicts our results with SB 747651A (47, 48). Third, our data are consistent with previous studies on MSK1-inhibition: exposure of mouse bone marrow-derived DCs to an MSK1 inhibitor (32) as well as silencing of MSK1 in human keratinocytes lead to reduction of pro-inflammatory cytokine production (49). Finally, the effects of SB 747651A on murine macrophage cytokine production closely resembled the phenotype observed in macrophages of MSK1/2 knockout mice (32). Taken together, our data suggest that MSK1 is an important player in cytokine production by cDC2s and its inhibition limits the pro-inflammatory phenotype of these cells.

Thus, using two independent cohorts of patients and controls we provide the first evidence of molecular dysregulation of cDC2s in pSS, including the decreased expression of miR-708 and miR-130a that is associated with TLR-activation. In addition, we show that miR-130a regulates the expression of MSK1, which is a pharmacologically targetable signaling protein that is overexpressed in pSS cDC2s and associated with enhanced production of pro-inflammatory cytokines. In view of its regulation by miR-130a and its central role in NF-κB signaling, MSK1 inhibition to impede pro-inflammatory cytokine production represents a novel therapeutic avenue for treatment of pSS.

Data Availability

All relevant data are contained within the manuscript, miRNA profiling data have been deposited in NCBI's Gene Expression Omnibus (GSE) and are accessible through GEO Series accession number GSE132842. All the raw data of this manuscript are available by the authors, without undue reservation, to any qualified researcher.

Ethics Statement

This study was carried out in accordance with the recommendations of the board of medical ethics committee of the University Medical Center Utrecht with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the board of medical ethics committee of the University Medical Center Utrecht (METC no. 13-697).

Author Contributions

AL, JvR, SB, EC, AK, MR, TR, and MH were involved in study conception and design. AL, SB, MW, EC, SH, KvdW-J, BB and MH were involved in acquisition of

data. Analysis and interpretation of data was performed by AL, MW, AK, MR, JvR, TR and MH. All authors were involved in drafting the article or revising it critically, and all authors approved the final version of the manuscript to be published.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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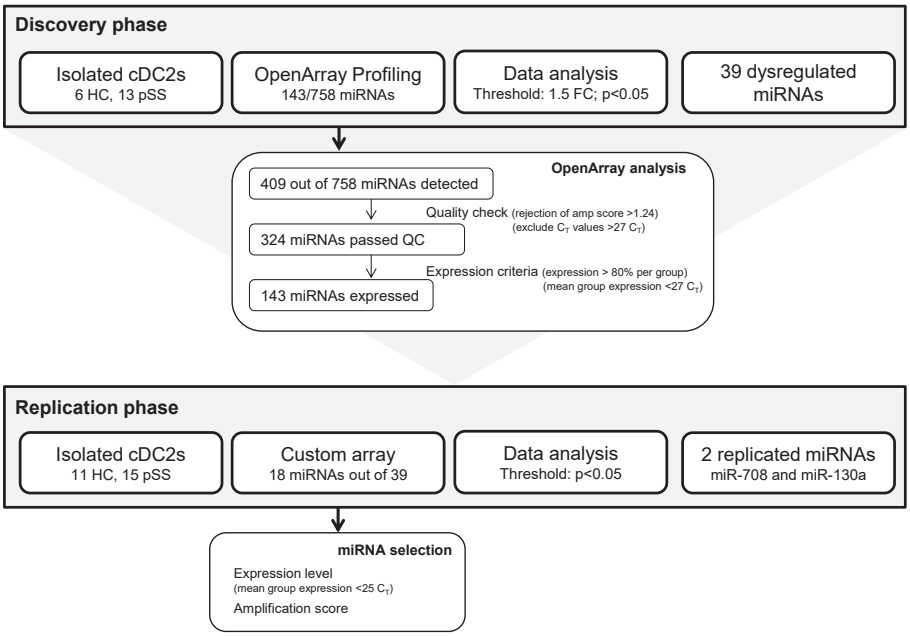
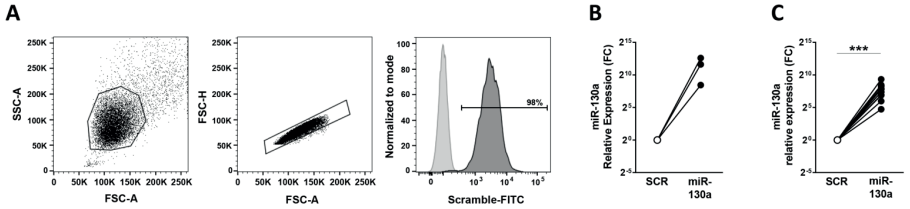
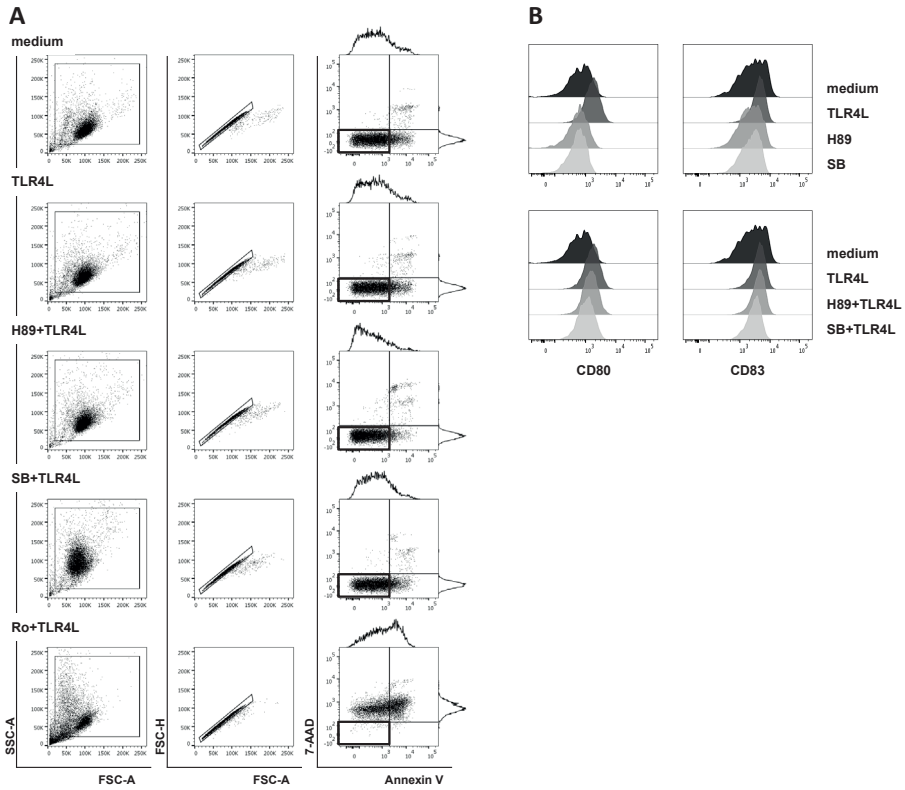
Supplementary material



Supplementary Figure 1. Workflow of the miRNA analysis in HC and pSS patients using two independent cohorts. In the replication phase, miRNAs were considered to be replicated when they showed a difference in the same direction (up/down regulated) as in the discovery phase at a p-value <0.05.



Supplementary Figure 2. miR-130a is efficiently transfected into HEK-293T cells and cDC2s. Transfection efficiency was measured by FACS in HEK-293T cells**.** The percentage of FITC-positive cells of one representative experiment out of three is depicted (**A**). miR-130a overexpression was assessed by TaqMan miRNA assays after transfection with miR-130a or non-targeting miRNA control (SCR) both in HEK-293T cells (**B**) and in cDC2s (**C**). *** indicates p≤0.001



Supplementary Figure 3. MSK1 inhibition with SB 747651A does not affect cDC2 viability and regulates the expression of co-stimulatory molecules. Gating strategy for analysis of cell viability in cDC2s isolated from buffy coats that were left untreated or were treated either with H89, SB 747651A and Ro 31-8220 for 1h prior to TLR4L stimulation (**A**). Representative histograms show flow cytometry staining of co-stimulatory molecules in viable (Annexin V and 7-AAD double negative) cDC2s for the different studied conditions (**B**).

Supplementary Table 1. miRNAs differentially expressed in both discovery and replication phase.

		Discovery phase		Replication phase	
	miR	Fold change pSS vs HC	p-value	Fold change pSS vs HC	p-value
Downregulated	**miR-708**	**0.190**	**0.001**	**0.428**	**0.032**
	miR-142-3p	**0.243**	**0.028**		
	miR-30e-3p	**0.377**	**0.009**	1.012	>0.999
	miR-378	**0.390**	**0.005**		
	miR-213	**0.398**	**>0.001**	4.666	0.601
	miR-30d	**0.403**	**0.031**	0.985	0.919
	miR-126-5p	**0.433**	**0.001**	1.611	0.879
	miR-29b	**0.434**	**0.018**	1.364	0.919
	miR-223-5p	**0.451**	**0.005**		
	miR-340	**0.464**	**0.009**		
	miR-21	**0.483**	**0.031**	1.105	0.799
	let-7g	**0.487**	**0.006**	0.907	0.357
	miR-25	**0.495**	**0.006**	0.896	0.377
	miR-223	**0.495**	**0.014**	0.946	0.646
	miR-26b	**0.506**	**0.009**		
	miR-140	**0.514**	**0.047**		
	miR-345	**0.520**	**0.007**		
	miR-150	**0.529**	**0.011**	0.731	0.799
	miR-29a	**0.529**	**0.007**	0.751	0.232
	miR-26a	**0.538**	**0.011**		
	miR-106b	**0.538**	**0.029**		
	miR-590-5p	**0.539**	**0.031**		
	miR-146a	**0.541**	**0.033**	1.028	0.959
	miR-532	**0.542**	**0.020**	0.953	0.683
	miR-16	**0.546**	**0.012**		
	miR-221	**0.547**	**0.011**		
	miR-374-5p	**0.561**	**0.027**		
	miR-103	**0.587**	**0.035**		
	let-7f	**0.590**	**0.019**	0.768	0.330
	miR-130a	**0.597**	**0.041**	**0.680**	**0.047**
	miR-629	**0.610**	**0.040**		
	miR-335	**0.616**	**0.012**		
	let-7e	**0.622**	**0.015**		
	let-7d	**0.625**	**0.032**	0.917	0.721
	miR-15b	**0.642**	**0.028**		
	miR-9	**0.647**	**0.041**	1.924	0.879
Upregulated	**miR-886-5p**	**2.377**	**>0.001**		
	miR-1248	**1.719**	**0.041**		
	miR-1274B	**1.995**	**0.003**		

Results are expressed as mean FC (p-value). Differences between groups that met the threshold (for discovery: FC difference of 1.5 at p-value of $p<0.05$; for replication: FC difference in same direction as seen in discovery at $p<0.05$) are indicated in bold. Thermofisher Cloud software was used to analyse the data from the discovery phase, while Mann-Whitney U test was used to compare the groups in the replication phase.

Supplementary Table 2. List of the miRNAs targets selected by the prediction algorithms and identified in the proteomic analysis.

Protein Name		Log2 (Ratio Reverse)	Log2 (Ratio Forward)	Unique/Total Peptides
miR-708				
IMPDH1	Inosine Monophosphate Dehydrogenase 1	0.568324	-0.659016	15/17
P4HA1	Prolyl 4-Hydroxylase Subunit Alpha 1	0.504671	-0.458287	5/5
miR-130a				
NPTN	Neuroplastin	0.550507	-0.592636	4/4
STX6	Syntaxin-6	0.512075	-0.613035	6/6
ARL6IP1	ADP-ribosylation factor-like protein 6-interacting protein 1	0.500496	-0.360657	5/5
DICER1	Endoribonuclease Dicer	0.475811	-0.731733	18/18
ACSL4	Long-chain-fatty-acid-CoA ligase 4	0.409690	-0.447171	14/17
MSK1	Mitogen- and stress-activated protein kinase-1	0.390998	-0.369334	12/13
RAB5A	Ras-related protein Rab-5A	0.389347	-0.384979	4/8

IMPDH1: Inosine monophosphate dehydrogenase 1; P4HA1: Prolyl 4-hydroxylase subunit α 1; NPTN: Neuroplastin; STX6: Syntaxin-6; ARL6IP1: ADP-ribosylation factor-like protein 6-interacting protein 1; DICER1: Endoribonuclease dicer; ACSL4: Long-chain-fatty-acid-CoA ligase 4; MSK1: Mitogen- and stress-activated protein kinase-1; RAB5A: Ras-related protein Rab-5A.

Supplementary Table 3. Sequences of primers used for RT-qPCR.

	Name	Sequence 5'- 3'
MSK1 FW	Mitogen- and stress-activated protein kinase-1	CAACAATCGTTCAAAAGGCCAA
MSK1 RV		CGACTGCCTAATGTGTTCCAG
IL-10 FW	Interleukin 10	GAGGCTACGGCGCTGTCAT
IL-10 RV		CCACGGCCTTGCTCTTGTT
TNF-α FW	Tumor necrosis factor alpha	GGAGAAGGGTGACCGACTCA
TNF-α RV		CTGCCCAGACTCGGCAA
IL-6 FW	Interleukin 6	TGCAATAACCACCCCTGACC
IL-6 RV		TGCGCAGAATGAGATGAGTTG
IL-12p35 FW	Interleukin 12 subunit p35	CTCCAGAAGGCCAGACAAAC
IL-12p35 RV		AATGGTAAACAGGCCTCCACT
IL-12p40 FW	Interleukin 12 subunit p40	TGCCGTTCACAAGCTCAAGT
IL-12p40 RV		TGGGTCAGGTTTGATGATGTCC
IL-8 FW	Interleukin 8	TGAGAGTGGACCACACTGCG
IL-8 RV		TCTCCACAACCCTCTGCACC
RPL32 FW	Ribosomal protein L32	AGGGTTCGTAGAAGATTCAAGG
RPL32 RV		GGAAACATTGTGAGCGATCTC
ACTB FW	Actin Beta	CATCGAGCACGGCATCGTCA
ACTB RV		TAGCACAGCCTGGATAGCAAC
B2M FW	Beta-2-microglobulin	GATGAGTATGCCTGCCGTGT
B2M RV		TGCGGCATCTTCAAACCTCC

Supplementary Methods

Stable isotope labelling of amino acids in cell culture (SILAC)

For SILAC labelling, HEK-293T cells were cultured in SILAC-labelled Dulbeccos Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) with 10% dialyzed fetal calf serum (FCS) and 1% penicillin/streptomycin (Thermo Fisher Scientific) containing L-arginine and L-lysine (light medium) or ^{13}C6-L-arginine and ^{13}C6-L-lysine (heavy medium) for at least 14 days to eliminate non-labeled arginine and lysine. The day before the transfection, 1.5x10^5 cells were seeded in a 6-well plate in a final volume of 3mL. The next day, the medium was changed and the 60-70% confluent cells were transfected with a specific miRNA mimic

either for miR-708 and miR-130a or a non-targeting miRNA control (SCR) (Thermo Fisher Scientific) at a final concentration of 30nM together with lipofectamine RNAiMAX and Opti-MEM (Thermo Fisher Scientific). 48h post-transfection cells were harvested by adding lysis buffer containing 8M urea, 1M ammonium bicarbonate, 10nM tris (2-carboxyethyl) phosphine, and 40nM chloroacetamide. Cell lysates were incubated at 95°C for 5 min, sonicated and diluted to 2M urea with 1M ABC. After protein quantification using the BCA protein assay, cell lysates from miR-130a or miR-708-transfected cells generated from heavy medium and SCR-transfected cells from light medium were mixed 1:1 (reverse mode) and vice versa (forward mode). Proteins were digested overnight with 2% (w/w) trypsin, peptides were fractionated based on their molecular mass using ultra performance liquid chromatography (UltiMate-3000 system, Thermo Fisher Scientific), and finally desalted and acidified on a C-18 cartridge (3M). C18-stagetips were activated with methanol, washed with buffer containing 0.5% formic acid in 80% ACN (buffer B) and then with 0.5% formic acid (buffer A). After loading of the digested sample, stage-tips were washed with buffer A and peptides were eluted with buffer B, dried in a SpeedVac, and dissolved in buffer A. Peptides were electro-sprayed directly into an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific) and analysed in Top Speed data-dependent mode. Raw files were analysed using Maxquant software. For identification, the human Uniprot was used with both the peptide and the protein false discovery rate set to 1%. Proteins identified were filtered for reverse and decoy hits, standard contaminants and selected to have more than 1 unique or razor peptide by using the Perseus software 1.5.1.6. Heavy/light normalized ratios (ratio reverse and ratio forward) were used to quantify protein expression and were further processed for comparative analysis of differential expression among the conditions.

Selection of in silico predicted miRNA targets

In silico predicted and experimentally validated targets of the identified miRNAs were retrieved using the miRWalk2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) to search in the miRANDA, PICTAR2, PITA, RNA22, miRDB and Targetscan databases. The presence of the miRNA seed-sequence in the target sequence was required. Targets were selected if present in 4 out of the 6 selected databases.

Quantitative real-time PCR

First-strand cDNA was synthesized from total RNA using Superscript IV kit (Thermo Fisher Scientific), and quantitative real-time PCR was performed on the QuantStudio

12k flex System (Life-Technologies), following manufacturer's instructions. Sequences of the primers used are listed in **Supplementary Table 3**. RT-qPCR data were normalized to the expression of the selected housekeeping gene and analysed using the comparative CT method as described before.

Cytokine analysis

Cytokines in cell-free supernatant were measured using enzyme-linked immunosorbent assay for IL-6 (#M9316, Sanquin), TNF-α (#851.570.020, Diaclone), IL-10 (#88-7106-88) and IL-12p70 (#88-7126) (both Thermo Fisher Scientific), following the manufacturer's instructions.

Chapter 4

Long non-coding RNA *HCP5* is a key regulator of cDC2s function: implications for Sjögren's Syndrome

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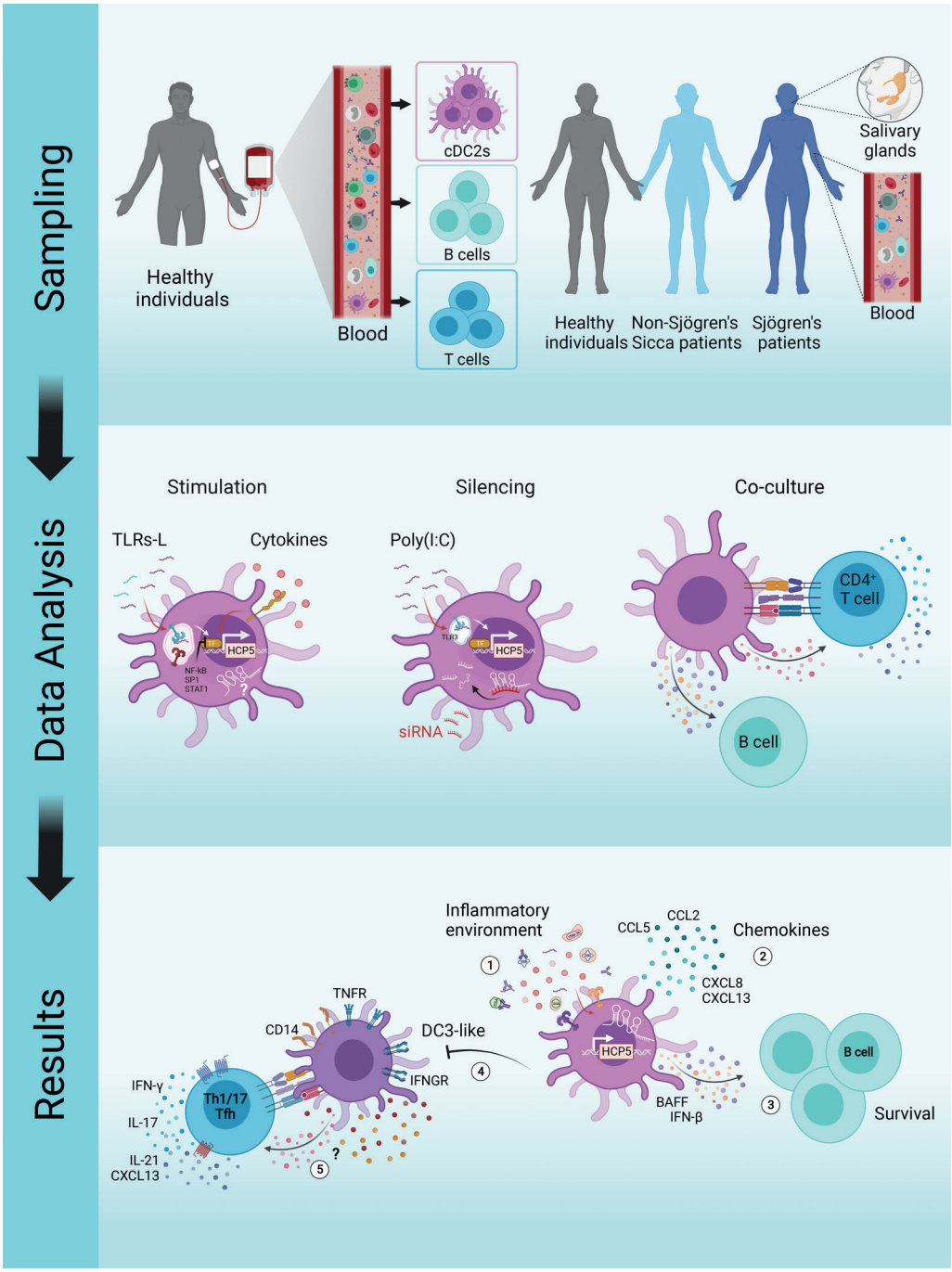
In preparation

Long non-coding RNAs are gene expression regulators that control immune cell functions during activation. We explored the role of *HCP5* in crucial functions of type 2 conventional dendritic cells (cDC2s) and identified that immune activation modulates *HCP5* expression and that SP1, NF-kB and STAT1 transcription factors are regulators of its expression. *HCP5* positively regulated interferon (IFN)-β, IFN–inducible genes and chemokine production. Moreover, we showed the impact of *HCP5* in driving B and T cell activation and found reduced B cell survival in line with reduced *TNFSF13B* expression and enhanced Tfh, Th1/17 cytokine production associated with modulation of PD-L1 expression and CD14+DC3 skewing. Lastly, *HCP5* expression was increased in inflamed tissues and circulating cDC2s of patients with primary Sjögren's syndrome, a prototypic type-I IFN disease. Our findings suggest that *HCP5* is an important mediator of cDC2s function and inflammation, and its targeting may yield novel avenues to halt immune activation.

Graphical abstract



Introduction

Type 2 conventional dendritic cells (cDC2s) are specialized antigen presenting cells and are required for the initiation of strong adaptive immune responses (1). cDC2s respond to a broad spectrum of microbial products (2) and upon activation, mature to instruct differentiation of different subsets of effector T cells, including Th1, Th2, and Th17 cells (3). cDC2s can also present antigens to B cells (4) and shape the microenvironment through the production of proinflammatory cytokines and/or chemokines to interact with other immune and nonimmune cells. This capacity represents a key feature of cDC2s and implicates them as potent and context-specific regulators of the innate and adaptive immune response (5). During activation, cDC2s rapidly alter their transcriptional profile through a dynamic reprogramming mechanism that relies on epigenetic changes to initiate precise immune responses (6).

Epigenetic mechanisms enable gene expression regulation by altering cellular function through changes in DNA accessibility and/or translation without changing the DNA sequence (7). Long non-coding RNAs (lncRNA) are important epigenetic regulators by regulating gene expression at multiple levels, including transcriptional and post-transcriptional regulation (8). As small RNA sponges and molecular scaffolds, lncRNAs can affect several biological processes, including cell proliferation, differentiation and apoptosis. In particular, they act as key regulators of innate immune responses and inflammation by affecting immune cell differentiation, activation, and repression of immune responses (9, 10).

Histocompatibility leukocyte antigen complex P5 (*HCP5*) is a lncRNA located in the Major Histocompatibility Complex (MHC) I region between the *MICA* and *MICB* genes (11, 12). At least half of the molecules encoded by this highly polymorphic locus are involved in antigen processing and presentation, regulation of inflammation and in innate and adaptive immune responses, highlighting the importance of the MHC region in immune-mediated autoimmune and infectious diseases (13). *HCP5* is aberrantly expressed in a variety of cancer cells and its dysregulation is closely related to cell proliferation, migration, invasion, apoptosis and metastasis (14, 15). Importantly, *HCP5* is mainly expressed in immune-related cells, suggesting a potential role in the regulation of certain immune functions (11). Several *HCP5* polymorphisms are associated with autoimmune diseases, including psoriatic arthritis, graves' disease and systemic lupus erythematosus (SLE) (16-19). In SLE patients *HCP5* is hypomethylated and associated with autoantibody

production (20). Interestingly, a single-nucleotide variant (SNV; rs3099839) in the *HCP5* was recently identified as susceptibility locus for primary Sjögren's Syndrome (pSS). The *HCP5* risk variant association with pSS was confirmed in an independent cohort of pSS patients, suggesting a potential role of *HCP5* in pSS (21). pSS is a chronic, systemic autoimmune disease characterized by immune cell infiltration of exocrine glands (22) and extraglandular manifestations associated with B cell hyperactivity and the presence of a type-I interferon (IFN)-signature (23). The immune mechanisms behind pSS are still not fully understood, yet a crosstalk between salivary epithelial cells, the innate and adaptive immunity has been implicated (24, 25).

Despite the association of *HCP5* with the innate and adaptive immune response, its expression, biological function, and regulatory mechanisms in immune cells are still unknown. In view of the central role of cDC2s as drivers of immune activation and their implication in the pathogenesis of type-I IFN-driven autoimmune diseases, such as pSS, we here studied the regulation of cDC2s function by *HCP5*. For this purpose, we investigated the modulation of *HCP5* expression and its contribution to cDC2s function. We assessed regulation of type-I IFN responses, cytokine/chemokine production, costimulatory molecule expression and B and $CD4^+$ T cell activation. Moreover, we investigated local and systemic *HCP5* expression in pSS, a prototypical type-I IFN-driven systemic autoimmune disease.

Material and Methods

cDC2s isolation and stimulation

cDC2s were isolated from buffy coats (Sanquin) by magnetic-activated cell sorting using $CD1c\ (BDCA\text{-}1)^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Consistent purity of isolated cDC2s was measured by flow cytometry, and the purity of the isolated samples was 98% [97-98] (median [range]).

cDC2s were cultured at 0.5×10^6 cells/mL in a 96-well round-bottom culture plate in complete medium [RPMI-glutamax (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Thermo Fisher Scientific)]. Cells were left unstimulated or were stimulated with different toll-like receptor (TLR) ligands and cytokines (**Supplementary Table 1**) for the indicated time points.

In addition, cDC2s were treated either with transcription factor inhibitor BAY 11-7082 [NF-κB (Invivogen)]; M6891 [SP1 (Merck-Sigma)]; Fludarabine [STAT1 (MedChemExpress)] or DMSO for 1h prior stimulation for 20h with TLR3 ligand (TLR3L; poly(I:C)) at a final concentration of 25 µg/mL and lysed for RNA analysis.

HCP5 silencing in cDC2s

cDC2s were transfected by nucleofection using the Amaxa™ Nucleofector™ II system (Lonza Basel, Switzerland), program Y-001 in combination with the Amaxa® Human Monocyte Nucleofector® Kit (Lonza), according to the manufacturer's protocol. cDC2s were transfected with 2 µM of either *HCP5* antisense LNA GapmeR (siHCP5; Sequence 5'-3': AGTAGGAGAGTCACAG) or antisense LNA GapmeR Control (siNC; Sequence 5'-3': AACACGTCTATACGC) (both from Qiagen). After transfection, cells were rested for 24h in 1:1 medium [RPMI-glutamax:IMDM (Lonza)] supplemented with 10% FCS and 0.5% penicillin/streptomycin and then washed, counted and cultured for 20h at 0.5×10^6 cells/mL in a 96-well round-bottom culture plate with TLR3L (25 µg/mL) for RNA analysis.

RNA isolation and quantitative PCR

Cells were lysed in RLTPlus buffer (Qiagen) and total RNA was purified using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was assessed with Qubit RNA Kit (Thermo Fisher Scientific). First-strand cDNA was synthesized using superscript IV kit (Thermo Fisher Scientific), and quantitative real-time PCR was performed on the QuantStudio 12k flex System (Life Technologies), following manufacturer's instructions. Sequences of the primers used are listed in **Supplementary Table 2**. The RT² profiler PCR array for human innate & adaptive immune responses (ID: PAHS-052 Qiagen) was performed according to manufacturer's instructions on the QuantStudio 12k flex System (Life Technologies). RT-qPCR data were normalized to the expression of the selected housekeeping genes and analyzed using the comparative CT method. The relative fold change (FC) of each sample was calculated in relation to the ΔCt of the unstimulated or siNC sample (reference) according to the formula $FC = 2^{-\Delta\Delta Ct}$.

cDC2s co-cultures with B cells or $CD4^+$ T cells

B cells and $CD4^+$ T cells were isolated from buffy coats (Sanquin) by magnetic-activated cell sorting using CD19 MicroBeads (CD1c (BDCA-1)$^+$ Dendritic Cell kit) or $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity (median [interquartile range]) of the isolated B cells (99%

[98–99%]) and CD4+ T cells (98% [97–99%]) was consistent between samples. siHCP5 or siNC transfected cDC2s were co-cultured in the presence of TLR3L (25 µg/mL) with autologous B cells plus sCD40L (200 ng/mL) and IL-21 (100 ng/mL) at a 1:2 ratio (cDC2s: B cells) or with autologous CD4+ T cells plus IL-2 (10 ng/mL) at a 1:5 ratio (cDC2s: T cells) in complete medium for 6 days. To assess the proliferation of B cells and CD4+ T cells, cells were labelled with 1.5 µM of CellTrace Violet dye (CTV) (Invitrogen), prior to co-culture.

Flow Cytometry

Cell suspensions were incubated with a fixable viability dye (eBioscience) to allow exclusion of dead cells and blocked with Fc receptor blocking reagent (Miltenyi Biotech) and then stained for 20 min at 4°C with fluorochome-conjugated monoclonal antibodies according to the panels described in **Supplementary Table 3**.

To measure the production of intracellular cytokines by CD4+ T cells after 6 days of co-culture with transfected cDC2s, cells were restimulated for 4h with phorbol myristate acetate (PMA; 50 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of brefeldin A (10 µg/mL; Sigma-Aldrich). After incubation with fixable viability dye (eBioscience) and Fc receptor blocking reagent (Miltenyi Biotech) cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (eBioscience), and stained according to the panel in **Supplementary Table 3** following the manufacturer's instructions.

Samples were acquired on a BD LSR Fortessa (BD Biosciences), or on a BD FACSCanto (BD Biosciences) using the BD FACSDiva software (BD Biosciences). FlowJo software (Tree Star) was used for data analyses. The percentage of proliferating B cells or CD4+ T cells was measured as the proportion of CTV negative cells.

Cytokine analysis

Levels of IFN-β, CCL2, CCL5, CXCL13, APRIL, IL-1α, IL-1β, IL-18, IL-12p70, IL-23 were measured using Luminex technology, as previously described (26).

Long-non coding RNAs expression from publicly available datasets

HCP5 expression on different cellular models following viral infections was retrieved from GEO Signatures of Differentially Expressed Genes for Viral Infections (https://maayanlab.cloud/Harmonizome) (27). *HCP5* expression on

minor salivary glands and parotid gland (PG) from a control group (CTR) with no subjective or objective evidence of oral or ocular dryness, non-Sjogren's Sicca (nSS) patients with subjective or objective evidence of oral or ocular dryness but not fulfilling the classification criteria for pSS, and pSS patients was retrieved from publicly available microarray datasets (GSE157159 (28) and GSE40611 (29), respectively). Data from differentially expressed long-non coding RNAs between HC and pSS was retrieved from different GEO datasets from our group (pDCs - GSE135635 (30); cDC2s - GSE200020; Monocytes - GSE173670 (31); B cells – GSE199868) available on the Gene Expression Omnibus (GEO–NCBI).

Patients and controls

All pSS patients fulfilled the 2016 ACR-EULAR classification criteria for pSS (32) and a group of age and gender-matched healthy controls (HC) was included as control group (**Supplementary Table 4**). The study was approved by the medical ethics committee of the University Medical Center Utrecht (METC no. 13-697). All patients gave their written informed consent in accordance with the declaration of Helsinki.

Statistical Analysis

Statistical analyses and data visualization were performed on Graphpad Prism (GraphPad Software). Heatmaps were generated using ClustVis software (http://biit.cs.ut.ee/clustvis/) (33) and clustered using Euclidean distance and Ward's linkage method. Paired t-test was used to analyze the differences between the siHCP5 and siNC transfected cDC2s. Differences between pSS patients and HC were assessed using unpaired t-test. Pearson correlation coefficient was used for correlation analyses. Differences were considered to be statistically significant at $p < 0.05$.

Results

LncRNA *HCP5* expression is modulated by inflammatory and viral mediators and regulates type-I IFN response

Recent high-throughput data show that immune activation alters the expression of hundreds of lncRNAs (34-36), suggesting that inflammatory stimuli regulates lncRNAs. As such, we explored the effect of TLR- and cytokine-mediated activation of cDC2s on the kinetics of *HCP5* expression. cDC2s stimulation with TLR3L and TLR7/8L upregulated *HCP5* expression in a time- and expression- dependent manner (**Figure 1A**). Moreover, IFN-α driven *HCP5* expression was marked by

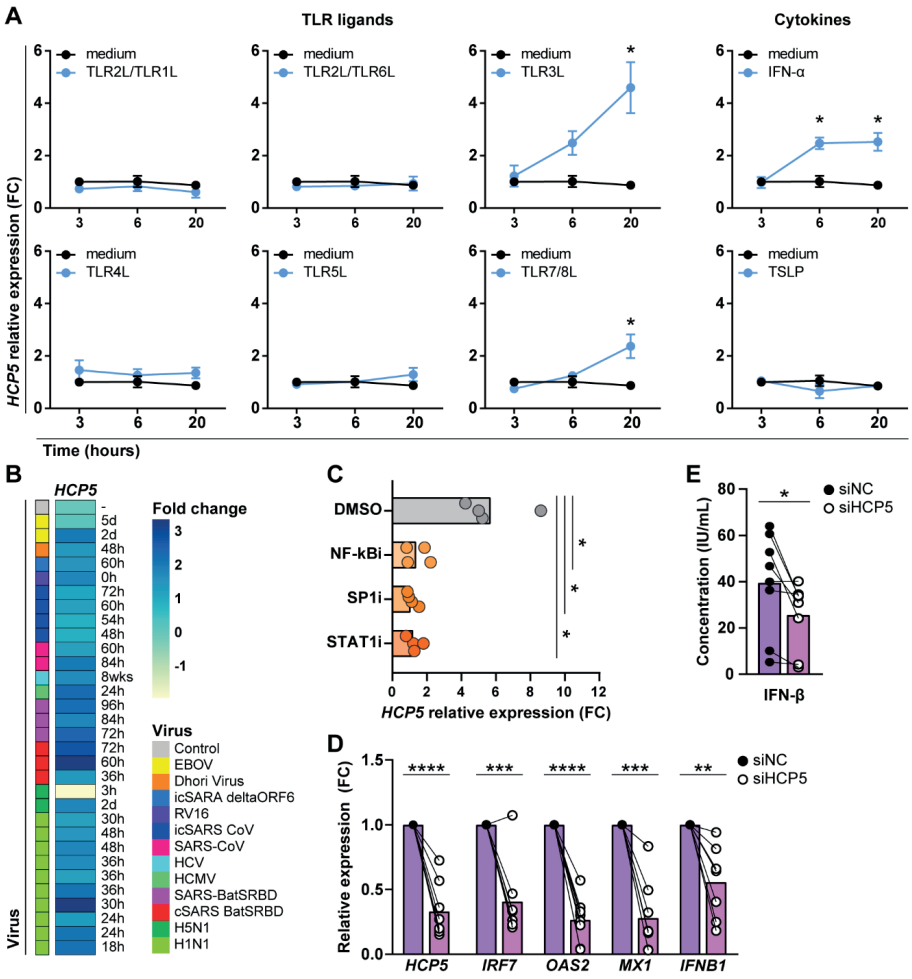


Figure 1. LncRNA *HCP5* expression is modulated by inflammatory mediators and controls type-I IFN response. (**A**) Kinetic of the *HCP5* expression in cDC2s stimulated with different TLR agonists and cytokines was assessed by qPCR at different time points. (**B**) *HCP5* expression in different cellular models upon viral infection [GEO Signatures of Differentially Expressed Genes for Viral Infections (https://maayanlab.cloud/Harmonizome)]. Colors depict the different viruses and *HCP5* expression in fold change. (**C**) *HCP5* expression assessed by qPCR in cDC2s pre-treated for 1 h with SP1 (M6891), NF-κB (BAY 11-7082) and STAT1 (Fludarabine) inhibitors or DMSO and stimulated with TLR3L (poly(I:C)) for 20h. (**D**) Isolated cDC2s were transfected either with non-targeting control (siNC) or *HCP5* siRNA (siHCP5) and rested for 24h. Transfected cDC2s were then stimulated with TLR3L (25μg/mL) for 20h. Expression of *HCP5* and type-I IFN inducible genes was measured by qPCR and (**E**) IFN-β production was measured by Luminex. Results are represented as mean with SEM or mean (bars); differences were considered statistically significant when $p < 0.05$ vs. medium or siNC (paired t-test). *, **, and *** represent nominal p-value < 0.05, $p < 0.01$, and $p < 0.001$, respectively.

an increased expression at an earlier time point (6h), and after, stable over time (**Figure 1A**). Given the observed modulation of *HCP5* expression by mediators involved in antiviral response, next we assessed the effect of viral infection on *HCP5* expression. To this end, we used publicly available data to screen *HCP5* expression in a wide range of viral infections in different cellular models (27). Independently of the time post-infection and viral classification, *HCP5* expression was overall upregulated upon viral infection (**Figure 1B**).

To gain further insights into the molecular mechanisms responsible for *HCP5* expression in cDC2s, we investigated its regulation by transcription factors. SP1 and NF-kB were previously described as transcriptional activators and were demonstrated to regulate its expression in osteosarcoma cells (37). In view of the observed *HCP5* upregulation by IFN-α and the presence of binding sites for STAT1 at the promoter region of *HCP5*, we also explored its role in the regulation of *HCP5*. Inhibition of SP1, NF-kB, and STAT1 reduced *HCP5* expression upon TLR3L activation (**Figure 1C**), suggesting that these transcription factors play a key role in regulating *HCP5* expression in cDC2s.

To better understand the link between viral/IFN- activation and *HCP5* expression, we silenced *HCP5* expression (siHCP5) in cDC2s and analyzed the expression of IFN-stimulated genes. *HCP5* silencing in cDC2s was consistent and on average led to a 67% reduction of *HCP5* expression (**Figure 1D**). No differences were found on cDC2s viability after transfection (**Supplementary Figure 1A-B**). Upon TLR3 stimulation, the expression of IFN regulatory factor (IRF)7, IFN-induced antiviral effectors (incl. *MX1* and *OAS2*), and *IFNB1* was reduced in siHCP5 cDC2s as compared to non-targeting control (siNC) (**Figure 1D**). In line, the production of IFN-β was also decreased in *HCP5*-silenced cDC2s after stimulation (**Figure 1E**). Thus, in cDC2s, *HCP5* expression is regulated by an IFN-dependent mechanism and its expression positively regulates IFN-signaling suggesting a positive feedback loop to auto sustain IFN production.

HCP5-silencing regulates cDC2s activation impacting B cell survival

To further explore the potential effect of *HCP5* in cDC2s function we silenced *HCP5* expression (siHCP5) in cDC2s and measured a panel of immune related genes involved in both innate and adaptive immune responses. Differential expression analysis revealed that 20 genes were consistently dysregulated in siHCP5-cDC2s compared with siNC-cDC2s, of which 2 genes (*CCL2*, *FN1*) were downregulated and 4 genes (*CD14*, *IL1R1*, *IFNGR1*, *NLRC4*) were upregulated

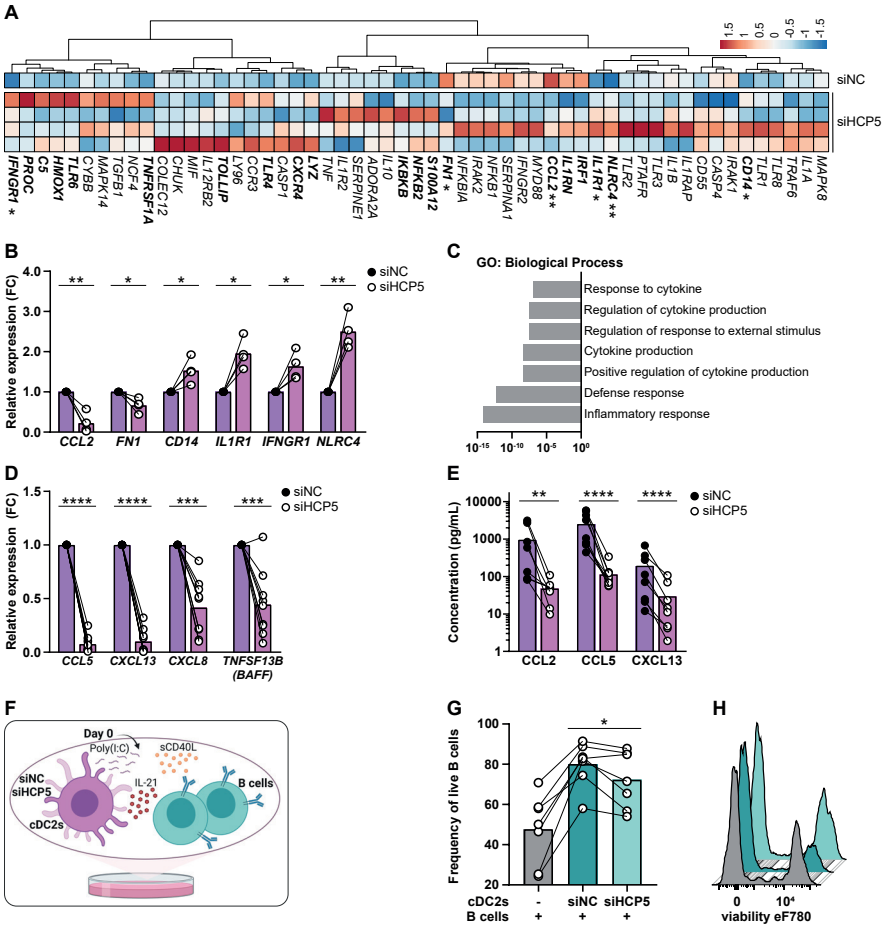


Figure 2. *HCP5* silencing in cDC2s regulates chemokines and BAFF expression affecting B cell survival. Isolated cDC2s were transfected either with non-targeting control (siNC) or *HCP5* siRNA (siHCP5), rested for 24h and then stimulated with TLR3L (Poly(I:C)) for 20h. (**A**) Heatmap visualization of the genes involved in innate & adaptive immune responses (columns) and the fold change (rows) measured by qPCR array. Colors depict gene expression fold change normalized to the respective siNC-cDC2s. Consistently dysregulated genes are depicted in bold. (**B**) Expression of the differential expressed genes (*p < 0.05) identified in A. (**C**) Biological Process Gene Ontology (GO) analysis of the consistently differentially expressed genes between siHCP5 vs. siNC. (**D**) Chemokines and B cell survival factor expression upon TLR3L stimulation of siNC- or siHCP5-transfected cDC2s measured by qPCR and (**E**) chemokines protein levels measured by luminex-based assay. (**F**) B cell survival after six days of co-culture with siNC or siHCP5 transfected cDC2s (cDC2s/B cell ratio of 1:2) in the presence of TLR3L (Poly(I:C)), sCD40L and IL-21. (**G**) Quantification and (**H**) representative histogram of viability staining on B cells after co-culture measured by flow cytometry. Results are represented as mean; differences were considered statistically significant when p < 0.05 vs. siNC (paired t-test). *, **, *** and **** represent nominal p-value < 0.05, p < 0.01, p < 0.001 and p < 0.0001, respectively.

with a nominal p-value ≤ 0.05 (**Figure 2A-B**). Gene Ontology (GO) analysis demonstrated that the 20 consistently dysregulated genes identified are enriched for inflammatory response, positive regulation of cytokine production, among others (**Figure 2C**).

Considering that CCL2 is a chemokine relevant for cell recruitment, which is a crucial event for efficient immune cell activation, and given the regulation of *CCL2* by *HCP5*, next we investigated the effect of *HCP5* on chemokine production, important for leukocyte recruitment to inflammatory sites (38). In addition to *CCL2*, the expression of *CCL5*, *CXCL13* and *CXCL8* was remarkable downregulated in siHCP5-cDC2s stimulated with TLR3L (**Figure 2D**). In line with this, we confirmed reduced CCL2, CCL5 and CXCL13 protein production in siHCP5-cDC2s upon TLR3 stimulation (**Figure 2E**). Thus, these results suggest that *HCP5* has a pivotal contribution to leukocyte recruitment to the inflammatory tissues by cDC2s.

Importantly, cDC2s can orchestrate adaptative responses by modulating B cell differentiation, activation, and survival through the production of soluble mediators (39). Given the reduced expression of *TNFSF13B* (B cell activating factor; BAFF), a key molecule for B cell survival (40), in siHCP5-cDC2s upon TLR3 stimulation (**Figure 2D**), we investigated the functional significance of *HCP5* silencing in cDC2s on B cell survival. To this end, B cells were cultured either alone, or with siHCP5- or siNC-transfected autologous cDC2s for 6 days (**Figure 2F and Supplementary Figure 2A**). B cell survival induced by cDC2s was reduced upon *HCP5* silencing, on average 10% when compared with siNC-cDC2s (**Figure 2G-H**). Whereas the frequency of proliferating B cells, plasmablasts or CD27 B cell subsets was not affected (**Supplementary Figure 2C-D**). Furthermore, we observed increased protein levels but not gene expression of a proliferation-inducing ligand (APRIL) (**Supplementary Figure 2E-F**). As such, *HCP5* expression in cDC2s can modulate B cell recruitment and survival thought the regulation of CXCL13 and BAFF production.

HCP5-silencing in cDC2s reshapes co-stimulatory molecule expression and affects $CD4^+$T cell activation

As cDC2s are central players in orchestrating $CD4^+$ T cell responses, we next investigated the effect of *HCP5* silencing on the expression of costimulatory molecules as they represent an important step in the regulation of T cell-cDC2s interactions. The frequency of cDC2s expressing CD80, CD86, CD40 and ICOSL, and the expression of CD86 and CD40 was significantly reduced in siHCP5-

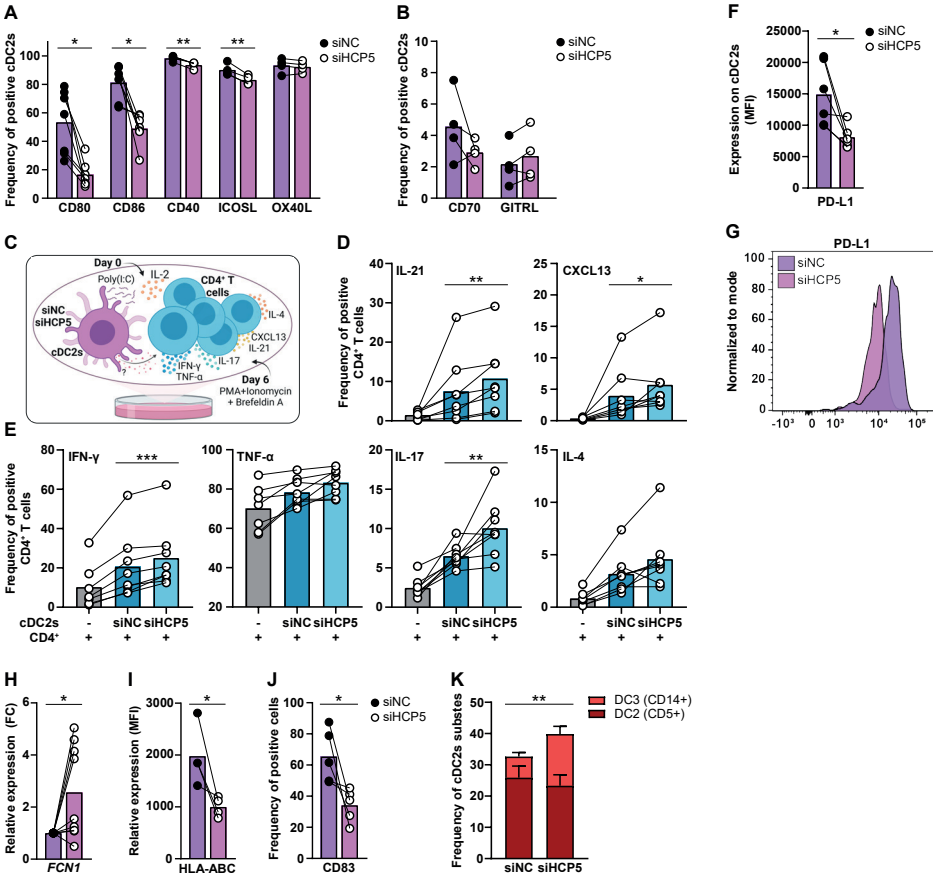


Figure 3. *HCP5* silencing increases Tfh, Th1 and Th17 cytokine secretion by modulation of cDC2s co-stimulatory profile and DC3 skewing. Non-targeting control (siNC) or HCP5 siRNA (siHCP5) transfected cDC2s were stimulated with TLR3L for 20h and (**A-B**) the surface expression (given by the percentage of positive cells) of costimulatory molecules was assessed by flow cytometry. (**C**) siNC or siHCP5 transfected cDC2s were co-cultured with $CD4^+$ T cells (cDC2s/$CD4^+$T cell ratio of 1:5) in the presence of IL-2 for six days and restimulated with PMA and ionomycin in the presence of brefeldin A. (**D-E**) The percentage of IL-21-, CXCL13-, IFN-γ-, TNF-α-, IL-17- and IL-4-producing $CD4^+$ T cells was measured by flow cytometry. (**F**) Quantification and (**G**) representative histogram of PD-L1 expression, represented as median fluorescence intensity (MFI), on transfected cDC2s stimulated with TLR3L was determined by flow cytometry. (**H**) *FCN1* gene expression was assessed by qPCR, (**I**) HLA-ABC expression represented as MFI and (**J**) CD83 expression, given by the percentage of positive cells was measured by flow cytometry on siNC or siHCP5 transfected cDC2s stimulated for 20h with TLR3L. (**K**) Frequency of DC2 and DC3 subsets in siNC or siHCP5 transfected cDC2s stimulated for 20h with TLR3L was determined by flow cytometry. Results are represented as mean (bars); differences were considered statistically significant when $p < 0.05$ vs. siNC (paired t-test). *, **, and *** represent nominal p-value < 0.05, $p < 0.01$, and $p < 0.001$, respectively.

cDC2s (**Figure 3A and Supplementary Figure 3A-B**). On the other hand, no differences were observed in the frequency of cDC2s expressing OX40L (**Figure 3A**). We did not observe differences in expression of CD70 and GITRL in siHCP5-cDC2s as compared to siNC-cDC2s (**Figure 3B and Supplementary Figure 3C**), though both proteins were low-expressed.

Considering that signals mediated by costimulatory molecules act to amplify or counteract the initial activating signals provided to T cells, we next assessed the capacity of cDC2s to activate autologous $CD4^+$ T cell (**Figure 3C**). The percentage of viable $CD4^+$ T cells was comparable in the absence or presence of cDC2s and independently of *HCP5* expression (**Supplementary Figure 3D-E**). In addition, no differences in $CD4^+$ T cell proliferation were found when *HCP5* expression was silenced in cDC2s (**Supplementary Figure 3F**). Next, we evaluated T cell-cytokine production linked to Tfh (IL-21, CXCL13), Th1 (IFN-γ, TNF-α), Th17 (IL-17), Th2 (IL-4), upon co-culture with siNC- or siHCP5-transfected cDC2s. Cytokine production by $CD4^+$ T cells was increased in the presence of cDC2s. However, when co-cultured with siHCP5-cDC2s, a greater percentage of IL-21-, CXCL13-, IFN-γ- and IL-17 producing $CD4^+$ T cells was observed as compared to siNC-cDC2s (**Figure 3D-E**). No differences were found in the production of TNF-α and IL-4 by $CD4^+$ T cells upon co-culture with siHCP5-cDC2s (**Figure 3D and Supplementary Figure 3D**). In line with increased $CD4^+$ T cell activation, we found a decreased expression of the immune inhibitory receptor PD-L1 on siHCP5-cDC2s when compared to siNC-cDC2s (**Figure 3F-G**). Thus, our results suggest that the induction of $CD4^+$ T cell cytokine production associated with effector T cell functions consists of co-stimulatory molecule dependent and independent mechanisms.

cDC2s are a heterogeneous population and recently two phenotypically distinct subsets, the DC2 and DC3 were described (41, 42). As *HCP5* silencing altered cDC2s phenotype and function, we next investigated whether the phenotype induced by *HCP5* silencing was identical to any of these cDC2 subtypes (41). siHCP5-cDC2s, showed a higher *CD14*, *IFNGR1* and *FCN1* expression (**Figure 2B and Figure 3H**) and lower *TNFSF13B* (BAFF), *CXCL8*, CD80 and CD86 (**Figure 2D and Figure 3A**), comparable to the DC3 subset (41). In addition, the expression of HLA-ABC and CD83 was also reduced in siHCP5-cDC2s (**Figure 3I-J**), in line with the previously described low *CD83*, *HLA-B* and *HLA*-C expression found in DC3 (41). We also confirmed an increased frequency of DC3 ($CD14^+$cDC2s) in *HCP5*-silenced cDC2s (**Figure 3K**). Thus, our data suggest that *HCP5* regulates $CD14^+$DC3-like skewing.

Reactivation of effector or memory T cells is less dependent of costimulatory signals, but mainly driven by the milieu of inflammatory cytokines present during T cell activation (43). As such, we next investigated secretion of cytokines by cDC2s associated with effector T cell activation upon *HCP5* silencing. The production of IL-1α, IL-1β and IL-18, as a read-out of NLRC4 inflammasome activation was relatively low after TLR3L stimulation and no differences were observed between the different transfection conditions (**Supplementary Figure 3G**). Moreover, the production of IL-12p70 and IL-23 by siHCP5-cDC2s was reduced as compared with siNC-cDC2s (**Supplementary Figure 3H**). As such, our results demonstrate that the regulation of cDC2s phenotype by *HCP5* expression is relevant to reshape $CD4^+$ T cell activation, reaffirming that *HCP5* regulation is of interest to determine the fate of adaptive immune responses.

HCP5 expression is increased in salivary glands and in circulating cDC2s of pSS patients

In light of *HCP5* role in cDC2s and that cDC2s are epigenetically altered in pSS, we examined the expression of *HCP5* in the inflamed exocrine glands of pSS patients. To this end, we retrieved publicly available transcriptional data of minor salivary glands (28) and parotid glands (29) from control patients (CTR), non-Sjögren's Sicca patients (nSS) and pSS patients. Bulk RNA-sequencing of minor salivary glands and parotid glands showed increased *HCP5* expression in both exocrine glands of pSS patients (**Figure 4A-B**), as compared to nSS patients and CTR. Moreover, *HCP5* expression correlated with immune cell infiltration (given by the expression of *PTPRC*, CD45 gene) and with the presence of cDC2s (characterized by *CD1C* expression which is restricted to cDC2s subset (**Supplementary Figure 4**) in both minor salivary glands (**Figure 4C-D**) and parotid glands of pSS patients (**Figure 4E-F**). Thus, the increased *HCP5* expression in the inflamed glands, associated to cDC2s, suggests that this lncRNA by regulating cDC2s function could contribute to maintain the inflammatory environment present in pSS patients.

Next, we sought to confirm whether *HCP5* expression was also dysregulated in the circulation of pSS patients. We used transcriptomic data from our group and found that in addition to cDC2s, *HCP5* expression was significantly increased in pDCs, B cells and monocytes of pSS patients compared to HC (**Figure 4G**). Strikingly, *HCP5* was the only lncRNA commonly dysregulated among the four studied circulating subsets and increased in pSS patients compared to HC (**Figure 4H**). Using an independent cohort of pSS patients and HC, we validated the increased *HCP5* expression in circulating cDC2s, pDCs and B cells of pSS

patients, but not monocytes (**Figure 4I**). In addition, we showed that B cells showed the highest *HCP5* expression followed by pDCs, cDC2s and monocytes (**Supplementary Figure 5**). Thus, considering the pivotal role of *HCP5* in cDC2s functions, its overexpression both in circulation and in the inflamed tissues suggests an active contribution in the regulation of inflammation in pSS patients.

Discussion

Here, we demonstrate that in cDC2s *HCP5* expression is increased upon stimulation and regulated by the transcription factors SP1, NF-kB and STAT1. In addition, we show that *HCP5* is critical for type-I IFN production and signaling, cell recruitment mediated by chemokines and reshaping of cDC2s phenotype and function to enhance B cell survival and decrease Tfh, Th1 and Th17 cytokine production. The regulation of T cell activation is associated with modulation of PD-L1 expression and phenotypic skewing towards DC3-like subset. Finally, we show that in pSS, a prototypic type-I IFN disease with B and T cell hyperactivity, *HCP5* is overexpressed in the salivary glands and in circulating cDC2s.

Given the regulation of IFN-inducible genes and IFN-β production by *HCP5* and its modulation by mediators involved in antiviral response, our results indicate that *HCP5* acts as a positive regulator of type-I IFN signaling. In fact, recent evidence shows that type-I IFN response is regulated at different levels by several lncRNAs (44, 45). The suppression of IFN-signaling in the absence of *HCP5* expression might be, at least in part, related to the regulation by SP1. SP1 activation induces an endogenous response that activates the OAS-RNase L axis, RIG-I pathway, the production of small self-RNAs (46, 47) and noncoding RNA genes including *HCP5* (48). As such, we hypothesize that *HCP5* expression regulated by SP1, together with other noncoding RNA genes, could lead to the activation of OAS-RNase L axis and the production of small self-RNAs that can serve as RIG-I ligands. Using loss-of-function experiments, we demonstrate that *HCP5* silencing reduced the levels of interferon-stimulated genes both at the mRNA and protein levels. Thus, our data suggest that induction of *HCP5*, e.g., upon viral infection, is an important mechanism that sustains type-I IFN responses.

Using a broad molecular analysis, we identified additional key functions for innate and adaptive immune responses regulated by *HCP5* in cDC2s, providing proof of concept to the previously described association of *HCP5* with immune

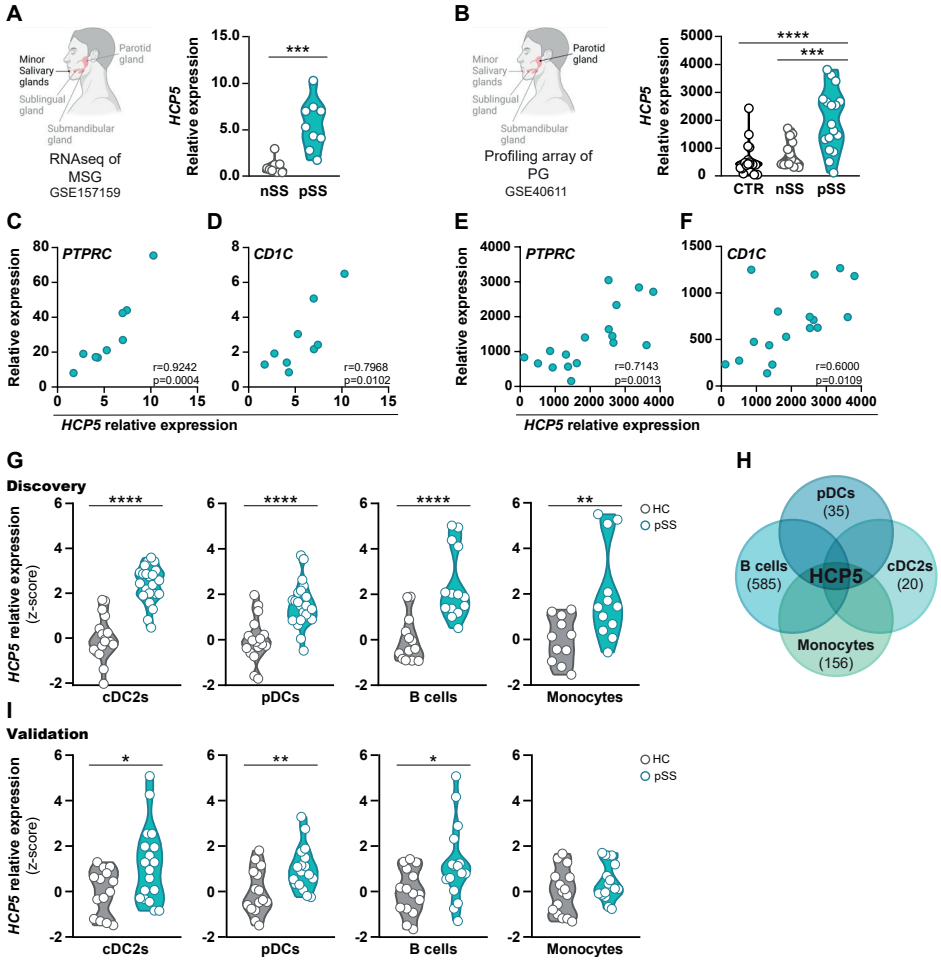


Figure 4. Increased *HCP5* expression in inflamed salivary gland tissue and in circulating cDC2s from pSS patients. *HCP5* expression in (**A**) minor salivary gland and (**B**) parotid gland of nSS, pSS patients and controls (CTR) was retrieved from publicly available datasets. The association of *HCP5* expression with *PTPRC* (CD45) and *CD1C* expression (**C-D**) in minor salivary gland and (**E-F**) parotid gland of pSS patients was assessed. (**G**) Violin plots depict *HCP5* expression from RNA sequencing data in cDC2s, pDCs, B cells and monocytes from HC and pSS patients retrieved from available datasets from our group. (**H**) Venn diagram shows the overlap of the differentially expressed lncRNAs with a nominal p-value < 0.05 between the studied cell subsets. (**I**) Violin plots depict *HCP5* expression in circulating cDC2s, pDCs, B cells and monocytes of an independent cohort of HC and pSS patients measured by qPCR. Differences were considered statistically significant when $p < 0.05$ (t-test and Pearson correlation coefficient, when applicable). *, **, *** and **** represent nominal p-value < 0.05, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

response (11). As CCL2, CCL5, CXCL13 and CXCL8 are essential for the recruitment of monocytes, DCs, T and B cells (49) and have a broad target cell selectivity, our results suggest that *HCP5* expression in cDC2s regulates the trafficking of immune cells in numerous inflammatory conditions including viral infections (50), autoimmunity (51) and cancer (52, 53). Although the signaling mechanisms by which *HCP5* regulates chemokine production in cDC2s need further confirmation, we hypothesize that the PI3K/AKT and ERK axes might be relevant, in view of the positive regulation of *HCP5* on AKT, PI3K, and ERK phosphorylation (54) and the role of these signaling pathways in chemokine production (55). Thus, positive regulation of chemokine production by *HCP5* in cDC2s supports the notion that *HCP5* plays a role in the recruitment of immune cells.

In addition to the essential role of DCs in T cell priming, they also play an important role in B cell function by promoting B cell chemotaxis and germinal center formation through CXCL13 production but also B cell survival and proliferation by secreting various stimulatory cytokines, including BAFF (56, 57). Given the regulation of *HCP5* in the production of these mediators by cDC2s, our findings reinforce the importance of these cells and more importantly *HCP5* expression to impact B cell recruitment and survival.

Expression of costimulatory and checkpoint molecules profile on DCs are important to control T cell fate and dictate T cell response (43). Interestingly, we found that *HCP5* modulation reduced CD80, CD86, CD40 and ICOSL expression but not T cell cytokine production associated with effector T cell functions. CD80 and CD86 reduction or blockade abrogates naive T cell activation, but not DC-T cell interaction or IFN-γ production, even when both CD80 and CD86 are blocked (58). Thus, suggesting that other molecules or soluble mediators can also significantly impact T cell activation. IL-12 is an important driver of IFN-γ production by Th1 (59), however its levels were decreased upon *HCP5* silencing in cDC2s. As autocrine IFN-β secretion is crucial for IL-12 production (60), we suggest that the reduction of IFN-β, upon stimulation, in *HCP5* silenced cDC2s could explain the lower IL-12 levels. Nonetheless, due to the increased *IFNGR1* expression on cDC2, in the presence of IFN-γ producing $CD4^+$ T cells, IL-12 production by cDC2s could be rescued to sustain a positive feedback loop and thus favor Th1 and Tfh expansion (59, 61). In addition, the increased Tfh-cytokine production could also be related with the unaltered OX40L expression, as its blocking reduces IL-21 and CXCL13 production by T cells (62) and OX40-deficient mice display reduced Tfh cells after infection (63).

PD-L1 expression in DCs has a critical role in limiting T cell responses and inhibition of T cell differentiation (64) and by reducing PD-L1 expression on cDC2s, *HCP5* expression can directly regulate cDC2-T cell interaction to license $CD4^+$ T cell activation. *HCP5* directly acts as a miR-150-5p sponge via a competing endogenous RNA mechanism to allow PD-L1 transcription and expression in cancer cell lines (65). In line with our hypothesis, PD-L1 silencing in DCs strongly increased IFN-γ production by stimulated T cells, with no effect on T cell proliferation (66) and induced the expansion of hyper activated pro-inflammatory $CD8^+$ T cells, with increased levels of IFN-γ and IL-17 (67). Another possible mechanism by which *HCP5* expression in cDC2s contributes to increased $CD4^+$ T cytokine production, in particular IL-17, might be related to the increased frequency of $CD14^+$DC3. Contact-dependent signals mediated by CD14 expression on DCs induce a Th17 profile, mainly dependent on IL-1β-priming of DCs, which upregulates CD14, and by itself upregulates the master regulator of Th17 cell differentiation (retinoic acid-related orphan receptor-γ thymus) and IL-17 production (68). Moreover, the $CD14^+$DC3 subset is transcriptionally programmed to favor Th17 polarization (42) and in psoriasis, a Th17 cell–related disease, $CD14^+$DC3 are increase in lesional skin and link to Th17 cell differentiation and activation (69). As such, *HCP5* by modulating the skewing towards a $CD14^+$DC3-like phenotype, can dictate the fate of T cell responses.

Given the modulation of *HCP5* expression by inflammatory mediators that are implicated in pSS (70, 71), we suggest that the increased *HCP5* expression in both inflamed tissues and circulation of pSS patients, might in part be, a consequence of the inflammatory environment. In addition, the positive regulation of *HCP5* in IFN-signaling and the increased *HCP5* expression in both cDC2s and pDCs from pSS patients might contribute to maintain the type-I IFN signaling observed in pSS. Moreover, *HCP5* strongly regulated chemokine production and therefore immune cell recruitment by cDC2s, which can explain the strong correlation of *HCP5* expression with immune infiltration observed in the inflamed tissues of pSS patients. In line with this, *HCP5* expression in the parotid glands of pSS patients was strongly associated with *CCL5* and *CXCL13* expression and therefore with immune infiltration (72). As such, the regulation by *HCP5* of mediators involved in IFN-signaling, immune cell trafficking, B cell survival and $CD4^+$ T cell fate, holds promise for *HCP5* as important player to link innate and adaptive immunity in pSS. Future research to determine the molecular mechanisms by which *HCP5* expression is upregulated and its direct functional role in pSS patients is needed and could reveal novel therapeutic options.

Taken together, we identified lncRNA *HCP5* as an important regulator of several key adaptive immune functions in cDC2s, affecting both arms of the immune response. Perturbation of this axis in pSS suggests that *HCP5* plays an important role in regulating inflammation and cell activation in this disease. Conceivably, these new insights may unveil novel therapeutic targets and hold promise for type-I IFN–associated autoimmune diseases and other immune-mediated diseases, where *HCP5* expression is dysregulated.

Author Contributions

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TR was a principal investigator in the immune catalyst program of GlaxoSmithKline, which was an independent research program. He did not receive any financial support other than the research funding for the current project. Currently, TR is an employee of Abbvie where he may hold stock. TR had no part in the design and interpretation of the study results after he started at Abbvie. The authors have no additional financial interests.

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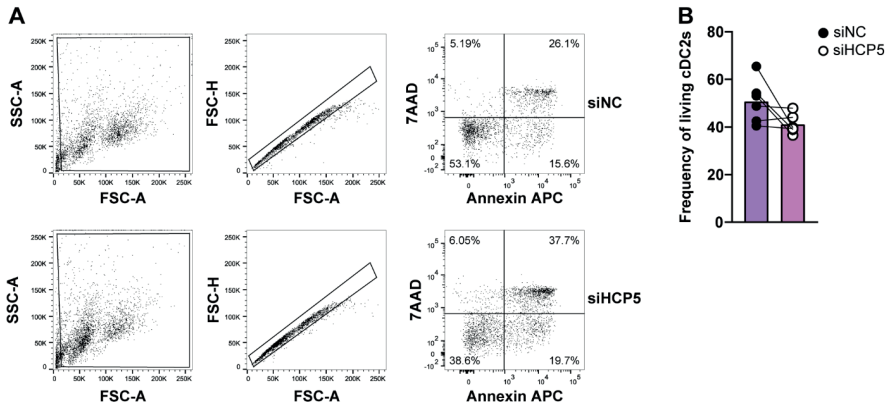
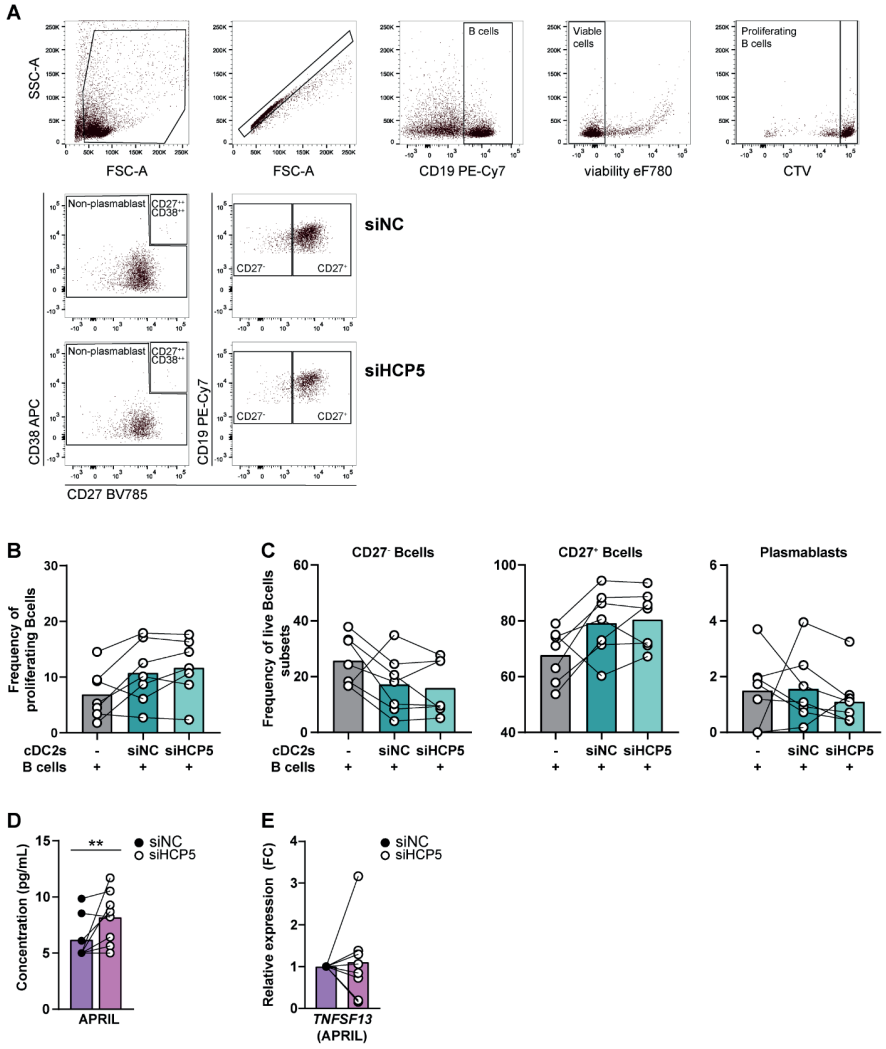
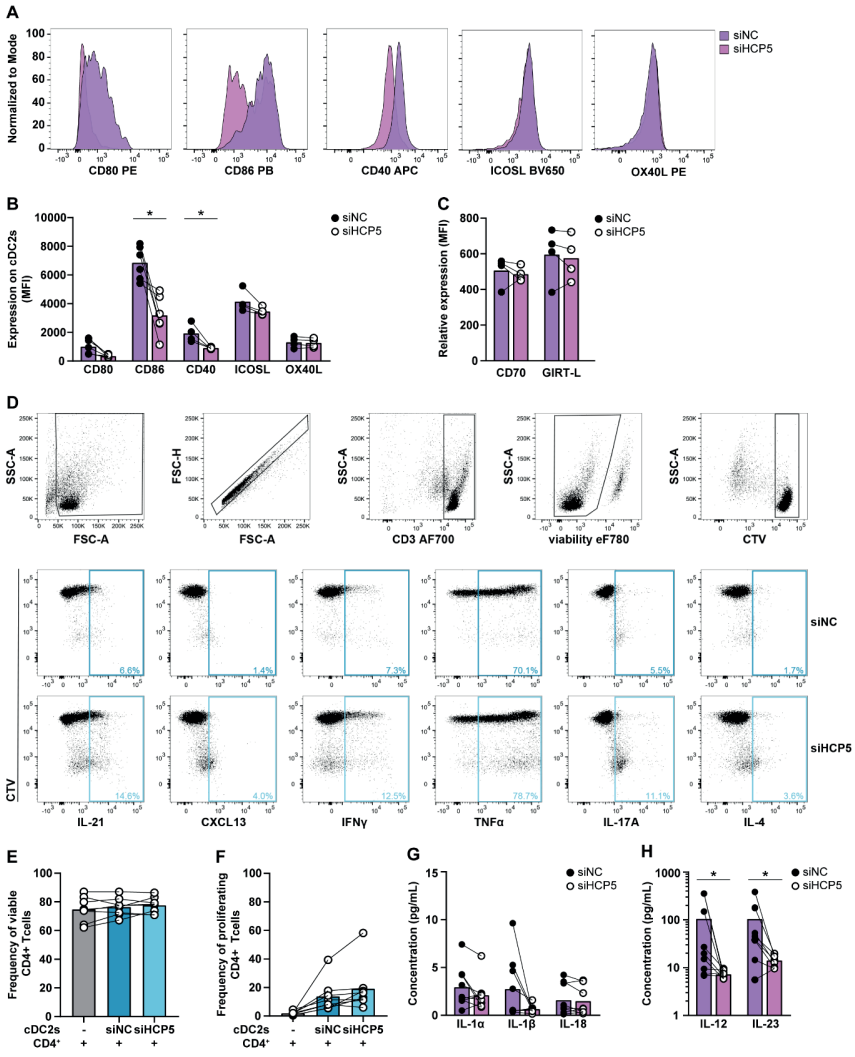
Supplementary material



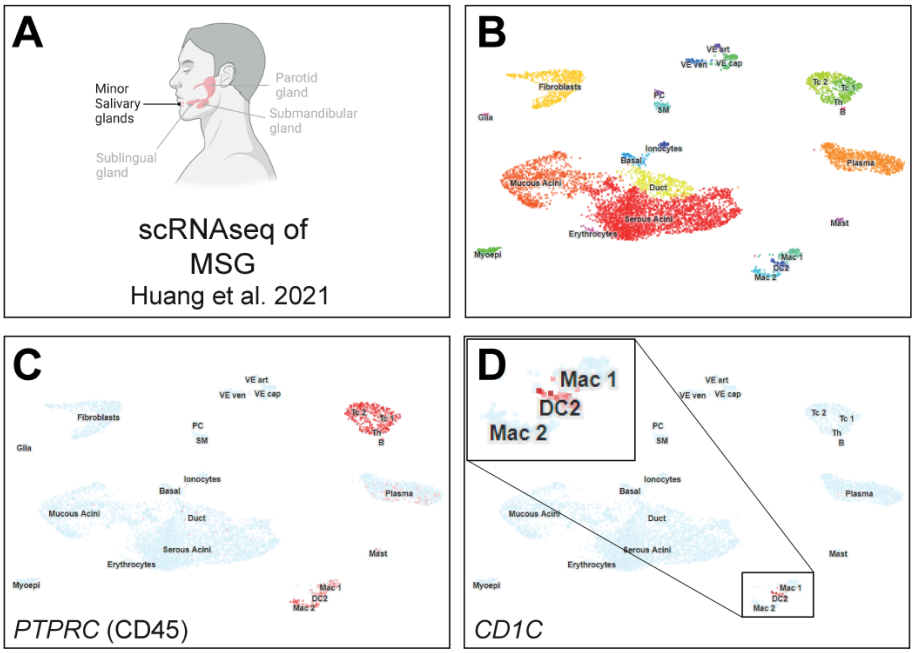
Supplementary Figure 1. *HCP5* transfection does not affect cDC2 viability. (A) Representative flow cytometry gating strategy analysis of cell viability in cDC2s isolated from buffy coats transfected either with *HCP5* antisense LNA GapmeR (siHCP5) or antisense LNA GapmeR Control (siNC) and stimulated with TLR3L for 20h. **(B)** Quantification of viable cells after 20h was assessed by flow cytometry. Results are represented as mean (bars).



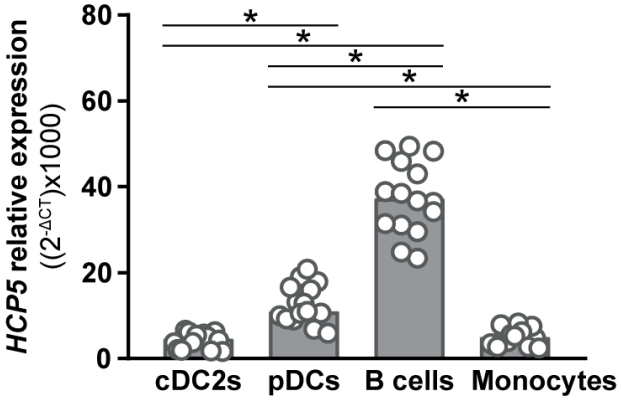
Supplementary Figure 2. B cell proliferation and differentiation is not affected by *HCP5* silencing in cDC2s. Isolated cDC2s from buffy coats were transfected either with *HCP5* antisense LNA GapmeR (siHCP5) or antisense LNA GapmeR Control (siNC) and co-cultured with B cells (cDC2s/B cell ratio of 1:2) for six days in the presence of TLR3L, sCD40L and IL-21. (**A**) Representative flow cytometry gating strategy analysis to identify viable and proliferating B cells as well as plasmablasts ($CD27^{++}CD38^{hi}$), naïve B cells ($CD27^{-}$) and memory B cells ($CD27^{+}$). Quantification of (**B**) proliferating B cells and (**C**) B cell subsets was assessed by flow cytometry after co-culture. APRIL production upon TLR3L stimulation of siHCP5 or siNC transfected cDC2s was measured by (**D**) Luminex-based assay (**E**) and qRT-PCR. Results are represented as mean (bars); differences were considered statistically significant when $p < 0.05$ vs. siNC (paired t-test). **represents nominal p-value < 0.01.



Supplementary Figure 3. Intracellular cytokine production by CD4⁺ T cells as well as co-stimulatory profile and cytokine production by siHCP5 silenced cDC2s. cDC2s were transfected either with *HCP5* antisense LNA GapmeR (siHCP5) or antisense LNA GapmeR Control (siNC) and stimulated with TLR3L for 20h. (**A**) Representative histogram and (**B-C**) quantification of co-stimulatory molecules represented as median fluorescence intensity (MFI). siHCP5 or siNC transfected cDC2s were co-cultured with CD4⁺ T cells (cDC2s/CD4⁺T cell ratio of 1:5) in the presence of IL-2 for six days and restimulated with PMA and ionomycin plus brefeldin A. (**D**) Representative flow cytometry gating strategy analysis to identify IL-21-, CXCL13-, IFNγ-, TNFα-, IL-17-, and IL-4-producing CD4⁺ T cells in siHCP5 and siNC -cDC2s. (**E**) Frequency of viable and (**F**) proliferating CD4⁺ T cells after PMA and ionomycin restimulation. (**G-H**) Cytokine production in transfected cDC2s was measured upon TLR3L stimulation using a Luminex-based assay. Results are represented as mean (bars); differences were considered statistically significant when $p < 0.05$ vs. siNC (paired t-test). *represent nominal p-value < 0.05.



Supplementary Figure 4. *CD1C* expression in minor salivary glands is restrict to the cDC2s subset. (**A**) Single-cell RNA-sequencing data from minor salivary glands from a control group (73) was retrieved using UCSC Cell Browser (74). (**B**) UMAP projections of single cell transcriptomic data from minor salivary glands. cDC2s are shown in dark blue, (**C**) cells expressing *PTPRC* (CD45) and (**D**) *CD1C* are indicated in red. Plots were generated with https://cells.ucsc.edu/?ds=oral-cavity+salivary-gland.



Supplementary Figure 5. *HCP5* expression in circulating cell subsets. Quantification of *HCP5* expression in isolated cDC2s, pDCs, B cells and monocytes from HC peripheral blood assessed by qPCR. Differences were considered statistically significant when $p < 0.05$ (t-test). * Represents nominal p-value < 0.05.

Supplementary Table 1. List of the TLR ligands and cytokines used for cDC2s stimulation.

Stimulus	Manufacturer	Concentration
Pam3CSK4 – TLR2/1	Invivogen	5 µg/mL
Pam2CSK4 – TLR2/6	Invivogen	1 µg/mL
Poly(I:C) – TLR3	Invivogen	25 µg/mL
LPS – TLR4	Invivogen	0.1 µg/mL
Flagellin – TLR5	Invivogen	2 µg/mL
R848 – TLR7/8	Invivogen	1 µg/mL
IFN-α	Cell Sciences	1000 U/mL
TSLP	R&D Systems	20 ng/mL

Supplementary Table 2. Sequences of primers used for RT-qPCR.

Gene	Primer forward 5' – 3'	Primer reverse 5' – 3'
GAPDH	GCCAGCCGAGCCACATC	TGACCAGGCGCCCAATAC
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
IRF7	TACCATCTACCTGGGCTTCG	AGGGTTCCAGCTTCACCA
OAS2	CTCAGAAGCTGGGTTGGTTTAT	ACCATCTCGTCGATCAGTGTC
MX1	GCATCCCACCCTCTATTACTG	CGCACCTTCTCCTCATACTG
IFNB1	CAGCAATTTTCAGTGTCAGAAGC	TCATCCTGTCCTTGAGGCAGT
CCL5	CCAGCAGTCGTCTTTGTCAC	CTCTGGGTTGGCACACACTT
CXCL13	CGACATCTCTGCTTCTCATGCT	AGCTTGTGTAATAGACCTCCAGAACA
CXCL8	TGAGAGTGGACCACACTGCG	TCTCCACAACCCTCTGCACC
BAFF	TGGGGATGAATTGAGTCTGGTG	GCAATGCCAGCTGAATAGCA
TNFSF13	CTCTGCTGACCCAACAAACAG	TTTTCCGGGATCTCTCCCCAT
FCN1	GGGCAGTGCGGGTAATTCTC	GAAGCATGACAGTCGGCGTA
HCP5	Designed ID 571823-2	

Supplementary Table 3. List of antibodies used for flow cytometry staining.

Target	Label	Manufacturer	Clone	cDC2s-B cell co-culture
CD24	PerCP-Cy5.5	BD Biosciences	ML5	x
CD38	APC	eBioscience	HIT2	x
Viability dye	eFluor 780	eBioscience	-	x
CD11c	PE-CF594	BD Biosciences	B-ly6	x
CTV	-	Invitrogen	-	x
CD19	PE-Cy7	Beckman Coulter	J3-119	x
CD21	BV711	BD Biosciences	B-ly4	x
CD27	BV785	Biolegend	O323	x
IL-17a	FITC	eBioscience	eBio64DEC17	
IFN-γ	PerCP-Cy5.5	eBioscience	4S.B3	
CXCL13	APC	R&D Systems	53610	
CD3	AF700	Biolegend	UCHT1	
IL-21	PE	BD Biosciences	3A3-N2.1	
TNF-α	PE-Cy7	eBioscience	MAb11	
IL-4	BV711	BD Biosciences	MP4-25D2	
Annexin	APC	BD Biosciences	-	
7AAD	-	BD Biosciences	-	
CD80	PE	BD Biosciences	L307.4	
CD83	FITC	Beckman Coulter	HB15a	
CD86	PB	Biolegend	IT2.2	
PD-L1	PE-Cy7	BD Biosciences	MIH1	
CD70	FITC	BD Biosciences	Ki-24	
OX40L	PE	Biolegend	11C3.1	
GITR-L	PerCP	R&D Systems	109101	
CD40	APC	Sony Biotechnology	HB14	
CD14	eFluor 450	eBioscience	61D3	
ICOSL	BV650	BD Biosciences	2D3/B7-H2	
CD5	PE	Biolegend	UCHT2	
CLEC10a	APC	Biolegend	H037G3	
HLA ABC	PE-Cy7	BD Biosciences	G46-2.6	
CD163	BV510	Biolegend	GHI/61	

	cDC2s-CD4⁺ T cell co-culture	cDC2s viability	Co-stimulatory molecules	DC3-like phenotype
	x		x	x
	x			
	x			
	x			
	x			
	x			
	x			
	x			
		x		
		x		
		x		
		x		
		x		
		x		
			x	
			x	
			x	
			x	
			x	x
			x	
				x
				x
				x
				x

Supplementary Table 4. Characteristics of the pSS patients and controls enrolled in the study.

	HC	pSS
N (M/F)	24 [0/24]	33 [2/31]
Age (yr.)	51 [26-67]	56 [22-82]
LFS (foci/4 mm^2)	-	2.5 [1.0-6.4]
ESSDAI	-	4.3 [0.0-13]
ESSPRI	-	5.5 [1.3-9.0]
Schirmer (mm/5 min)	-	9.4 [0.0-28]
ANA (no. positive [%])	-	25 [76%]
SSA (no. positive [%])	-	24 [73%]
SSB (no. positive [%])	-	18 [55%]
RF (no. positive [%])	-	14 [58%]
Serum IgG (g/L)	-	15 [8.5-29]
ESR (mm/hour)	-	17 [2-99]
C3 (g/L)	-	1.0 [0.8-1.6]
C4 (g/L)	-	0.2 [0.1-0.4]
Not treated (no. [%])	-	23 [70%]
Only HCQ (no. [%])	-	6 [18%]
Other (no. [%])	-	4 [12%]

HC: healthy control; pSS: primary Sjögren's syndrome; LFS: lymphocytic focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: anti-nuclear antibodies; SSA: anti-SSA/Ro; SSB: anti-SSB/La; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; HCQ: hydroxychloroquine. Other treatment group includes azathioprine in combination with prednisone (n=2); mesalazine (n=1); prednisone in combination with HCQ (n=1). Values are median [range] unless stated otherwise.

Supplementary References

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Chapter 5

Deciphering the role of cDC2s in Sjögren's Syndrome: transcriptomic profile links altered antigen processes with IFN-signature and autoimmunity

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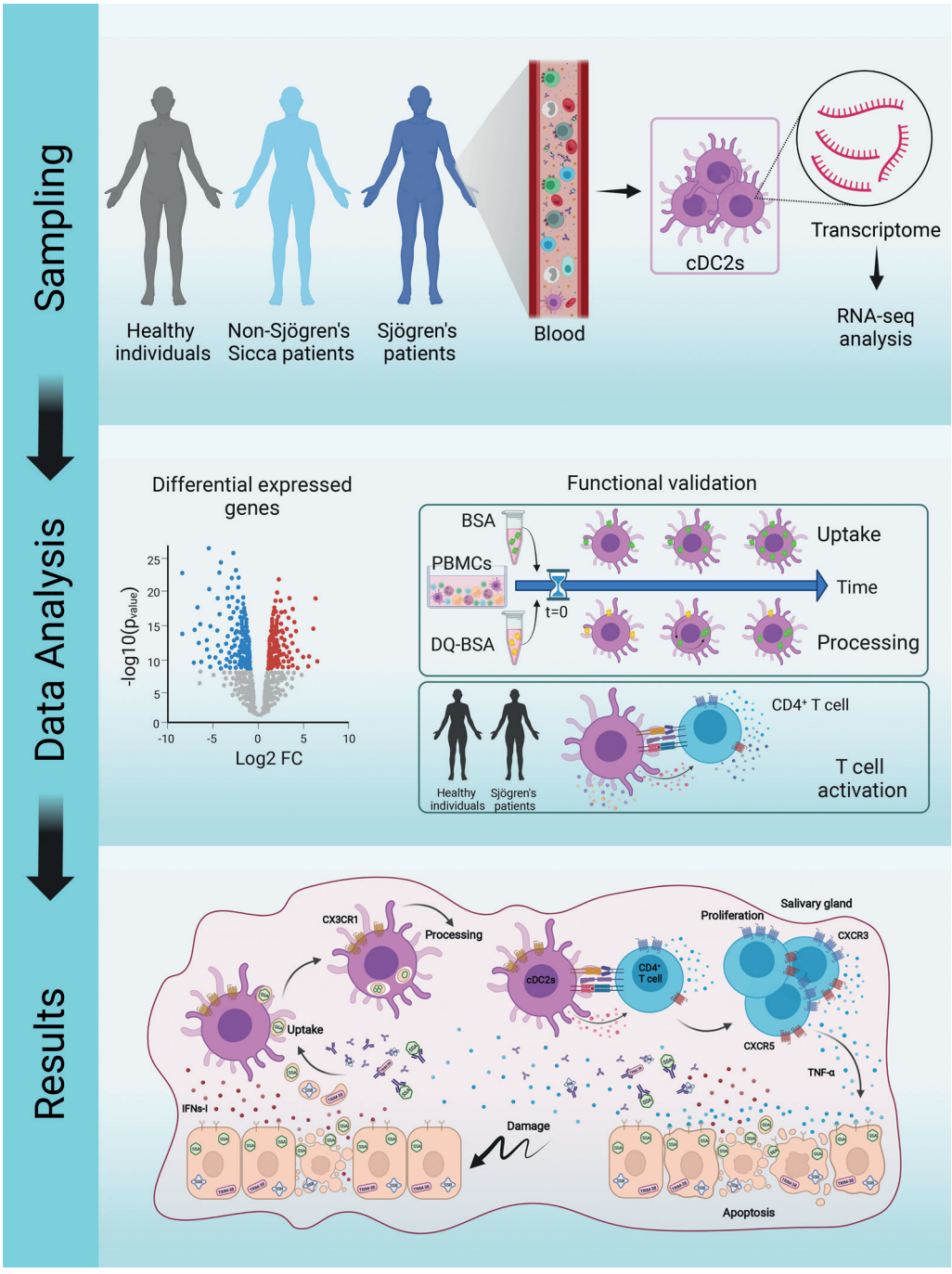
Objective Type 2 conventional dendritic cells (cDC2s) are key orchestrators of inflammatory responses, linking innate and adaptative immunity. Here we explored the regulation of immunological pathways in cDC2s from patients with primary Sjögren's syndrome (pSS).

Methods RNA sequencing of circulating cDC2s from pSS, non-Sjögren's sicca (nSS) patients, and healthy controls (HC) was exploited to establish transcriptional signatures. Phenotypical and functional validation was performed in independent cohorts.

Results Transcriptome of cDC2s from pSS patients revealed alterations in type-I interferon (IFN), toll-like receptor (TLR), antigen processing and presentation pathways. Phenotypic validation showed increased CX3CR1 expression and decreased integrin beta-2 and plexin-B2 on pSS-cDC2s. Functional validation confirmed impaired capacity of pSS-cDC2s to degrade antigens and increased antigen uptake, including self-antigens derived from salivary gland epithelial cells. These changes in antigen uptake and degradation were linked to anti-SSA autoantibodies and the presence of type-I IFNs. In line, *in vitro* IFNα priming enhanced the uptake of antigens by HC-cDC2s, reflecting the pSS-cDC2 profile. Finally, pSS-cDC2s compared to HC-cDC2s increased the proliferation and the expression of CXCR3 and CXCR5 on proliferating $CD4^+$ T cells.

Conclusions pSS-cDC2s are transcriptionally altered, and the aberrant antigen uptake and processing, including (auto-)antigens, together with increased proliferation of tissue-homing $CD4^+$ T cells suggests altered antigen presentation by pSS-cDC2s. These functional alterations were strongly linked to anti-SSA positivity and the presence of type-I IFNs. Thus, we demonstrate novel molecular and functional evidences for the role of cDC2s in orchestrating immune response in pSS, which may yield novel avenues for treatment.

Graphical abstract



Key messages

What is already known on this topic

Type 2 conventional dendritic cells (cDC2s) are central in the initiation and control of immune responses, but their functional role in primary Sjögren's syndrome is poorly understood.

What this study adds

- Transcriptomic profile of cDC2s reveals changes in important pathways in pSS patients consistent with cell activation, presence of type-I interferon and altered antigen response.
- Phenotypical validation shows increased fractalkine receptor (CX3CR1) expression on cDC2s from pSS patients
- cDC2s from pSS patients with anti-SSA autoantibodies demonstrate altered antigen uptake and processing, modulated by type-I interferon.
- cDC2s from pSS patients activate and increase CXCR3 and CXCR5 expression on $CD4^+$ T cells, facilitating migration to the inflammatory sites.

How this study might affect research, practice or policy

Considering their key role in orchestrating inflammatory responses understanding the underlying molecular mechanisms that drive cDC2s function and activation may disclose novel targets to halt immunopathology in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by prominent T and B lymphocyte infiltration of the exocrine glands, which is associated with glandular destruction and dysfunction [1, 2]. The immunological hallmarks of pSS include B cell hyperactivity, the presence of anti-Ro/SSA and anti-La/SSB antibodies [3] and a type-I interferon (IFN) signature. Furthermore, the presence of a type-I IFN signature in pSS patients is associated with higher disease activity and higher levels of autoantibodies [4] and reinforces the involvement of the innate immune system [5].

Type 2 conventional dendritic cells (cDC2s) are professional antigen presenting cells with a unique ability to induce potent T and B cell responses [6]. Upon stimulation, cDC2s take up antigens and migrate into the T cell area of the draining lymph node to initiate immune responses [7]. The internalization, processing and presentation of antigens is a critical step for T cell priming. cDC2s are potent activators of $CD4^+$ T cells and induce T helper (Th) cell polarization, thus directing the immune system in distinct directions [8]. Moreover, cDC2s can affect B cell differentiation and survival, mainly through the production of B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) [9]. Activation and maturation of cDC2s to initiate adaptive immune responses can be potently amplified by inflammatory mediators, particularly type-I IFNs [10, 11].

Despite the important role of cDC2s to activate T and B cells, their contribution to pSS immunopathology has been poorly studied. Recently, transcriptomic analysis of minor salivary glands from pSS patients confirmed the presence of a cDC2 gene signature in the inflamed salivary glands, which was strongly associated with $CD4^+$ T cells [12, 13]. In pSS patients, cDC2s are epigenetically altered with decreased miR-130a expression and increased expression of its target mitogen- and stress activated protein kinase-1 (MSK1), important for pro-inflammatory cytokine production. Furthermore, upon stimulation cDC2s from pSS patients produce more IL-12 and TNFα [14]. In addition, in nonobese diabetic (NOD) and IQI/Jic, both spontaneous mouse models of Sjögren's syndrome, DC influx precedes the presence of focal lymphocytic infiltrates into the submandibular glands [15, 16]. Furthermore, in aged mice lacking *Dcir* expression, a crucial negative regulator of DC function, mice spontaneously develop sialadenitis with elevated serum autoantibodies levels including anti-Ro/SSA, anti-La/SSB, and anti-nuclear antibodies [17].

Thus, cDC2s seem to play an important role in pSS pathogenesis as well as in T and B cell activation and in driving salivary gland inflammation. Here, we set out to investigate the role of cDC2s in pSS by exploiting RNA sequencing to identify the transcriptional profile of circulating cDC2s from pSS and non-Sjögren's sicca (nSS) patients as compared to healthy controls (HC). In addition, we performed phenotypical and functional validation to confirm identified altered pathways and mechanisms through which cDC2s could drive pSS.

Material and methods

Patients and controls

Patients and controls were age and gender-matched and randomly allocated across the different experiments. All pSS patients fulfilled the AECG classification-criteria for pSS [18]. Patients that did not fulfill the pSS classification-criteria, but presented with dryness-complaints without a known cause, in the absence of any generalized autoimmune disease were classified as non-Sjögren's sicca (nSS) patients and subjected to minor salivary gland biopsy. Two independent cohorts were selected to establish the transcriptional profile of cDC2s: a discovery cohort (14 pSS, 9 nSS, 8 HC) and a replication cohort (9 pSS, 6 nSS, 10 HC) (**Table 1**). For the validation experiments, additional independent cohorts of HC and pSS patients were recruited (**Supplementary Table 1**). The study was approved by the medical ethics committee of the University Medical Center Utrecht (METC no. 13-697). All patients gave their written informed consent in accordance with the declaration of Helsinki.

Patients and public involvement

Patients and healthy controls recruited for this study were not involved in the design, or conduct, or reporting, or dissemination plans of our research.
Detailed information for all the methods, including phenotypic and functional validation can be found in the online supplementary material and methods.

Results

The transcriptome of cDC2s from pSS patients is distinct from nSS patients and HC

In the discovery cohort, the majority of differentially expressed genes (DEGs) were found between pSS and HC cDC2s (**Figure 1A**). Similarly, in the replication

cohort the larger number of DEG was also observed between pSS and HC cDC2s (**Figure 1B**). In addition, cDC2s from nSS patients, partly exhibited changes in gene expression similar to pSS cDC2s in both cohorts. However, the magnitude of differences was generally larger in cDC2s from pSS patients compared to nSS patients. Overall, the cDC2s transcriptomic profile of pSS patients overlaps to some extent to nSS patients, but both are distinct from HC (**Figure 1A-B**).

Table 1. Characteristics of the patients and controls enrolled in the RNAseq cohort.

	RNAseq profiling					
	Discovery cohort (n=31)			Replication cohort (n=25)		
	HC	nSS	pSS	HC	nSS	pSS
N (M/F)	8 [0/8]	9 [0/9]	14 [3/11]	10 [1/9]	6 [0/6]	9 [1/8]
Age (yr.)	58 [54-67]	43 [25-68]	54 [29-70]	51 [29-59]	46 [24-68]	55 [26-76]
LFS (foci/4 mm^2)	-	0 [0.0-1.0]	1.9 [1.0-4.0]	-	0.1 [0.0-0.5]	2.1 [1.0-4.0]
ESSDAI	-	-	2.0 [0.0-19]	-	-	5.0 [1.0-13]
ESSPRI	-	-	3.7 [2.0-8.8]	-	-	2.9 [1.0-8.0]
Schirmer (mm/5 min)	-	3.3 [0.0-21]	5.0 [0.5-25]	-	10 [0.0-32]	13 [1.0-28]
ANA (no. positive [%])	-	1 [11%]	10 [71%]	-	3 [50%]	6 [67%]
SSA (no. positive [%])	-	2 [22%]	8 [57%]	-	2 [40%]	5 [56%]
SSB (no. positive [%])	-	0 [0%]	4 [29%]	-	0 [0%]	2 [22%]
RF (no. positive [%])	-	0 [0%]	5 [42%]	-	0 [0%]	4 [50%]
Serum IgG (g/L)	-	12 [6.8-17]	14 [8.3-30]	-	13 [11-15]	14 [8.5-42]
ESR (mm/ hour)	-	11 [4.0-17]	11 [5.0-36]	-	7 [5.0-23]	14 [7.0-77]
C3 (g/L)	-	1.2 [0.6-2.0]	1.1 [0.7-1.3]	-	1.1 [0.8-1.3]	1.1 [0.5-1.6]
C4 (g/L)	-	0.3 [0.2-0.4]	0.3 [0.1-0.3]	-	0.2 [0.1-0.4]	0.3 [0.1-0.4]
Not treated (no. [%])	-	6 [86%]	11 [79%]	-	6 [100%]	6 [67%]
Only HCQ (no. [%])	-	1 [14%]	1 [7%]	-		1 [11%]
Other (no. [%])	-	-	2 [14%]	-		2 [22%]

HC: healthy control; pSS: primary Sjögren's syndrome; LFS: lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: anti-nuclear antibodies; SSA: anti-SSA/Ro; SSB: anti-SSB/La; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein, HCQ: hydroxychloroquine. Other treatment group includes for discovery: azathioprine (n=1); mesalazine (n=1) and for replication: azathioprine (n=1); prednisone in combination with HCQ (n=1). Values are median [19] unless stated otherwise.

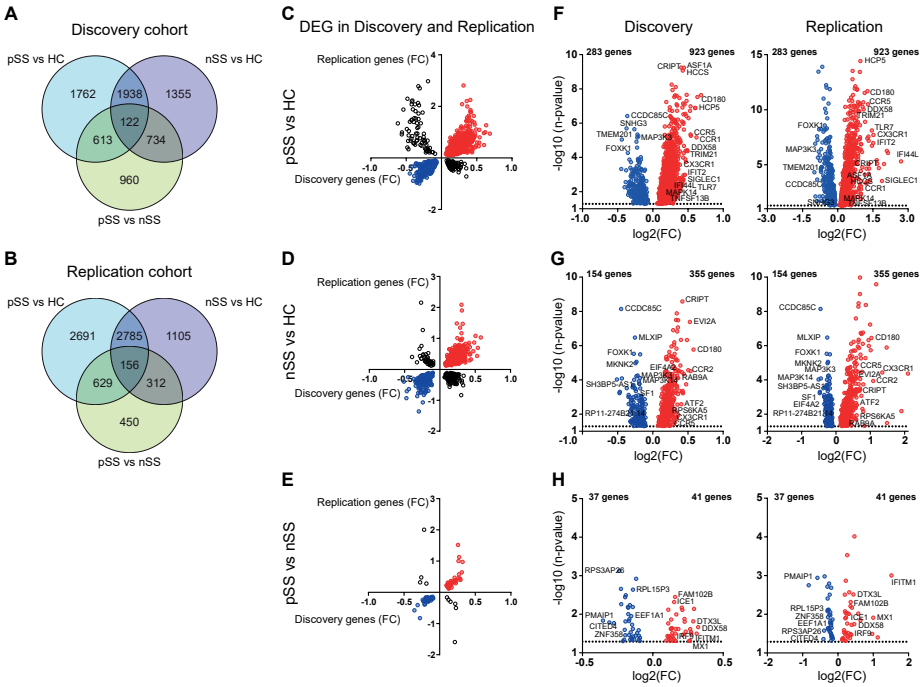


Figure 1. Transcriptomic characterization of circulating cDC2s from pSS and nSS patients. RNA sequencing of circulating cDC2s was performed independently for both discovery and replication cohort. Venn diagrams show the overlap of the differentially expressed genes (DEGs) with a nominal p-value < 0.05 between any of the three groups for the discovery cohort (**A**) and replication cohort (**B**). Scatterplots show the fold change (log2) (FC) of the DEGs between the two cohorts for the different comparisons, pSS vs. HC (**C**), nSS vs. HC (**D**) and pSS vs. nSS (**E**). Volcano plots display the relationship between the fold change (log2; x-axis) and the nominal p-value (-log10; y-axis) of the DEGs consistently downregulated or upregulated in both cohorts for each comparison (**F-H**).

To identify the most robust and consistently altered genes, those genes differentially expressed with a nominal p-value <0.05, with an average base mean expression (defined as the mean of normalized counts of all samples normalizing for sequencing depth) higher than 100 in both cohorts were selected (**Figure 1C-E**). Of the DEGs identified in both cohorts, a large fraction (87% in pSS vs. HC; 66% in nSS vs. HC and 87% in nSS vs. pSS) exhibited the same directionality (**Figure 1F-H**) and therefore was considered to be replicated. The majority of the replicated DEGs were found between pSS and HC and out of these 30% (356 genes) were differentially expressed between nSS and HC (**Supplementary Figure 2**). For the DEGs between nSS patients and HC cDC2s, functional annotation did not reveal enriched pathways (**Supplementary Figure 3A**). As for the DEGs between pSS vs.

nSS (**Figure 1E**), functional annotation indicated that these genes were associated with viral and IFN-related pathways, non-sense mediated decay and translation processes (**Supplementary Figure 3B-C**).

Transcriptomic analysis of pSS-cDC2s reveals impaired expression of genes involved in cell trafficking and activation

To gain further insight into the pSS-cDC2s transcriptional profile, we identified the top 100 DEGs in both cohorts, based on fold-change differences. The majority of upregulated genes identified in pSS-cDC2s included IFN-inducible genes *(e.g., MX1, IFITM1* and *DDX58*), molecules involved in cell migration (e.g., *CCR2, CX3CR1* and *CCR5*) and activation (*TLR7*). Likewise, the top downregulated genes in pSS-cDC2s comprise important regulators of cell activation like *NFKBIA*, a member of the NF-κB inhibitor family; *PLXNB2 and PLXND1,* both negative regulators of IL-12p40 production [20]; *ITGB2*, a negative regulator of TLR activation [21]; and *PELI1*, which negatively regulates noncanonical NF-κB signaling [22] (**Figure 2A**).

As DC migration, cell-cell interaction and activation are crucial steps in the initiation and regulation of the immune response, we sought to further investigate the expression of potential mediators of these processes at protein level (**Figure 2B** and **Supplementary Figure 4A**). To this end, the protein expression of *CX3CR1*, a key chemokine receptor involved in trafficking of cDC2s [23], *TNFSF13B* (B cell activating factor; BAFF), *ITGB2* (integrin beta-2) and *PLXNB2* (plexin-B2), both regulators of cell activation, was assessed by flow cytometry. In line with the transcriptomic data, the surface expression of CX3CR1 was significantly higher and the expression of integrin beta-2 and plexin-B2 was significantly lower on cDC2s from pSS patients when compared to HC. BAFF surface expression was not significantly different (**Figure 2C**). Together, these results corroborate transcriptional changes in key mediators of migration and activation in pSS-cDC2, and identify a possible mediator of cDC2 recruitment to the inflamed salivary glands.

To further understand the functional pathways altered in pSS-cDC2s, we performed annotation of the consistently DEGs (**Figure 1F**). Pathways involved in inflammation, including interferon signaling, class I major histocompatibility complex (MHC) mediated antigen processing and presentation, toll-like receptors (TLR) cascade, and mitochondrial translation were enriched in pSS-cDC2s compared to HC-cDC2s (**Figure 3A**). As TLRs are crucial receptors for cDC2 activation, we sought to investigate the phosphorylation profile of downstream TLR signaling mediators. However, we did not observe changes in the

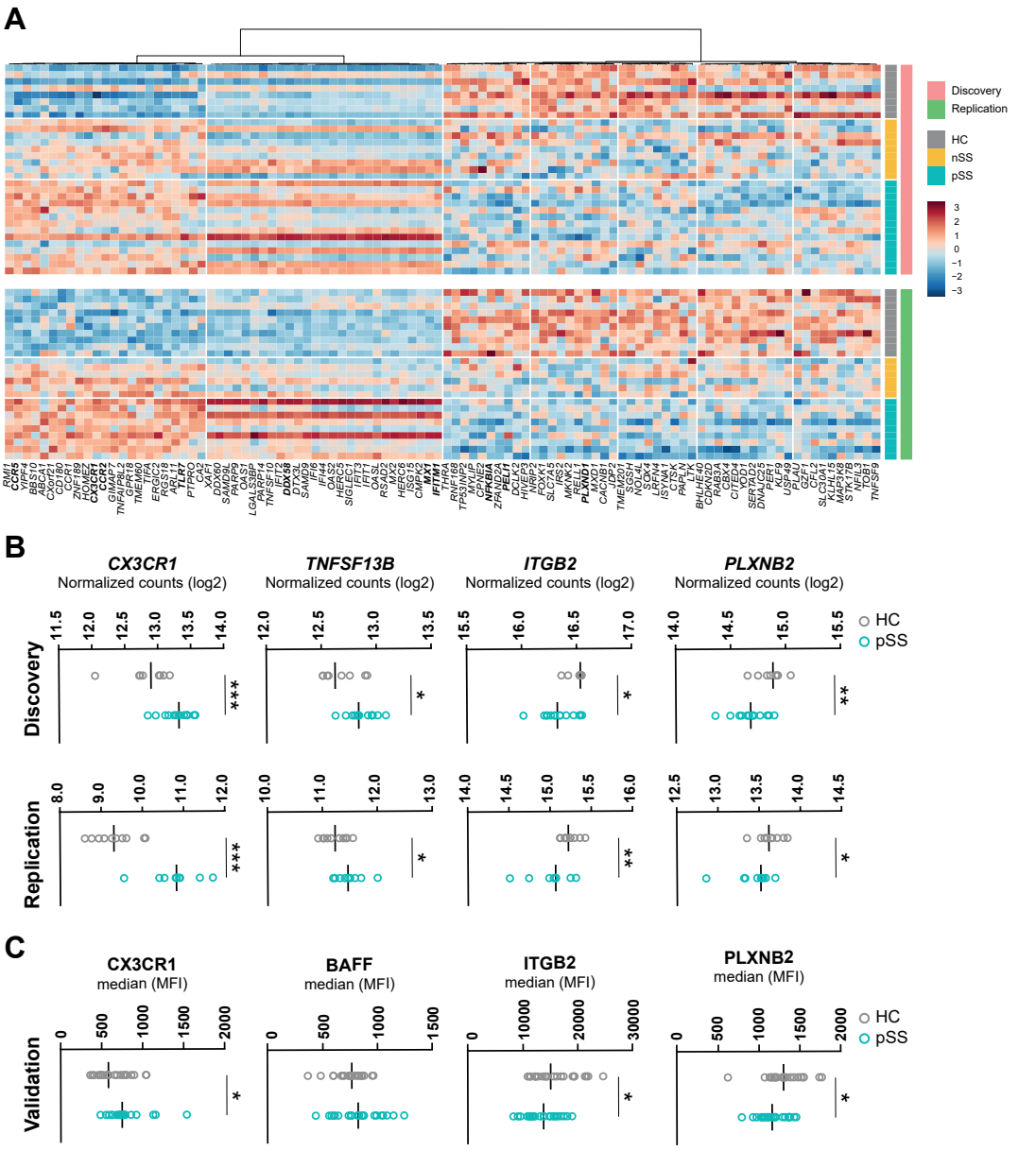


Figure 2. Transcriptomic profile and protein validation of cDC2s from pSS patients display altered expression of key molecules involved in cell trafficking, activation and interferon signaling. Heatmap visualization of the top 100 DEGs (50 upregulated and 50 downregulated genes; rows) across the two cohorts (discovery and replication) and the studied groups (HC, nSS and pSS; columns) clustered by Euclidean distance and Ward's method (**A**). Dot plots depict the expression of selected DEGs in discovery and replication cohorts in both HC and pSS patients (**B**). Protein expression of the selected differentially expressed genes was assessed on cDC2s by flow cytometry in HC (n=22) and pSS patients (n=22) (**C**). *, **, and *** represent nominal p-value < 0.05, p < 0.01, and p < 0.001, respectively.

phosphorylation profile of p38, ERK1/2, JNK, ATF2, and NF-kB p65 between pSS-cDC2s and HC-cDC2s, both *ex-vivo* and after TLR4 activation (**Supplementary Figure 5**).

cDC2s from pSS patients display a less effective antigen processing capacity

Antigen uptake and processing are crucial pathways in cDC2s that affect their antigen presentation to $CD4^+$ and $CD8^+$ T cells. As functional annotation of pSS-cDC2 DEGs indicated altered antigen processing in pSS-cDC2s, we performed *in vitro* validation experiments to assess the capacity of pSS-cDC2s to degrade phagocytosed protein. To this end, we used as an antigen model BSA protein labelled with a fluorescent BODIPY dye (DQ-BSA). DQ-BSA is not fluorescent due to self-quenching, but upon endocytosis and degradation the self-quenching is abolished and a fluorescent signal can be detected using flow cytometry (**Figure 3B**). As immuno-suppressive treatment affects antigen processing, patients who were on treatment at the time of sample collection were excluded from this analysis (**Supplementary Figure 6A**). We observed significantly reduced antigen processing in cDC2s from pSS patients as compared to HC, particularly at t=60 minutes (**Figure 3C-D** and **Supplementary Figure 4A**). Next, we investigated the association between the presence of anti-SSA–autoantibodies and the antigen processing capacity of cDC2s. Interestingly, cDC2s from pSS patients with anti-SSA-autoantibodies (pSS-SSA+) displayed a significantly lower processing capacity compared to HC, while patients without anti-SSA-autoantibodies (pSS-SSA-) demonstrated antigen processing similar to that of HC (**Figure 3E**). No differences were found in the frequency of circulating cDC2s between pSS patients and HC (**Supplementary Figure 4B**).

As decreased capacity to process antigen has been associated with prolonged antigen survival, facilitating MHC-I supply [24], and enhanced cross-presentation to $CD8^+$ T cells, we further investigated the expression of genes involved in class I mediated processing. The expression of *TAP1*, a key transporter associated with MHC-I loading [25], the peptidase *LNPEP*, implicated in endosomal trimming of cross-presented peptides and interaction with MHC-I molecules [26], as well as the expression of proteasome subunits like *PSMA3*, involved in protein degradation in a ubiquitin-independent manner, were upregulated in cDC2s from pSS patients compared to HC (**Figure 3F**). Thus, our results suggest that pSS-cDC2s are possibly more efficient at storing (auto-)antigens, enhancing cross-presentation to $CD8^+$ T cells.

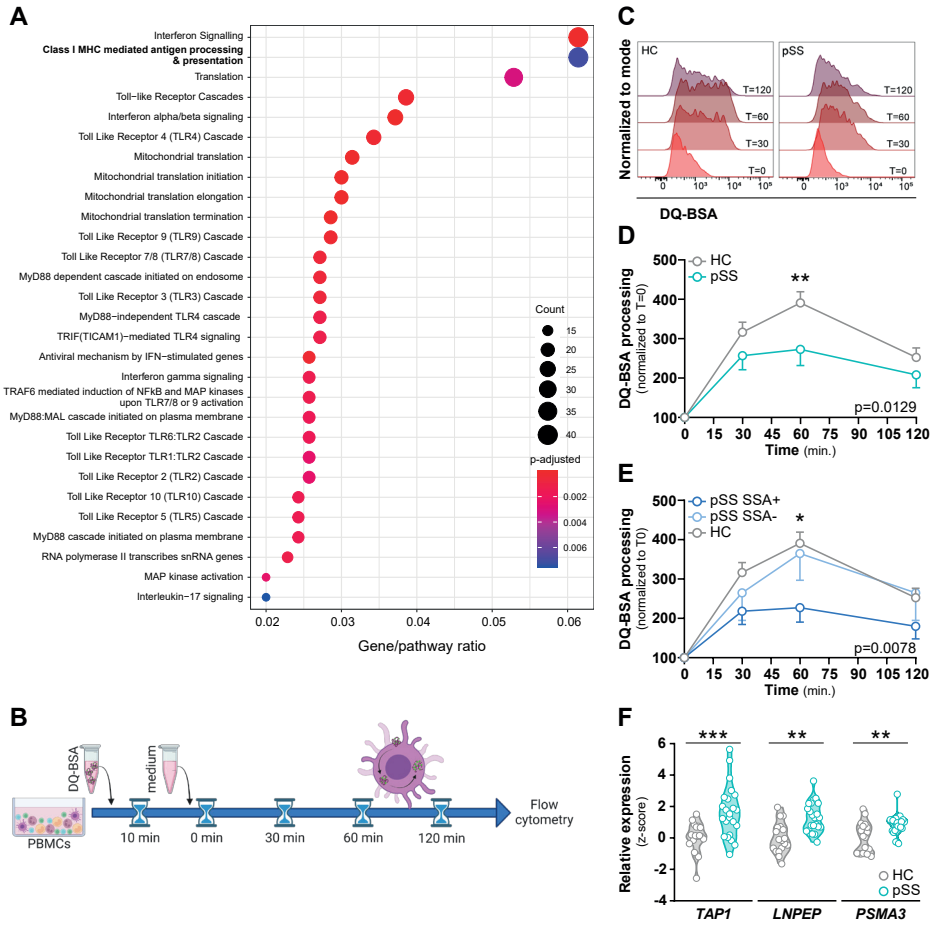


Figure 3. cDC2s from pSS patients are functionally different in interferon-associated pathways and in antigen processing. Reactome pathway enrichment analysis was used for functional annotation of the DEGs between pSS vs. HC (selected in Figure 1F). The top significantly enriched reactome pathways are depicted. X-axis show the number of DEGs found within the pathway over the total number of pathway components (ratio), dot-size depicts the number of genes used for enrichment and color indicates the statistical significance (**A**). Isolated peripheral blood mononuclear cells (PBMCs) were incubated with DQ–BSA for 10 minutes and antigen processing was followed for the indicated time points (**B**). Representative histograms (**C**) and quantification (**D**) of processed DQ-BSA, represented as median fluorescence intensity (MFI) normalized to T=0, in HC (n=11) and non-treated pSS patients (n=6) at different time points determined by flow cytometry. Quantification of DQ-BSA processing in pSS patients with (pSS-SSA+; n=4) or without (pSS-SSA-; n=2) anti-SSA antibodies (**E**). Violin plots depicts *TAP1*, *LNPEP* and *PSMA3* gene expression in HC and pSS patients from discovery and replication cohort combined (**F**).

Increased antigen uptake of pSS-cDC2s is related with anti-SSA-autoantibodies and type-I IFNs

As cDC2 antigen processing and presentation are importantly impacted by antigen uptake, we next investigated the capacity of cDC2s from pSS patients and HC to uptake BSA (**Figure 4A-B**). Time-course analyses of BSA uptake demonstrated that pSS-cDC2s have an increased antigen uptake compared to HC-cDC2s, particularly at later time points (t=60 and t=120min) (**Figure 4C** and **Supplementary Figure 4A**). No differences were observed in cDC2 antigen uptake between patients who were treated with immune-suppressive treatment and those who were not (**Supplementary Figure 6B**). Interestingly, cDC2s from pSS-SSA+ uptake more antigen compared to pSS-SSA- and HC. A significantly higher uptake capacity was observed in pSS-SSA+ compared to HC at t=60 min and this further increased at t=120 min (**Figure 4D**). In addition, at t=120 min, pSS-SSA+ cDC2s uptake more antigen compared to pSS-SSA-, who show similar BSA uptake to HC (**Figure 4D**).

Given the strong association between the presence of anti-SSA-autoantibodies and the IFN-signature [27], we confirmed that the majority of the pSS-SSA+ patients (8 out of 10) exhibited an IFN-signature (pSS-IFN+), reflected by a higher IFN score (**Figure 4E**). In addition, as functional annotation of DEGs in pSS-cDC2s indicated altered IFN signaling, we investigated whether the presence of type-I IFN affects cDC2s antigen uptake and processing. For this, HC-cDC2s were left untreated or primed with IFNα for 3h to mimic the IFN-signature and next challenged with labeled BSA (**Figure 4F**). IFNα priming of HC-cDC2s increased antigen uptake by HC cDC2s to similar levels as those seen for pSS-SSA+ cDC2s (**Figure 4G**), but did not alter antigen processing of cDC2s (data not shown). In addition, an increased antigen uptake was also observed in cDC2s from pSS-IFN+ patients compared to pSS-IFN- patients, particularly at t=120min (**Figure 4H**). Together, these results show that cDC2s from anti-SSA+ pSS patients have an increased antigen uptake capacity related to the IFN-signature, as exposure of HC-cDC2s to type-I IFN induces an increase uptake profile similar to pSS-SSA+ cDC2s.

Next, we tested whether pSS-SSA+ cDC2s also uptake increased amounts of auto-antigens derived from apoptotic salivary gland (HSG)-epithelial cells, which is a relevant mechanism to drive pSS. To this end, PBMCs from pSS patients and HC were incubated with apoptotic CFSE-labeled HSG-epithelial cells for 2h and the phagocytic capacity of cDC2s was assessed by flow cytometry (**Supplementary Figure 7**). Similar to BSA uptake, pSS-cDC2s displayed a significantly enhanced

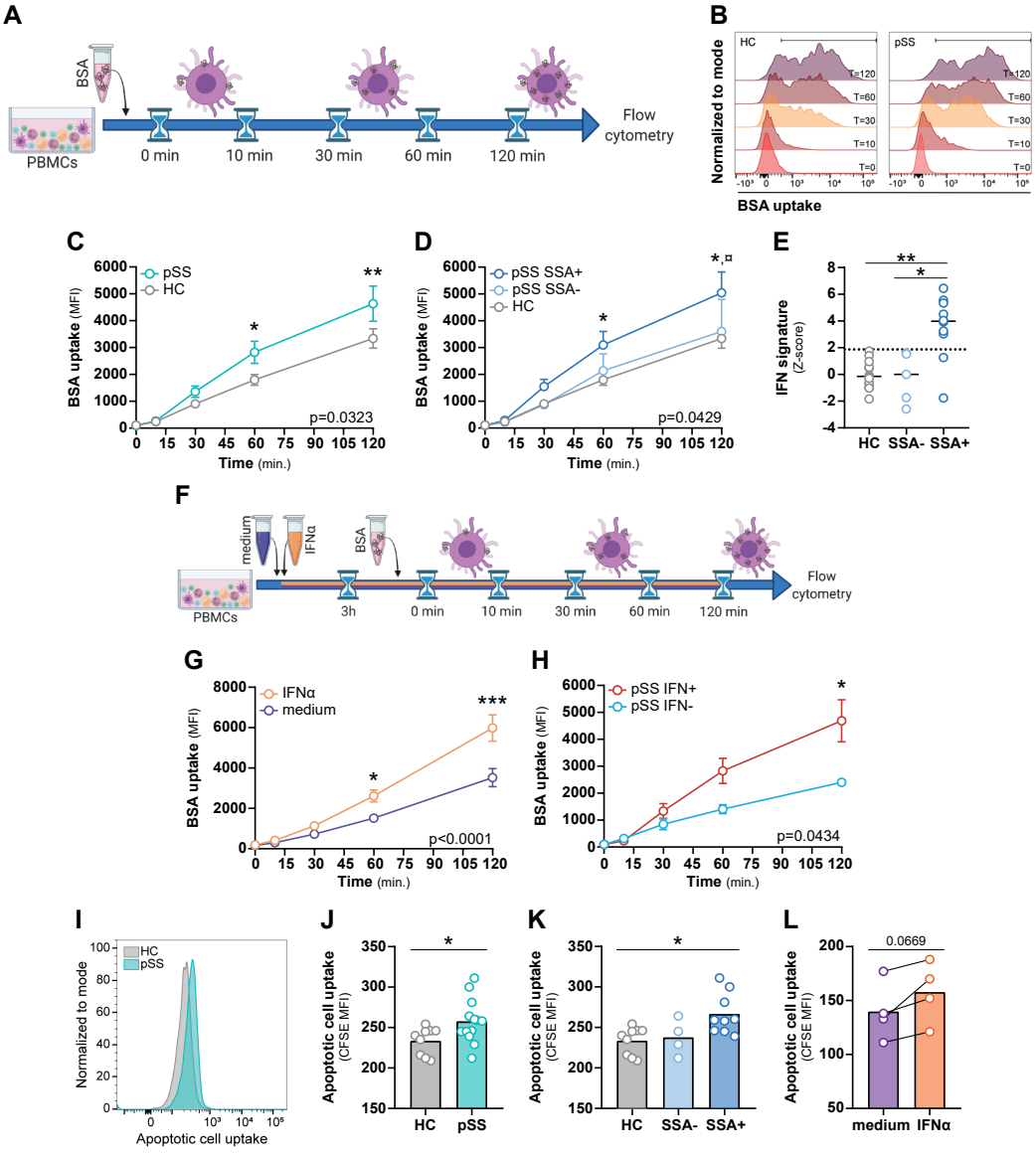


Figure 4. Enhanced uptake of antigen and apoptotic cells by pSS-cDC2s is associated with autoimmunity and type-I IFN. PBMCs from HC and pSS patients were incubated with AF647–BSA for the indicated time points and the uptake by cDC2s, represented as median fluorescence intensity (MFI), was assessed by flow cytometry (**A**). Representative histograms (**B**) and quantification of BSA uptake by cDC2s of HC (n=11) and pSS patients (n=14) (**C**) and pSS patients with (pSS-SSA+; n=10) or without (pSS-SSA-; n=4) anti-SSA antibodies (**D**). IFN-signature calculated as the mean Z-score of five IFN-induced genes was determined by qPCR in HC (n=13), pSS-SSA- (n=5) and pSS-SSA+ (n=10) (**E**). HC-PBMCs were primed for 3h without (medium) or with IFNα, exposed to AF647–BSA and chased for the indicated times by flow cytometry (**F**). The effect of IFNα priming on BSA uptake was

analyzed in HC-cDC2s (n=5) by flow cytometry (**G**). Quantification of BSA uptake by cDC2s of pSS patients with (pSS-IFN+; n=9) or without (pSS-IFN-; n=3) IFN-signature (**H**). Apoptotic CFSE labeled HSG-epithelial cells were added to PBMCs from HC (n=9) and pSS patients (n=13) at a 1:1 ratio for 120 min. Representative histogram (**I**) and quantification of apoptotic cell uptake of cDC2s from HC and pSS patients (**J**) and pSS-SSA- (n=4) and pSS-SSA+ (n=9) (**K**) measured by flow cytometry. HC-PBMCs were primed for 3h with IFNα or without (medium) and exposed to apoptotic CFSE labeled HSG-epithelial cells for 2h. The effect of IFNα priming on apoptotic cells uptake was analyzed in HC-cDC2s (n=4) by flow cytometry (**L**). Results are represented as mean ± SEM. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

ability to uptake apoptotic HSG-epithelial cells (**Figure 4I-J**) compared with HC-cDC2s. Furthermore, the increased uptake of apoptotic HSG-epithelial cells was only observed in cDC2s of pSS-SSA+ patients (**Figure 4K**). Moreover, IFNα priming of HC-cDC2s increased uptake of apoptotic HSG-epithelial cells by HC-cDC2s, as observed in BSA uptake, although without statistical significance (**Figure 4L**). Thus, cDC2s from pSS patients with anti-SSA antibodies also uptake auto-antigens more efficiently, possibly associated with the presence of type I IFN.

pSS-cDC2s increase proliferation of CD4⁺ T cells with a tissue homing signature

In the context of the enhanced antigen uptake observed in pSS-cDC2s, we next evaluated whether *in vivo* priming by these cells differently affects CD4⁺ T cell activation *in vitro*. CD4⁺ T cells from HC were allogenic co-cultured with cDC2s from either pSS patients or HC for 3 days (**Figure 5A**). No significant differences were observed among the CD4⁺ T cell subsets between the different donors at the start of the experiment (**Figure 5B** and **Supplementary Figure 8A**). Co-culture of CD4⁺ T cells with pSS-cDC2s significantly increased the frequency of proliferating CD4⁺ T cells compared to co-culture with HC-cDC2s (**Figure 5C-D** and **Supplementary Figure 8B**). In addition, we observed an increased percentage of proliferating CD4⁺ T cells expressing CXCR3 and CXCR5 in the presence of pSS-cDC2s when compared with HC-cDC2s. No differences were observed on CCR4 expression (**Figure 5E-F**). Furthermore, CD4⁺ T cells co-cultured with pSS-cDC2s produced substantially increased levels of TNFα, but not IFNγ (**Figure 5G**). Thus, the altered transcriptional profile and observed differences in antigen uptake and processing in pSS-cDC2s are associated with an increased capacity to activate CD4⁺ T cells to express markers that allow them to migrate to the salivary glands and produce proinflammatory cytokines to drive local autoimmune response.

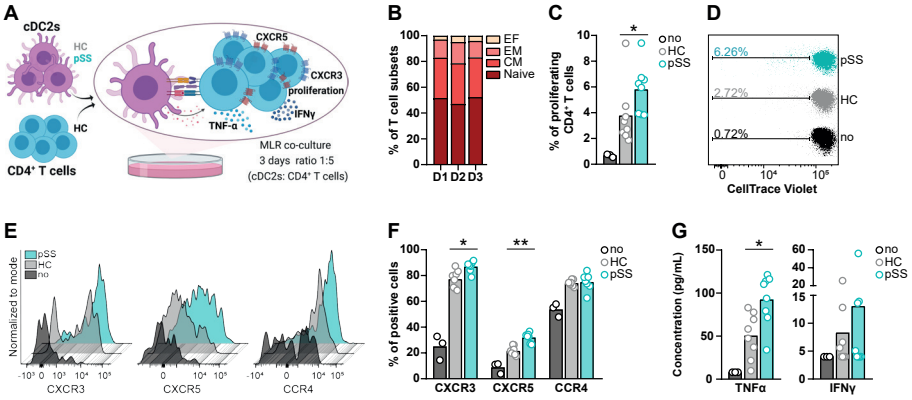


Figure 5. cDC2s from pSS patients efficiently induce CD4⁺ T cell proliferation with a tissue homing signature. Total CD4⁺ T cells from HC were cultured alone (n=3) or co-cultured either with cDC2s from HC (n=3) or pSS patients (n=3) at a 5:1 ratio (T cells: cDC2s) for 3 days (**A**). The frequency of CD4⁺ T cell subsets (Naïve; CM: central memory; EM: effector memory; EF: effector) was assessed by flow cytometry directly after cell isolation (**B**). Quantification (**C**) and representative flow cytometry dot plot (**D**) of proliferating CD4⁺ T cells measured by flow cytometry at day 3. Representative histograms of CXCR3, CXCR5 and CCR4 expression on proliferating CD4⁺ T cells (**E**) and quantification of the percentage of chemokine receptor expressing CD4⁺ T cells measured by flow cytometry (**F**). TNFα and IFNγ production during T cells: cDC2s co-culture was measured by ELISA (**G**).

Discussion

In-depth transcriptional profiling was performed in two independent cohorts and identified consistent transcriptional alterations in cell trafficking, activation, interferon signaling, and class I mediated antigen processing and presentation in cDC2s from pSS patients compared to nSS patients and HC. Using in vitro cultures with primary isolated cDC2s from pSS patients, we confirmed that pSS-cDC2s display altered uptake and processing of (auto-) antigens. We observed that these differences were most pronounced in pSS patients with anti-SSA antibodies, linked with type-I IFN and possibly associated with enhanced antigen presentation to CD4⁺ and CD8⁺ T cells. Indeed, pSS-cDC2s mediated increased proliferation of CD4⁺ T cells, associated with increased expression of chemokine receptors and enhanced production of TNFα.

Our transcriptomic analysis revealed that the altered molecular signature found in cDC2s from pSS patients is, at least in part, shared by cDC2s from nSS patients. Although nSS patients do not display signs of systemic autoimmune disease, this heterogeneous group of patients share severe objective dryness,

and occasionally present single systemic features similar to pSS patients. The overlap of clinical features and DEGs between pSS and nSS, suggests that nSS patients might have a low-grade inflammatory environment similar to pSS patients and thus might share immune mediated pathological processes driving symptom burden. In accordance, we previously showed that in nSS patients both the transcriptomic profile of plasmacytoid dendritic cells and monocytes display an intermediate phenotype that largely overlaps with that of pSS patients [28, 29]. In addition, nSS patients also share locally and systemically signs of immune activation with pSS patients such as presence of effector T cells (Th1, Th17 and Tfh cells) in the salivary glands and similar proteomic profile and increased circulating U6-sncRNA in circulation [30-32]. However, differences in IFN-signaling and non-sense mediated decay pathways in cDC2s of nSS patients could indicate that these patients still maintain some regulatory mechanisms to ensure cellular homeostasis [33]. Altogether, the transcriptional profile of nSS patients reveals an intermediate phenotype between HC and pSS patients. As such, longitudinal studies and further investigation in the regulation of the immune profile of nSS patients is essential to gain more insight into the mechanisms driving immune activation and development of pSS immunopathology.

The presence of cDC2s in the salivary glands of pSS patients is clear [12, 13], however the mechanisms that regulate the migration of cDC2s to the inflamed glands is unknown. Given the increased CX3CR1 expression in pSS-cDC2s and since fractalkine levels (the CX3CR1 ligand; CX3CL1) are increased in the salivary gland of pSS patients [34], the CX3CL1-CX3CR1 axis could mediate the presence of cDC2s in the inflamed glands of pSS patients. In fact, recruitment and adhesion of CX3CR1-expressing cDC2s mediated by fractalkine lead to their accumulation in inflamed kidneys [23]. Furthermore, reduction of plexin B2 (PLXNB2), a negative regulator of IL-12/IL-23p40 response [20], is in line with increased IL-12 levels found in pSS patients [35]. Hence, a reduction of PLXNB2 expression may contribute to increased IL-12 production in pSS patients. Moreover, the decreased integrin beta-2 (ITGB2) expression in pSS-cDC2s is in accordance with an activated state of these cells, in view of its role as negative regulator of TLR activation [21] and mediator of T cell priming by DC [36]. As such, the enhanced CX3CR1 expression combined with changes in cell activating pathways indicates that cDC2s could significantly contribute to the maintenance and possibly the initiation of inflammatory responses in pSS.

The less efficient antigen processing observed in cDC2s from pSS patients can possibly lead to long-term antigen storage, shown to occur in mature DCs [37]. This antigen retention capacity by cDC2s together with their migratory profile suggests that pSS-cDC2s favor the coordination of an efficient antigen presentation/cross-presentation to T cells at specific inflammatory sites. Long-term antigen storage can promote more effective cross-presentation via MHC-I, which is in-line with the increased *TAP1* and *LNPEP* expression observed in pSS-cDC2s. These molecules can potentiate cross-presentation by enhancing the transport of antigens-derived peptides for binding to MHC-I [38] and by trimming peptides in optimal size for loading on MHC-I [39], providing potent $CD8^+$ T cell activation that in turn can contribute to exacerbate the immune inflammation in the salivary glands. Hence, the activated $CD8^+$ T cells present in the salivary glands of pSS patients, which are associated with increased lymphocytic focus score, disease severity and autoimmunity [40] can be, in part, a consequence of an impaired regulation of cross-presentation by cDC2s.

Type-I IFNs are pleiotropic cytokines that can affect the maturation and activation of cDC2s [11], but also antigen-related processes. We showed that IFNα priming increased the uptake capacity of HC-cDC2s to levels observed in pSS-SSA+ patients and that impaired antigen processing was also related with type-I IFNs. Although antigen processing by cDC2s remained unaltered after short IFNα priming (3 hours), stimulation for 24 hours was effective to up-regulate genes involved in class I antigen processing and cross-presentation in a subset of cDC2s [11], suggesting that the modulation by IFN might be a long-lasting process. In line, IFNα–driven monocyte-derived DCs were characterized by up-regulation of genes linked to antigen processing and degradation, resembling cDC2 from pSS patients with anti-SSA antibodies [24, 41]. Thus, the auto-sustaining feedback loop that in part, maintains IFN production and anti-SSA antibodies alters cDC2 functions and possibly cDC2s-mediated T cell activation.

Apoptosis represents an important source of self-antigens for DCs, and it has recently emerged as a possible mechanism to expose auto-antigens in pSS [42, 43]. In fact, this process can trigger immune cell activation to self-proteins [44] as challenging of DCs with apoptotic cells induces their maturation, IL-12 production and Th1/Th17 responses [45, 46]. Moreover, apoptosis of epithelial cells leads to cleavage and translocation of Sjögren's autoantigens, including α-fodrin and SSA, into apoptotic particles [47]. The increased uptake of apoptotic cells by cDC2s of anti-SSA+ pSS patients could represent a mechanism through

which cDC2s activate CD4⁺ T cells and B cells to maintain the autoantibody production observed in pSS. Although the mechanisms underlying the antigen uptake and processing by pSS-cDC2s need to be further clarified, our results suggest that these processes may be driven by type-I IFN and could significantly contribute to the chronic inflammatory response observed in pSS.

Interestingly, the increased CD4⁺ T cell proliferation induced by pSS-cDC2s with a tissue homing signature corroborates that cDC2s are also important in reshaping the CD4⁺ T migratory profile in pSS. Blocking of CXCR3 function in a NOD mouse model of pSS reduces effector CD8⁺ T cell infiltration and TNFα expression in the salivary glands [48]. Moreover, the increased CXCR5 expression induced by pSS-cDC2s is in line with an increase frequency and recruitment to the salivary glands of pSS patients, despite the differences observed in the peripheral blood [30, 49-51]. Recruitment of CXCR5 expressing CD4⁺ T cells is mediated by CXCL13 to assure preferential B cell interaction and activation [52]. In the salivary glands of pSS patients CXCR5-expressing Tfh cells strongly correlated with *CXCL13* expression and were associated with B cell frequency and lymphocytic infiltration [30]. Thus, these findings suggest that pSS-cDC2s through the modulation of CD4⁺ T cell chemokine receptors, favor their migration to the salivary glands contributing to B cell hyperactivity. However, whether CD4⁺ T cells undergo expansion in the gland, or whether it occurs elsewhere, e.g., in the lymphoid organs, with subsequent migration still remains unclear.

In conclusion, cDC2s from pSS patients are transcriptionally altered, display an aberrant antigen uptake and processing, including self-antigens derived from salivary gland epithelial cells, and induce increased proliferation of tissue-homing CD4⁺ T cells. Although our study has limitations related with sample size and heterogeneity of the RNA-sequencing analysis cohorts, we were able to successfully corroborate these results using an independent set of pSS patients and HC to experimentally validate our hypothesis. Future studies in larger cohorts of patients with integrated analysis of different multi-omics data from different tissues would be valuable to support our study. These data represent the first evidence of molecular and functional alteration of cDC2s in pSS, highlighting a novel pathway via which cDC2s contribute to the pSS pathogenesis.

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Competing Interests

TR was a principal investigator in the immune catalyst program of GlaxoSmithKline, which was an independent research program. He did not receive any financial support other than the research funding for the current project. Currently, TR is an employee of Abbvie where he may hold stock. TR had no part in the design and interpretation of the study results after he started at Abbvie.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

AL, MH, TR, and JR were involved in conception and design of the study. AL, MH, AH, SB, CB and AK were involved in data acquisition. AL, MH, CB, AP, AK, TR, and JR were involved in data analysis and interpretation. AL drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Supplementary material and methods

cDC2s isolation and RNA isolation

cDC2s were isolated from peripheral blood mononuclear-cells (PBMCs) by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^{+}$ Dendritic Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. In brief, CD19+ cells were depleted using anti-CD19-coated magnetic beads, and after CD1c+ cells were isolated using biotinylated anti-CD1c and anti-biotin-beads. cDC2s purity measured by flow cytometry (**Supplementary Table 2** and **Supplementary Figure 1**) was 90% [83–97%] (median [interquartile range]), and there were no significant differences between groups.

Cells were lysed in RLTplus buffer (Qiagen) supplemented with 1% beta-mercaptoethanol for transcriptional analyses. Total RNA was purified using AllPrep Universal Kit (Qiagen), according to the manufacturer's instructions. RNA concentration was assessed with Qubit RNA Kit (Thermo Fisher Scientific) and RNA integrity was measured by capillary electrophoresis using the RNA 6000 Nano Kit (Agilent Technologies); all samples had a RIN-score >7.0.

RNA sequencing analysis

RNA sequencing was performed at the Beijing genomics institute, at two different time points, using a NextSeq 500 sequencer (Illumina) for the discovery cohort and an Illumina HiSeq 4000 sequencer (Illumina) for the replication cohort, following the standard manufacturer's protocols. For both cohorts about 20 million paired-end (91 bp for discovery; 100 bp for replication) reads were generated for each sample. RNA-sequencing data analysis was performed as previously described [1]. Briefly, FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), was used to assess the quality control of the reads. All samples passed the quality check. Next, reads were aligned to the human genome assembly (GRCh38 build 79) [2] using STAR aligner [3]. The aligned reads (mapping quality > 30) were used to calculate the read counts using Python package HTSeq [4] for each annotated gene. To remove unwanted variance (k=1 parameter) related with technical aspects RUVSeq was used [5]. A total of 65217 genes were analyzed in both cohorts. Differentially expressed genes (DEGs) were identified using Bioconductor/R package DESeq2 [6]; Wald's test was used to identify DEGs in each pair-wise comparison performed between the three groups (HC, nSS, and pSS) and likelihood ratio test to identify DEGs considering multiple groups. Differences in gene expression with a nominal p-value <0.05 were considered differentially expressed. Variance stabilizing

transformation was applied to obtain normalized gene counts (variance stabilized data), which were used for subsequent analyses. Raw and processed RNA sequencing data are available in NCBI's Gene Expression Omnibus under the following accession number GSE200020.

Pathway enrichment analysis

Pathway enrichment analysis, was performed for the consistently DEGs in both cohorts using the R/Bioconductor package ReactomePA [7] to identify the processes and pathways dysregulated between patients and HC.

Flow cytometry

For validation of the selected targets identified by RNA sequencing, PBMCs were cryopreserved in complete medium (RPMI glutamax (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich) and 1% penicillin/streptomycin (Thermo Fisher Scientific)) and freezing medium (20% (v/v) DMSO in FCS) at a 1:1 ratio. PBMCs were thawed and washed prior to incubation with the fixable viability dye eFluor506 (eBioscience) to allow exclusion of dead cells and blocked with Fc receptor blocking reagent (Miltenyi Biotech). Hereafter cells were stained for 20min at 4°C with the fluorochome-conjugated monoclonal antibodies described in **supplementary Table 2** and measured by flow cytometry. cDC2s were identified based on the expression of HLA-DR, CD1c, CD11c and FcεR1 (**Supplementary Figure 4A**).

Antigen processing and uptake by cDC2s

To study processing by cDC2s, PBMCs were thawed as previously described and directly incubated in complete medium with 0.5 µg/mL of DQ-green bovine serum albumin (BSA; Biovision) for 10 min at 37°C. Next, cells were washed, resuspended in complete medium and chased for 10, 30, 60 and 120 min. At the specific time points, samples were placed on ice and when all time points were collected, cells were washed twice with cold phosphate-buffered saline (PBS) (Sigma) with 0.1% FCS and 0.05% NaN3 (Immunosource), stained on ice for 15 min with the antibodies described in **supplementary Table 2** and measured by flow cytometry (**Supplementary Figure 4A**). BSA processing by cDC2s was calculated as the median fluorescence intensity (MFI) of DQ-BSA normalized to T=0.

For BSA uptake assay, PBMCs were incubated in complete medium with 0.5µg/mL of BSA conjugated with Alexa Fluor 647 (Invitrogen) at 37°C. In addition, HC-PBMCs were primed with 1000U/mL of IFNα2a (Cell Sciences) or left in complete medium for 3h before BSA exposure. BSA uptake was monitored at 10, 30, 60

and 120 minutes. At the specific time point, samples were placed on ice and after all time points were collected, cells were washed twice with cold PBS (Sigma) with 0.1% FCS and 0.05% NaN3 (Immunosource), stained on ice for 15 min with the antibodies described in **supplementary Table 2** and measured by flow cytometry (**Supplementary Figure 4A**). BSA uptake was calculated as the median fluorescence intensity (MFI) of BSA-AF647 on cDC2s.

Interferon signature assessment

To determine the type I IFN-score, PBMCs were lysed in RLTPlus buffer (Qiagen) and total RNA was purified using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using Superscript IV kit and quantitative-PCRs were performed on the QuantStudio 12k flex system (both Thermo Fisher Scientific). The expression of each IFN-induced genes (*IFI44L*, *IFI44*, *IFIT3*, *LY6E*, and *MX1*) was normalized to that of the endogenous *GAPDH* (**Supplementary Table 3**). The average IFN-score was calculated as previously described [8].

Uptake of apoptotic salivary gland epithelial cells by cDC2s

The human submandibular salivary gland (HSG) epithelial cell line (HTB-41) was kindly provided by Dr. Lynne Bingle (School of Clinical Dentistry, University of Sheffield). HSG cells were cultured in McCoy's 5A media (Thermo Fisher Scientific) supplemented with 1% penicillin/streptomycin (Thermo Fisher Scientific) and 10% FCS (Sigma-Aldrich).

HSG cells were stained with 0.1 µM of carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific) and induced to apoptosis during 24h with 1 µM of staurosporine (Merck Chemicals BV), as previously described [9]. After that, supernatant was collected to preserve floating cells and adherent cells were rinsed with PBS (Sigma-Aldrich) and harvested by standard trypsinization. The frequency of apoptotic HSG cells was assessed by annexin V staining according to the manufacturer's protocol. Cryopreserved PBMCs were thawed, rested and then either primed with or without IFNα2a, as previouly described, or co-cultured at a 1:1 ratio with apoptotic HSG cell suspension for 2h at 37°C. Cells were washed twice with cold PBS (Sigma) with 0.1% FCS and 0.05% NaN3 (Immunosource), stained on ice for 15 min with the antibodies described in **supplementary Table 2** and measured by flow cytometry (**Supplementary Figure 7**). Uptake of apoptotic HSG cells was quantified using the CFSE median fluorescence intensity (MFI).

cDC2s and CD4$^+$ T cell allogenic co-cultures

CD4$^+$ T cells were isolated from buffy coats (Sanquin) of 3 different donors by MACS using CD4$^+$ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. To ensure consistent purity and comparable T cell compartments of the isolated CD4$^+$ T cell, cells were stained with the antibodies described in **supplementary Table 2**. The purity of the isolated CD4$^+$ T cells was consistently above 90% for all the donors (**Supplementary Figure 8A**). cDC2s were isolated as described before and co-cultured in complete medium with allogenic CD4$^+$ T cells at a 1:5 ratio (cDC2s: T cells) for 3 days. To assess the proliferation rate of CD4$^+$ T cells, cells were labelled with 1.5 μM of CellTrace Violet (CTV) dye (Invitrogen), prior to co-culture. After 3 days of co-culture, cells were stained at 4°C for 10 min with fixable viability dye eFluor780 (eBioscience) to allow exclusion of dead cells, washed and stained at 4°C for 15 min with the antibodies described in **supplementary Table 2** and measured by flow cytometry (**Supplementary Figure 8B**). The percentage of proliferating CD4$^+$ T cells was measured as the proportion of CTV negative cells, and the expression of chemokine receptors on the cell-surface was evaluated within the proliferating CD4$^+$ T cells.

Flow cytometry data acquisition of all experiments was performed using a BD LSR Fortessa (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Cytokine analysis

TNFα (Diaclone) and IFNγ (Thermo Fisher Scientific) levels were measured in cell-free supernatant of cDC2s-CD4$^+$ T cell co-culture using enzyme-linked immunosorbent assay following the manufacturer's instructions.

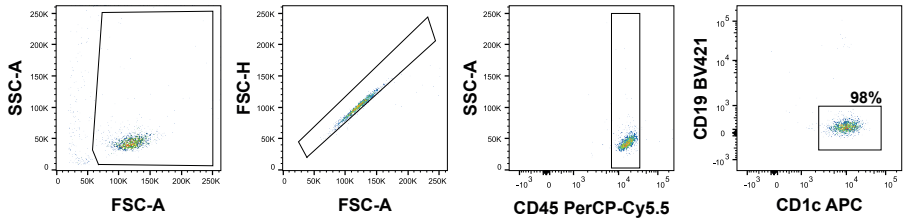
Phospho-Epitope Staining for Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated and directly stained (T=0) or cultured at 1 × 10^6 cells/mL in complete medium. Cells were left unstimulated or were stimulated with toll-like receptor (TLR)4 ligand [Lipopolysaccharide (Invivogen)] for 15, 30 and 60 minutes. Next, PBMC were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 15 minutes at room temperature. After washing with cold PBS, cells were resuspended in cold PBS before drop wise addition of ice-cold methanol (ratio PBS: methanol; 1:9) and incubated on ice for 30min. Next, cells were washed with FACS Buffer and stained for 30min on ice with the antibodies described in **supplementary Table 2**. Data acquisition was performed using a

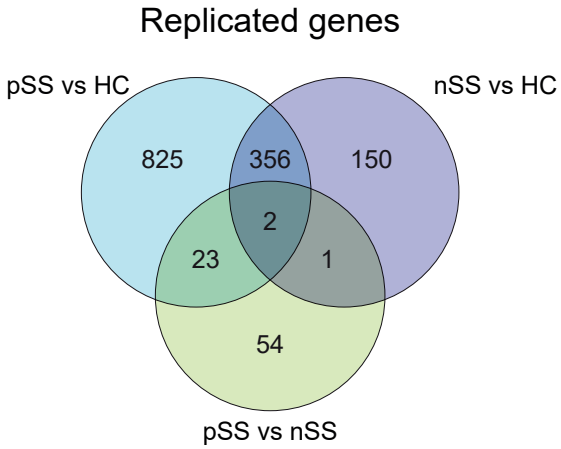
BD LSR Fortessa (BD Biosciences) and data were analysed using FlowJo software (Tree Star). cDC2s were identified based on the expression of HLA-DR, CD1c, CD11c and FcεR1 (**Supplementary Figure 4A**).

Statistical Analysis

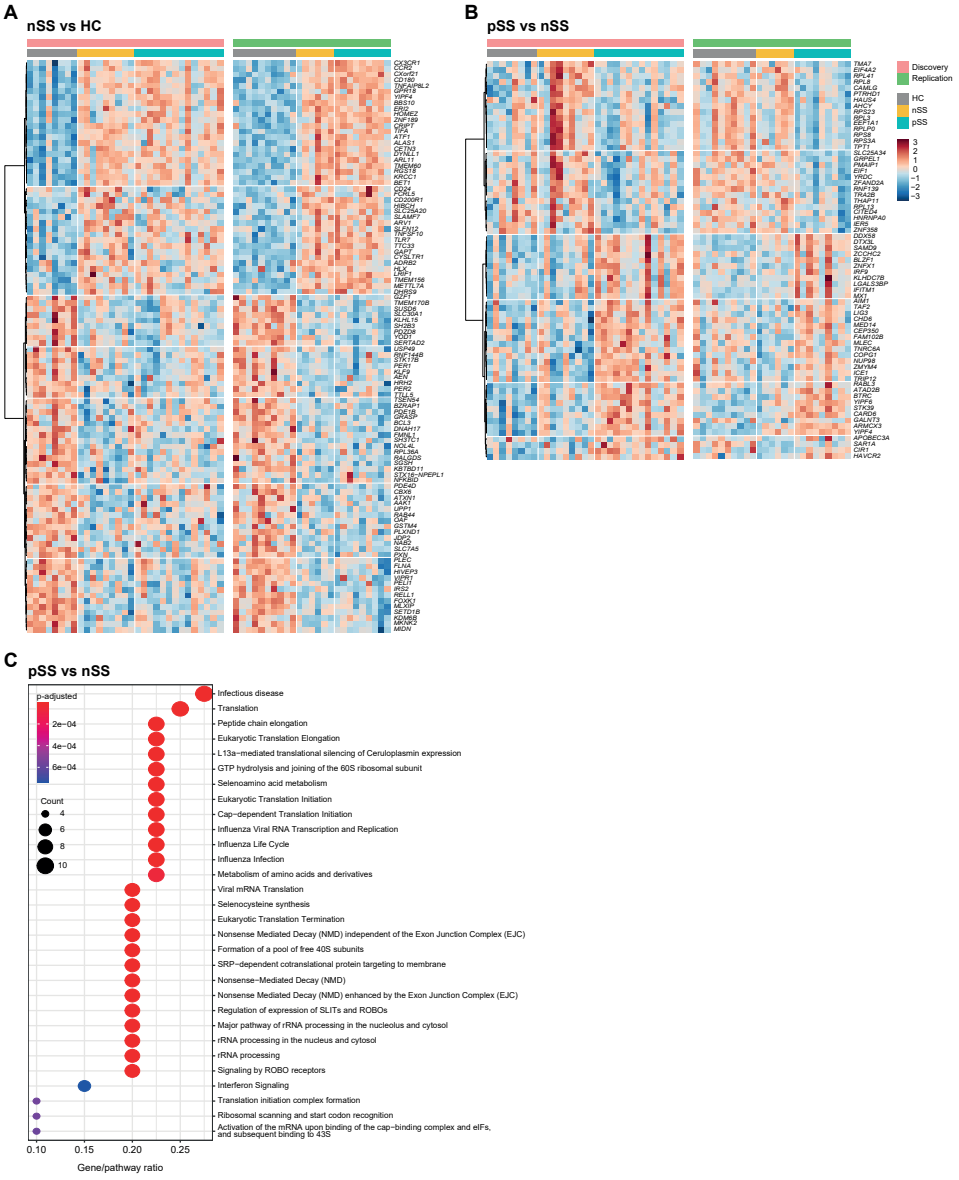
For RNA sequencing analysis, the Wald's test was used to identify DEGs in each pair-wise comparison performed between HC, nSS and pSS and the likelihood ratio test to identify DEGs considering multiple groups. Differences between the groups were analyzed by non-parametric test Mann-Whitney U-test and Kruskal-Wallis test, when appropriate. For the uptake and processing experiments at multiple time points the multiple comparison 2way ANOVA test with FDR correction was used. For the uptake experiments after IFNα priming at a single time point the paired t test was used. Statistical analyses and data visualization were performed using Python and R language, Graphpad Prism (GraphPad Software), MetaboAnalyst 4.0 [10] and ClustVis software [11]. Differences were considered to be statistically significant at $p < 0.05$.



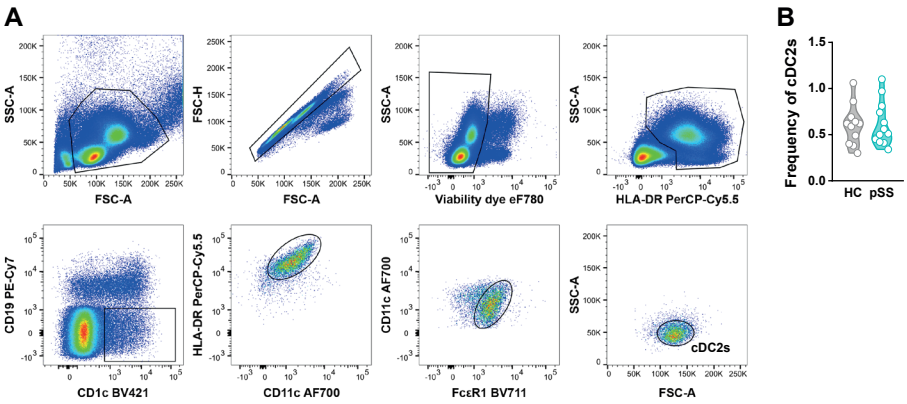
Supplementary Figure 1. Analysis strategy used to assess cDC2s purity. Flow cytometry gating strategy analysis to assess cDC2s purity after MACS isolation.



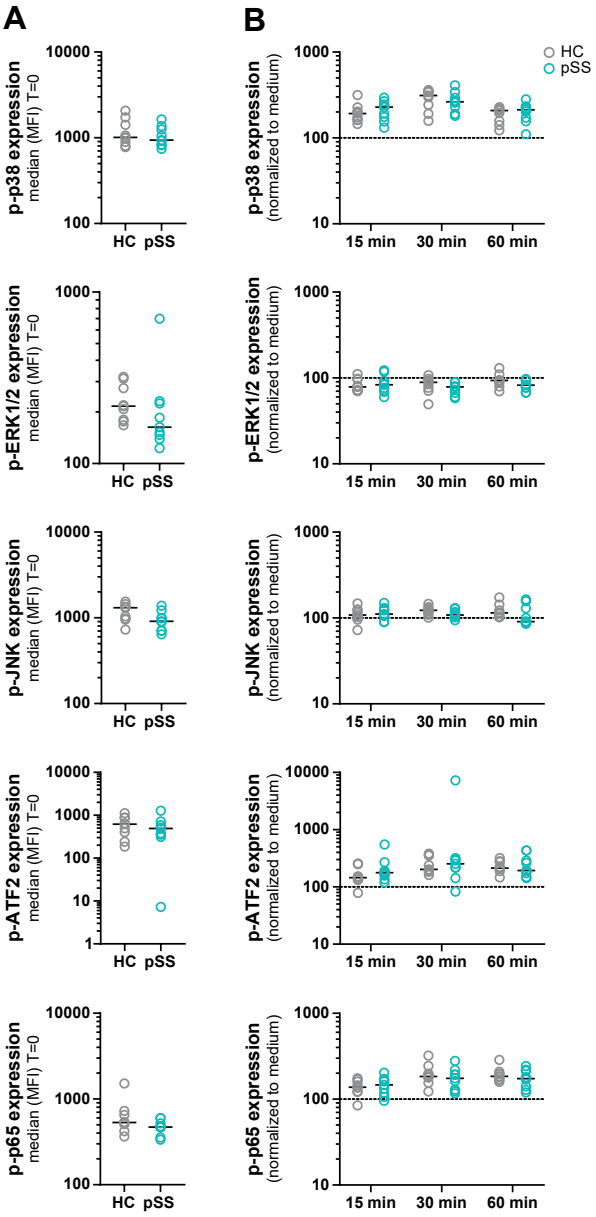
Supplementary Figure 2. Overlap of the replicated differentially expressed genes between pSS, nSS and HC. RNA sequencing of circulating cDC2s was performed independently for both discovery and replication cohort. Differentially expressed genes (DEGs) with a nominal p-value <0.05, average base mean expression >100 and with the same directionality in both cohorts were considered to be replicated. Venn diagrams show the overlap of the replicated differentially expressed genes (DEGs).



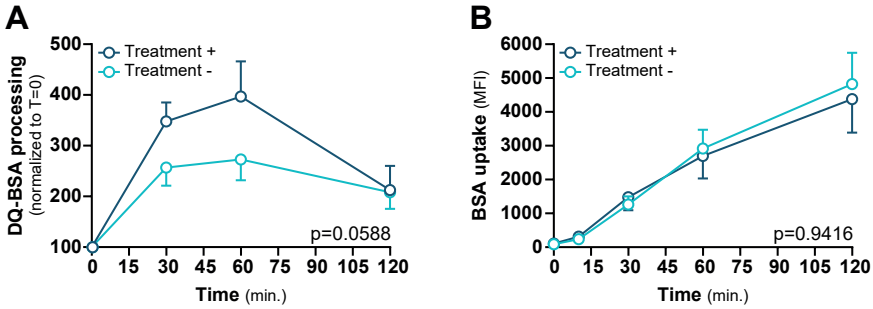
Supplementary Figure 3. Transcriptomic characterization of circulating cDC2s from nSS patients. RNA sequencing of circulating cDC2s was performed independently for both cohorts. Heatmap shows the top 100 protein coding differentially expressed genes (DEGs) in both cohorts between nSS vs. HC in both cohorts (**A**). Heatmap of protein coding DEGs with a nominal p-value < 0.05 between pSS vs. nSS in both cohorts (**B**). Pathway enrichment analysis of the DEGs between pSS vs. nSS depicted in Supplementary Figure 3B. Columns show the number of DEGs found within the pathway over the total number of pathway components (ratio), dot-size depicts the number of genes used for enrichment analysis and color indicates the statistical significance (**C**).



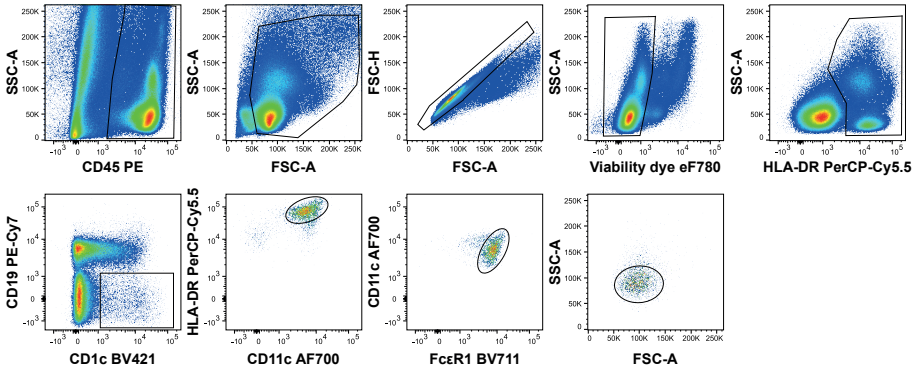
Supplementary Figure 4. Analysis strategy used to identify cDC2s in PBMCs. Representative flow cytometry gating strategy analysis to identify cDC2s in peripheral blood mononuclear cells (**A**). Violin plots depicts the frequency of circulating cDC2 in HC (n=11) and pSS patients (n=13) determined by flow cytometry (**B**).



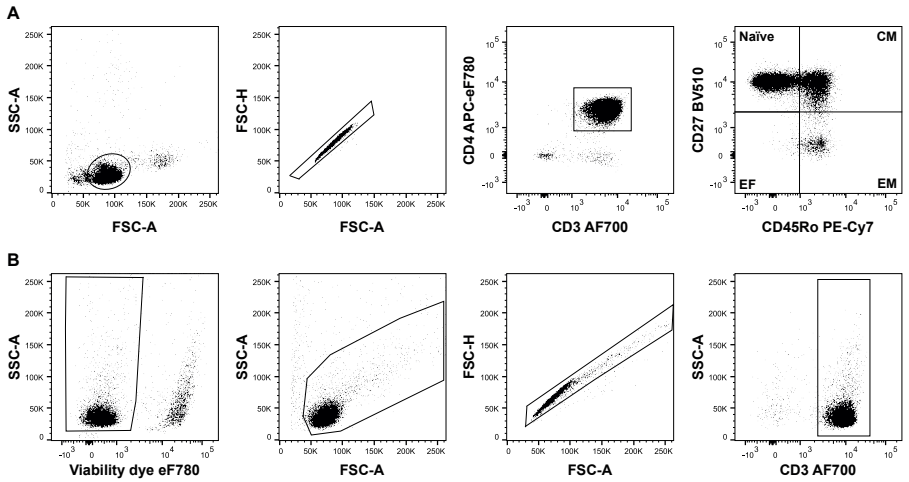
Supplementary Figure 5. Phosphorylation profile of cDC2s upon TLR4 activation. Phosphorylation levels of P38, ERK1/2, JNK, ATF2 and NF-kB were analyzed by flow cytometry in cDC2s of HC (n=9) and pSS patients (n=9) *ex-vivo* (basal level; T=0) (**A**) and after TLR4 stimulation at different time points. Phosphorylation results at the different time points was normalized to the respective medium condition (**B**).



Supplementary Figure 6. Influence of pSS treatment on antigen processing and uptake by cDC2s. Peripheral blood mononuclear cells (PBMCs) were incubated with DQ–BSA for 10 minutes and the antigen processing by cDC2s was followed at the indicated time points. DQ-BSA processing represented as median fluorescence intensity (MFI) normalized to T=0, in treated pSS patients (Treatment +; n=7) and non-treated pSS patients (Treatment -; n=6) at different time points assessed by flow cytometry (**A**). Isolated PBMCs were incubated with AF647–BSA for the indicated time points and the uptake by cDC2s was assessed by flow cytometry. BSA uptake in treated pSS patients (Treatment +; n=8) and non-treated pSS patients (Treatment -; n=6) at different time points assessed by flow cytometry (**B**).



Supplementary Figure 7. Analysis strategy used to identify cDC2s in co-culture with apoptotic salivary gland (HSG)-epithelial cells. Representative flow cytometry gating strategy analysis to identify cDC2s in peripheral blood mononuclear cells after co-culture with apoptotic salivary gland (HSG)-epithelial cells.



Supplementary Figure 8. Analysis strategy used to identify CD4⁺ T cells subsets after MACS isolation and after co-culture with cDC2s. Representative flow cytometry gating strategy analysis of the different CD4⁺ T cell subsets: naïve (CD27⁺CD45Ro⁻); CM: central memory (CD27⁺CD45Ro⁺); EM: effector memory (CD27⁻CD45Ro⁺); EF: effector (CD27⁻CD45Ro⁻) (**A**). Representative flow cytometry gating strategy analysis to identify CD3⁺ T cells in cDC2s co-cultures (**B**).

Supplementary Table 1. Characteristics of the patients and controls enrolled in the validation experiments.

	Protein validation		BSA uptake and processing		cDC2 – $CD4^+$ T cell co-culture	
	(n=44)		(n=29)		(n=6)	
	HC	pSS	HC	pSS	HC	pSS
N (M/F)	22 [1/21]	22 [1/21]	13 [0/13]	16 [0/16]	3 [0/3]	3 [0/3]
Age (yr.)	56 [25-78]	56 [21-76]	58 [35-63]	58 [41-78]	55 [43-60]	60 [56-65]
LFS (foci/4 mm^2)	-	3.0 [1.0-7.0]	-	1.7 [1.0-7.0]	-	1.4 [1.1-1.6]
ESSDAI	-	5.5 [1.0-15]	-	8.0 [0.0-16]	-	7.0 [0.0-7.0]
ESSPRI	-	7.0 [1.0-8.0]	-	7.0 [3.0-8.0]	-	6.0 [4.0-7.0]
Schirmer (mm/5 min)	-	3.0 [0.0-28]	-	1.5 [0.0-26]	-	12 [0.0-24]
ANA (no. positive [%])	-	18 [86%]	-	12 [75%]	-	2 [67%]
SSA (no. positive [%])	-	18 [82%]	-	12 [75%]	-	2 [67%]
SSB (no. positive [%])	-	13 [59%]	-	10 [63%]	-	1 [33%]
RF (no. positive [%])	-	12 [71%]	-	7 [78%]	-	2 [100%]
Serum IgG (g/L)	-	14 [7.0-33]	-	15 [8.4-26]	-	15 [9.0-16]
ESR (mm/hour)	-	15 [2.0-54]	-	18 [3.0-75]	-	10 [5.0-28]
C3 (g/L)	-	1.1 [0.8-1.6]	-	1.1 [0.9-1.4]	-	1.3 [1.2-1.4]
C4 (g/L)	-	0.2 [0.0-0.4]	-	0.2 [0.2-0.4]	-	0.3 [0.2-0.3]
Not treated (no. [%])	-	13 [59%]	-	8 [50%]	-	3 [100%]
Only HCQ (no. [%])	-	2 [9%]	-	3 [19%]	-	-
Other (no. [%])	-	7 [32%]	-	5 [31%]	-	-

HC: healthy control; pSS: primary Sjögren's syndrome; LFS: lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: anti-nuclear antibodies; SSA: anti-SSA/Ro; SSB: anti-SSB/La; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein, HCQ: hydroxychloroquine. Other treatment group includes for protein validation: azathioprine, alone (n=2) or in combination with prednisone (n=1); prednisone in combination with HCQ (n=2); methotrexate (n=2). For BSA uptake and processing: azathioprine (n=1), methotrexate, alone (n=1) or in combination with hydroxychloroquine (n=1), hydroxychloroquine in combination with leflunomide (n=1) and hydroxychloroquine in combination with azathioprine and prednisone (n=1). Values are median [range] unless stated otherwise.

Supplementary Table 2. List of antibodies used for the flow cytometry stainings

Target	Label	Manufacturer	Clone	cDC2s Purity	Protein validation
BDCA1	APC	eBioscience	L161	x	
CD14	FITC	Miltenyi	TÜK4	x	
CD19	BV421	Biolegend	HIB19	x	
CD20	PE	eBioscience	2H7	x	
CD45	PerCP	Biolegend	HI30	x	
FcεR1	BV711	Biolegend	AER-37		x
CX3CR1	FITC	Biolegend	2A9-1		x
BAFF	PE	Biolegend	T7-241		x
CD18 (ITGB2)	FITC	Biolegend	TS1/18		x
IFNAR1	PE	Thermo Scientific	85228		x
PLXND1	FITC	FAB4160G			x
PLXNB2	APC	FAB53291A			x
HLA-DR	PerCP-Cy5.5	Biolegend	L243		x
CD11c	AF700	eBioscience	3.9		x
CD14	APC-eF780	eBioscience	61D3		x
CD19	PE-Cy7	Beckman Coulter	J3-119		x
BDCA1	BV421	Biolegend	L161		x
BDCA1	AF488	Sony Biotechnology	L161		
CD14	APC-H7	BD Biosciences	MϕP9		
CD11c	PE-CF594	BD Biosciences	B-ly6		
CD19	BV711	Biolegend	HIB19		
Phospho-p38	AF647	Cell signaling Techn	28B10		
Phospho-ATF2	PE	Anbnova	G3		
P-p44/42 (ERK1/2)	PB	Cell signaling Techn	197G2		
P-NF-kB p65	AF647	BD Biosciences	K10-895		
Phospho-JNK	PE	BD Biosciences	N9-66		
CD16	V500	BD Biosciences	3G8		
FceR1	FITC	eBioscience	AER-37		
CD11c	PE	BD Biosciences	B-LY6		
FcεR1	PE	eBioscience	AER-37		
CD8	PerCP-Cy5.5	Biolegend	RPA-T8		
CD4	APC-eF780	eBioscience	RPA-T4		
CD56	PE-CF594	BD Biosciences	B159		
CD27	BV510	BD Biosciences	L128		
CXCR3	FITC	Biolegend	G025H7		
CXCR5	PerCP-Cy5.5	Biolegend	J252D4		
CD27	APC	BD Biosciences	L128		
CD3	AF700	Biolegend	UCHT1		
Viability dye	eF780	eBioscience	n.a.		
CCR4	PE	BD Biosciences	1G1		
CD45Ro	PE-Cy7	BD Biosciences	UCHL1		

Phospho flow	BSA uptake	DQ-BSA processing	$CD4^+$ T cell purity	cDC2s-$CD4^+$ T cell
x	x	x		
		x		
	x	x		
	x	x		
	x	x		
x				
x				
x				
x				
x				
x				
x				
x				
x				
	x	x		
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		x		
			x	
			x	
			x	
			x	
				x
				x
				x
			x	x
				x
				x
			x	x

Supplementary Table 3. Sequences of primers used for RT-qPCR

Gene	Primer forward 5′ – 3′	Primer reverse 5′ – 3′
IFI44L	CCACCGTCAGTATTTGGAATGT	ATTTCTGTGCTCTCTGGCTT
IFI44	TTTGCTCTTTCTGACATCTCGGT	TCCTCCCTTAGATTCCCTATTTGC
IFIT3	ACTGTTTCAACGGGTGTTGG	CCTTGTAGCAGCACCCAATC
LY6E	ATCTGTACTGCCTGAAGCCG	GTCACGAGATTCCCAATGCC
MX1	GCATCCCACCCTCTATTACTG	CGCACCTTCTCCTCATACTG
GAPDH	GCCAGCCGAGCCACATC	TGACCAGGCGCCCAATAC

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Chapter 6

The transcriptomic profile of monocytes from patients with Sjögren's syndrome is associated with inflammatory parameters and is mimicked by circulating mediators

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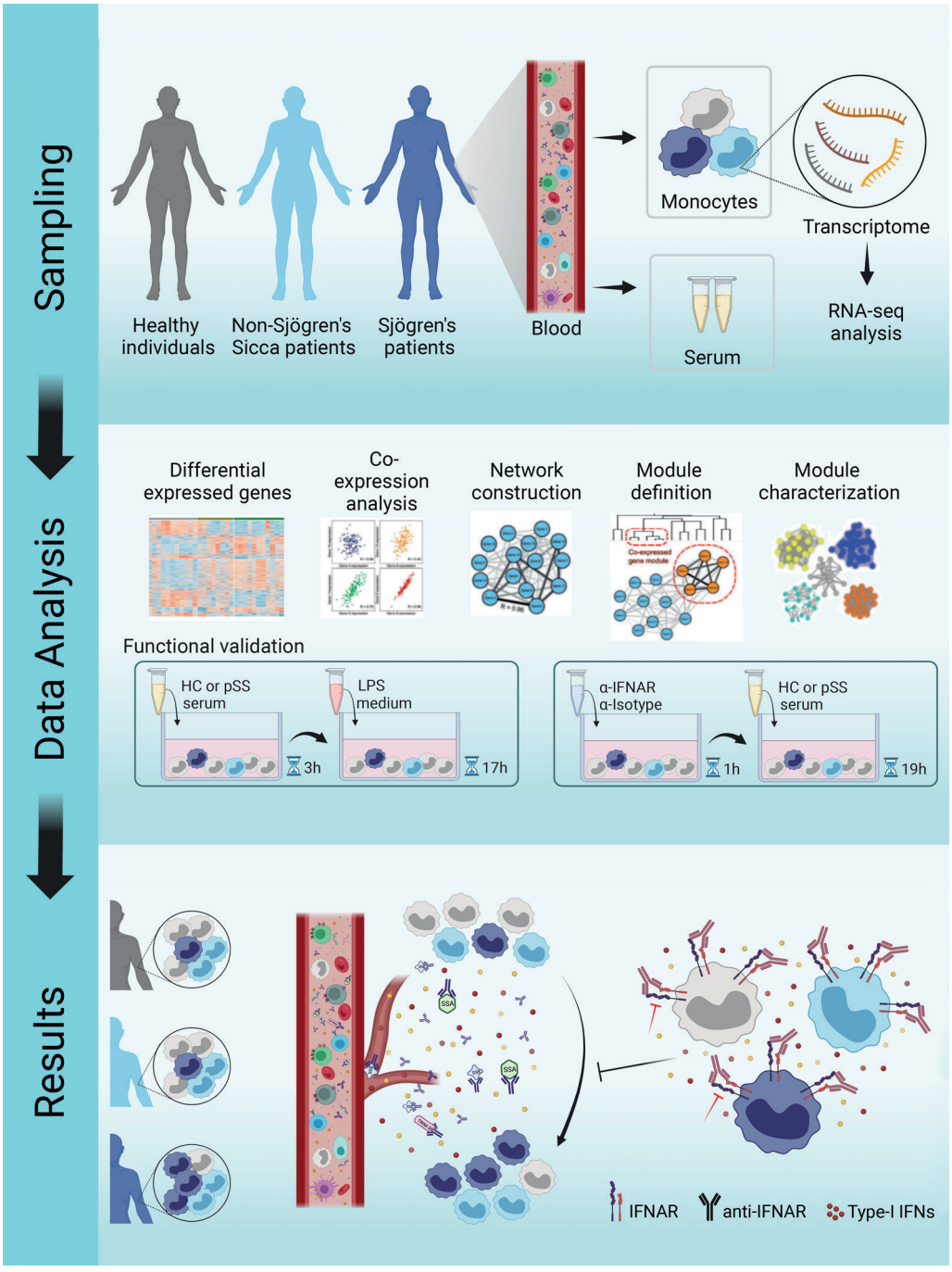
Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by infiltration of the exocrine glands and prominent B cell hyperactivity. Considering the key role of monocytes in promoting B cell hyperactivity, we performed RNA-sequencing analysis of CD14+ monocytes from patients with pSS, non-Sjögren's sicca (nSS), and healthy controls (HC). We demonstrated that the transcriptomic profile of pSS patients is enriched in intermediate and non-classical monocyte profiles, and confirmed the increased frequency of non-classical monocytes in pSS patients by flow-cytometry analysis. Weighted gene co-expression network analysis identified four molecular signatures in monocytes from pSS patients, functionally annotated for processes related with translation, IFN-signaling, and toll-like receptor signaling. Systemic and local inflammatory features significantly correlated with the expression of these signatures. Furthermore, genes highly associated with clinical features in pSS were identified as hub-genes for each signature. Unsupervised hierarchical cluster analysis of the hub-genes identified four clusters of nSS and pSS patients, each with distinct inflammatory and transcriptomic profiles. One cluster showed a significantly higher percentage of pSS patients with higher prevalence of anti-SSA autoantibodies, interferon-score, and erythrocyte sedimentation rate compared to the other clusters. Finally, we showed that the identified transcriptomic differences in pSS monocytes were induced in monocytes of healthy controls by exposure to serum of pSS patients. Representative hub-genes of all four signatures were partially inhibited by interferon-α/β receptor blockade, indicating that the circulating inflammatory mediators, including type I interferons have a significant contribution to the altered transcriptional profile of pSS-monocytes. Our study suggests that targeting key circulating inflammatory mediators, such as type I interferons, could offer new insights into the important pathways and mechanisms driving pSS, and holds promise for halting immunopathology in Sjögren's Syndrome.

Graphical abstract



Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of the salivary and lachrymal glands leading to glandular destruction and dysfunction and B cell hyperactivity (1). Although pSS pathogenesis remains to be fully uncovered, the important contribution of monocytes is evident, not only in the initial immune response but also in the maintenance of chronic inflammation (2–4). Monocytes are highly specialized in phagocytosis and antigen presentation, secrete a large range of different cytokines and chemokines, and migrate to the tissues in response to infection and injury. Once recruited to tissues, monocytes are capable of differentiating into macrophages and dendritic cells and thus contribute to local inflammation (5, 6).

Circulating blood monocytes are a heterogeneous population with a key role in regulation of inflammation (7), which can be subdivided into three major subsets: the classical monocytes (CD14+CD16-), the intermediate monocytes (CD14+CD16+), and the non-classical monocytes (CD14-CD16+), with the majority of the intermediate and non-classical monocytes emerging sequentially from the pool of classical monocytes (8, 9). Classical monocytes are primed for phagocytosis, innate sensing/immune responses, and migration, and are known to be important scavenger cells (10, 11). Intermediate monocytes represent a transitional population between the classical and non-classical monocyte subsets, with the highest capacity to present antigen (10), and induce CD4+ T cell proliferation (12). Non-classical monocytes have a pro-inflammatory behavior, secrete inflammatory cytokines in response to infection, and are involved in antigen presentation and T cell stimulation (10, 13). Recent studies have shown that this subset also contributes to the pathogenesis of several diseases, including autoimmune diseases like systemic lupus erythematosus (SLE) (14, 15). This contribution seems to be related with their patrolling behavior, Fc receptor-mediated phagocytosis, and higher secretion of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β (10, 16).

Several pieces of evidence point towards the contribution of monocytes to the immunopathology of Sjögren's syndrome. The frequency of intermediate and non-classical monocytes (3, 17) is increased in the circulation of pSS patients, pointing to their activation and differentiation. In-line with this, the intermediate monocyte subset is increased in pSS salivary glands and their numbers directly correlated with the expression of IL-34 (17), a cytokine that acts as regulator of

monocyte differentiation, proliferation, and survival (18). Further corroborating this, macrophages also accumulate in the salivary glands of pSS patients, and their presence was associated with lymphocytic infiltration and lymphoma (19). Furthermore, an abnormal activation of the NF-kB signaling pathway was observed in pSS monocytes (20), and the levels of pro-inflammatory mediators that are produced by monocytes and drive B cell activation, such as IL-6, type I interferon (IFN), B cell activating factor (BAFF), and chemokine C-X-C motif ligand 13 (CXCL13), are significantly increased in the inflamed tissues and circulation of pSS patients (21–24). Moreover, there is a marked upregulation of type I IFN-inducible genes (25) in pSS monocytes, reflecting the presence of a type I IFN signature in the salivary glands and circulation of pSS patients (26, 27). Type-I IFNs stimulate monocytes to produce inflammatory mediators (28) that drive B cell survival and maturation and ultimately sustains auto-antibody production (29). Thus, pSS monocytes seem to be activated, driving B cell hyperactivity, local inflammation, and ultimately immunopathology of pSS. Yet, the molecular mechanisms underlying the contribution of monocytes to pSS immunopathology have been poorly studied.

In-depth characterization of the dysregulated transcriptional profile of monocytes from pSS patients could offer new insights into the important pathways and mechanisms driving pSS, and ultimately might lead to the development of new therapeutic opportunities. We here exploited RNA-sequencing to investigate the transcriptional profile of circulating monocytes of pSS patients to unravel their role in the pathogenesis of pSS, and compared them to profiles of patients with non-Sjögren's sicca (nSS) and healthy controls (HC).

Materials and methods

Patients and Controls

Patients and controls were age and gender-matched and randomly allocated across the different experiments. All pSS patients fulfilled the AECG classification-criteria for pSS (30). Patients that did not fulfill the pSS classification-criteria, but presented with dryness-complaints without a known cause, in the absence of any generalized autoimmune disease were classified as non-Sjögren's sicca (nSS) patients (**Table 1**). The study was approved by the medical ethics committee of the University Medical Center Utrecht (METC no. 13-697). All patients gave their written informed consent in accordance with the declaration of Helsinki.

Table 1. Characteristics of the patients and controls enrolled in the study.

	RNA sequencing		
	HC	nSS	pSS
N (M/F)	11 [1/10]	8 [0/8]	12 [2/10]
Age (yr.)	51 [29-59]	46 [24-69]	55 [26-76]
LFS (foci/4 mm^2)	-	0.1 [0.0-0.6]	2.1 [1.0-4.0]
ESSDAI	-	-	5.0 [1.0-13]
ESSPRI	-	-	5.0 [1.0-8.0]
Schirmer (mm/5 min)	-	8.5 [1.5-32]	14 [0.0-28]
ANA (no. positive [%])	-	4 [50%]	10 [83%]
SSA (no. positive [%])	-	3 [38%]	9 [75%]
SSB (no. positive [%])	-	0 [0%]	4 [33%]
RF (no. positive [%])	-	1 [17%]	6 [60%]
Serum IgG (g/L)	-	13 [6.9-15]	16 [8.5-42]
ESR (mm/hour)	-	8 [5.0-23]	15 [3.0-77]
CRP (mg/L)	-	1.6 [0.2-5.2]	1.2 [0.0-4.2]
C3 (g/L)	-	1.2 [0.9-1.4]	1.0 [0.5-1.6]
C4 (g/L)	-	0.3 [0.2-0.4]	0.2 [0.1-0.4]
Not treated (no. [%])	-	7 [88%]	7 [58%]
Only HCQ (no. [%])	-	-	-
Other (no. [%])	-	-	4 [33%]

HC: healthy control; nSS: non-Sjögren's sicca; pSS: primary Sjögren's syndrome; LFS: lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: anti-nuclear antibodies; SSA: anti-SSA/Ro; SSB: anti-SSB/La; RF: rheumatoid factor; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein, HCQ: hydroxychloroquine. Other treatment group includes methotrexate (n=1); azathioprine, alone (n=1) or in combination with prednisone (n=2); prednisone, alone (n=1) or in combination with HCQ (n=2). Values are median [range] unless stated otherwise.

Whole blood		Serum stimulation	
HC	pSS	HC	pSS
15 [0/15]	15 [0/15]	12 [0/12]	11 [0/11]
57 [25-63]	59 [22-81]	59 [52-71]	67 [33-75]
-	3.3 [1.0-6.4]	-	3.0 [1.0-6.5]
-	5.0 [0.0-13]	-	9.0 [0.0-16]
-	6.5 [2.0-9.0]	-	7.0 [3.0-9.0]
-	4.3 [0.0-24]	-	0.0 [0.0-15]
-	14 [93%]	-	11 [100%]
-	13 [87%]	-	11 [100%]
-	11 [73%]	-	11 [100%]
-	7 [78%]	-	9 [90%]
-	12 [7.9-22]	-	19 [15-41]
-	14 [2.0-27]	-	39 [11-76]
-	1.9 [0.6-22]	-	2.6 [0.9-8.1]
-	1.0 [0.9-1.1]	-	1.0 [0.8-1.4]
-	0.2 [0.1-0.3]	-	0.2 [0.0-0.3]
-	7 [47%]	-	11 [100%]
-	5 [33%]	-	-
-	3 [20%]	-	-

Monocyte Isolation and RNA Isolation

Monocytes were isolated from peripheral blood mononuclear cells by magnetic-activated cell sorting using the CD14+ isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The frequency of monocytes subsets and the purity of the isolated monocytes was measured by flow-cytometry to insure consistent results (**Supplementary Table 1**). Monocytes subsets were defined as the following: classical monocytes (CD14+CD16-), intermediate monocytes (CD14+CD16+), and non-classical monocytes (CD14-CD16+) (**Supplementary Figure 1**). The purity of the isolated samples was (median [range]) 96% [89–99%], and there were no significant differences in cell purity between any of the groups. Cells were lysed in RLTplus buffer (Qiagen) supplemented with 1% beta-mercaptoethanol and total RNA was purified using AllPrep Universal Kit (Qiagen), according to the manufacturer's instructions. RNA concentration was assessed with Qubit RNA Kit (Thermo Fisher Scientific) and RNA integrity was measured by capillary electrophoresis using the RNA 6000 Nano Kit (Agint Technologies); all samples had RIN-score >7.0.

RNA Sequencing and Data Analysis

RNA sequencing was performed at the Beijing genomics institute, using an Illumina HiSeq 4000 sequencer (Illumina) following the standard manufacturer's protocols. About 20 million 100bp paired-end reads were generated for each sample. RNA-sequencing data analysis was performed as previously described (31).

Briefly, quality control of the reads was assessed with FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and all samples passed the quality check. Next, reads were aligned to the human genome using STAR aligner (32). The Python package HTSeq (33) was used to calculate the read counts for each annotated gene. Differentially expressed genes (DEGs) were identified using Bioconductor/R package DESeq2 (34); Wald's test was used to identify DEGs in each pair-wise comparison performed between the three groups (HC, nSS, and pSS) and likelihood ratio test to identify DEGs considering multiple groups. P-values were corrected using a false discovery rate (FDR) of 5% according to the Benjamini and Hochberg method. Differences in gene expression with a corrected p-value<0.05 were considered differentially expressed. Variance stabilizing transformation was applied to obtain normalized gene counts (variance stabilized data), which were used for subsequent analyses.

Gene Set Enrichment Analysis

Gene set enrichment analysis was conducted with Broad Institute software (35), to explore the contribution of each monocyte subset to the transcriptomic profile identified in monocytes from pSS patients. Thousand random permutations were performed to calculate the normalized enrichment score and FDR-corrected p-value. Gene sets were considered significantly enriched with a corrected p-value<0.05. Gene sets were obtained from a publicly available microarray dataset which identified the representative gene signature of each monocyte subsets (classical, intermediate, and non-classical monocytes) (10).

Weighted Gene Co-Expression Network Analysis

To study the inter-dependence between genes and their interactions, a gene co-expression network was performed using weighted gene co-expression network analysis (WGCNA) Bioconductor/R package (36). Gene co-expression networks were constructed using all differentially expressed genes (corrected p<0.05) in both comparisons, nSS or pSS vs. HC (**Supplementary Figure 2**). Networks were defined by first performing an unsigned pairwise spearman's correlation between genes and subsequently transforming the co-expression similarities into an adjacency matrix and scaling the adjacency matrix to achieve a scale-free topology (scaling power = 9, selected to have a network that fits the scale-free topology criterion). The identified networks were labelled using a conventional color scheme (blue, brown, turquoise and yellow) and further designated as gene signatures.

Pathway Enrichment Analysis

To understand the biological meaning of the identified signatures, pathway enrichment analysis was performed in the different sets of genes using the compareCluster function in the ReactomePA package (37) to compare and plot the pathways enriched in the different signatures.

Identification of Key Signatures and Hub-Genes Associated With Clinical Traits

For each signature, the expression profile was summarized by the first principal component of the signature expression level to calculate the module eigengene expression, which represents the average expression level of the genes within the signature. Next, to identify signatures that were correlated with relevant clinical features of pSS patients, a module-trait association was calculated using the pearson's correlation coefficient between the module eigengene and the clinical traits.

To gain further insight into the signatures and the genes that have the closest relationship with the clinical features, an intramodular analysis was performed. Module membership defined as the degree of correlation between the gene and the signature, and gene significance calculated as the absolute value of the association between the gene expression profile and each clinical trait were used. Correlation with a r value higher than 0.3 and a p-value<0.05 were plotted and selected for hub-gene identification. Hub-genes were identified in the brown, yellow and turquoise signature and defined by a module membership higher than 0.8 and a gene significance higher than 0.4. Next, hierarchical cluster analysis of the hub-gene expression based on Ward's method with Euclidian distance was performed using the MetaboAnalyst5.0 online software (http://www.metaboanalyst.ca/) to identify patients with a similar inflammatory profile.

The IFN-score was calculated using the gene expression of five IFN-induced genes (*IFI44L*, *IFI44*, *IFIT3*, *LY6E*, and *MX1*), after z-score normalization, as previously described (22).

TNF-α Production Upon Toll Like Receptor Activation

As functional annotation of the turquoise signature revealed enrichment in genes involved in TLR-signaling we set out to confirm these alterations on a functional level. For this purpose, whole blood was diluted 1:1 in RPMI-1640 medium with 1% L-glutamine (both from Thermo Fisher Scientific) and stimulated with different TLR ligands (**Supplementary Table 2**). 1h after stimulation, 10µg/mL of Brefeldin A (Sigma) was added. After a total of 6h of stimulation cells were stained with the extracellular antibodies and after washing, fixation and permeabilization with FIX&PERM (Thermo Fisher Scientific), cells were stained with anti-TNF-α (**Supplementary Table 1**). Data acquisition was performed using a BD LSRFortessa (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

In Vitro Monocyte Stimulation and IFNα/β Receptor Blockade

Monocytes from HC were isolated as previously described, and treated with serum of HC or pSS patients (20% v/v) for 20h, either alone or in the presence of TLR4 ligand (0.1µg/mL of LPS; Invivogen) for the last 17h of culture. Simultaneously, monocytes were pre-incubated for 1h at 37°C with 5µg/ml of either anti-IFNα/βR2 (clone MMHAR-2; PBL Assay Science) or its respective isotype control (IgG2a, Thermo Fisher Scientific), and then treated with serum of pSS patients (20% v/v) for 24h. Supernatants were harvested and cells lysed in RLTplus buffer (Qiagen). RNA isolation was performed as previously described. Quantitative-PCR reactions were performed using SYBR Select Master Mix (Applied Biosystems) on the

Quantstudio 12k system (Thermo Fisher Scientific). Relative gene expression levels were normalized to the geometric mean expression of two housekeeping genes: *B2M* and *ACTB* (**Supplementary Table 3**). The relative fold change (FC) of each sample was calculated in relation to the average ΔCt of the HC group or to the ΔCt of the respective isotype condition (reference), according to the formula FC = 2−ΔΔCt, where ΔΔCt = ΔCt sample —ΔCt reference. TNF-α levels were measured in the supernatants by ELISA (Diaclone), following the manufacturer's instructions.

Statistics

Differences in gene expression with a corrected p-value<0.05 were considered statistically significant. Kruskal-Wallis H test was used to analyze the differences between the nSS, pSS, and HC groups and between patient clusters. Fisher's exact test was used to compare categorical variables. Differences between pSS patients and HC were assessed using Mann-Whitney U-test. Changes observed after IFNα/βR2 blocking were analyzed using Wilcoxon matched-pairs signed rank test. Statistical analyses and data visualization were performed using Python and R, Graphpad Prism (GraphPad Software), and MetaboAnalyst 5.0 (38). Differences were considered to be statistically significant at p<0.05.

Results

Transcriptome of pSS Monocytes Is Enriched for Gene Expression Profiles Associated With Intermediate and Non-Classical Monocytes

To better understand the contribution of monocytes to the development of pSS and to identify monocyte-specific processes dysregulated in pSS, we performed RNA-sequencing profile of circulating monocytes of pSS and nSS patients compared to HC (**Table 1**). Differential expression analysis revealed that among the studied groups, 6.036 genes were differentially expressed with a nominal p-value ≤ 0.05, of which 3.126 were upregulated and 2.910 downregulated. The majority of the differentially expressed genes (3.408 DEGs) were found in the pSS vs. HC comparison, representing 56% of all the DEGs, and out of these 1.731 were uniquely differentially between pSS and HC. In addition, 29% off all DEGs (1.757 genes) were differentially expressed in nSS patients vs. HC, and only 465 (26%) were exclusively found differentially expressed between nSS and HC. Furthermore, in the comparison between pSS and nSS, out of the 871 DEGs only a small number of genes (280/32%) was uniquely differential between pSS vs. nSS (**Figure 1A**). Overall, the transcriptomic profile of pSS monocytes revealed clearly distinct

expression profiles compared to HC monocytes, but relatively similar profiles to nSS monocytes. To identify the most robust and consistently altered genes, those differentially expressed with an FDR corrected p-value ≤ 0.05 and with at least moderately high expression levels (base mean expression >100) were selected for further analysis (**Supplementary Figure 2**).

To gain further insights into the molecular signature of pSS monocytes, we performed gene set enrichment analysis to evaluate the contribution of each monocyte subset to the transcriptional profile found in pSS monocytes. To test this, we compared the DEGs in pSS patients that met the selection criteria (**Figure 1B**) with publicly available gene expression profiles of the three different monocyte subsets (10). Enrichment analysis revealed that genes highly expressed in intermediate and non-classical monocytes were significantly enriched in the transcriptional profile of pSS monocytes (**Figure 1C**). In line with this, flow-cytometry analysis of the monocyte subsets confirmed that the non-classical monocyte subset was increased in the circulation of pSS patients when compared with nSS patients and HC (**Figure 1D**). Thus, the transcriptomic alterations found in pSS monocytes are consistent with an increased frequency of non-classical monocytes.

Network Analysis Identifies Signatures of Co-Expressed Genes Associated With Systemic Inflammatory Parameters

To further analyze the functional consequences of the transcriptomic changes in pSS monocytes, we used weighted gene co-expression network analysis (WGCNA) to construct gene-correlation networks and identify signatures of co-expressed genes with pSS-associated biological functions. Network analysis using the genes that were differential between any of the three groups compared (**Supplementary Figure 2**) identified 4 gene signatures: blue (358 genes), brown (239 genes), turquoise (737 genes), and yellow (148 genes), each with distinct expression patterns (**Figure 2A and Supplementary Table 4**). 18 of the DEGs were not allocated to any of the previous signatures and therefore excluded from further analysis.

Functional annotation indicated that the blue signature was associated with translation mechanisms, the brown signature with IFN-signaling, and the turquoise signature with TLR signaling (**Figure 2B**). No pathways were enriched for the yellow signature. Next, we further characterized the identified signatures, by assessing the module eigengene expression, given by the first principal component of the signature expression level, in HC, nSS, and pSS patients. Overall, the expression of

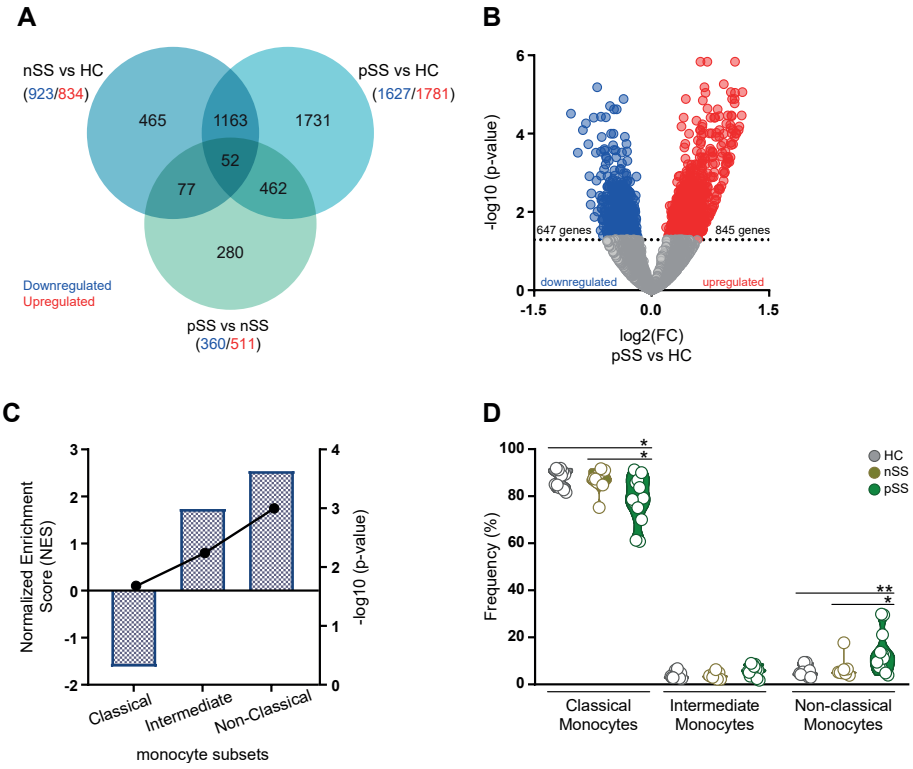
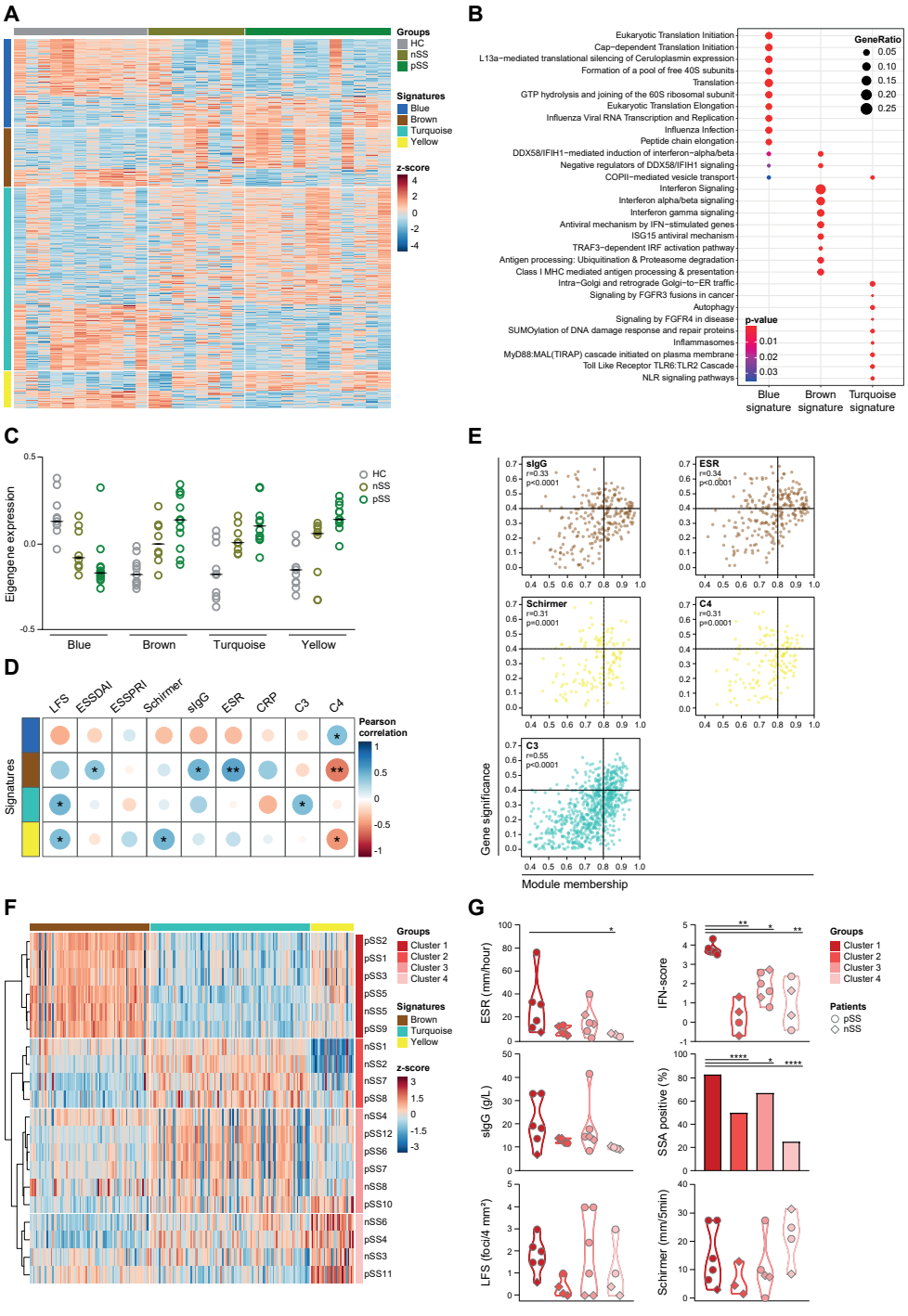


Figure 1. Transcriptomic profile of monocytes from pSS patients is enriched for genes associated with intermediate and non-classical monocytes. RNA sequencing of peripheral blood isolated monocytes of nSS and pSS patients and HC was performed and differentially expressed genes (DEGs) were identified. Venn diagram shows the overlap of the DEGs between the different comparisons with a nominal p-value < 0.05, downregulated (blue) or upregulated (red) genes are indicated for each comparison (**A**). The relationship between the fold change (log2) and the corrected p-value (-log10) of the DEGs in pSS vs. HC is displayed. DEGs, with a corrected p-value < 0.05, downregulated (blue) or upregulated (red) in pSS-monocytes are indicated (**B**). Gene-set enrichment analysis was performed comparing the DEGs identified in pSS patients to the molecular signature of the different monocyte subsets. Columns and left y-axis show the normalized enrichment score, black connected dots and right y-axis displays the FDR-corrected p-value (-log10) (**C**). Frequency of monocyte subsets assessed by flow-cytometry in HC, nSS and pSS patients after cell isolation is shown (**D**). * and ** represent p < 0.05 and p < 0.01, respectively.

genes from the blue signature was decreased in nSS and pSS patients and contains a large number of ribosomal protein genes, and a range of initiation factors and RNA polymerase subunit genes. Conversely, the expression of the brown, turquoise, and yellow signatures was largely increased in both patient groups, represented by an increased eigengene expression. The expression patterns of all


A
Groups
HC
nSS
pSS
Signatures
Blue
Brown
Turquoise
Yellow
z-score
4
2
0
-2
-4
B
Eukaryotic Translation Initiation
Cap−dependent Translation Initiation
L13a−mediated translational silencing of Ceruloplasmin expression
Formation of a pool of free 40S subunits
Translation
GTP hydrolysis and joining of the 60S ribosomal subunit
Eukaryotic Translation Elongation
Influenza Viral RNA Transcription and Replication
Influenza Infection
Peptide chain elongation
DDX58/IFIH1−mediated induction of interferon−alpha/beta
Negative regulators of DDX58/IFIH1 signaling
COPII−mediated vesicle transport
Interferon Signaling
Interferon alpha/beta signaling
Interferon gamma signaling
Antiviral mechanism by IFN−stimulated genes
ISG15 antiviral mechanism
TRAF3−dependent IRF activation pathway
Antigen processing: Ubiquitination & Proteasome degradation
Class I MHC mediated antigen processing & presentation
Intra−Golgi and retrograde Golgi−to−ER traffic
Signaling by FGFR3 fusions in cancer
Autophagy
Signaling by FGFR4 in disease
SUMOylation of DNA damage response and repair proteins
Inflammasomes
MyD88:MAL(TIRAP) cascade initiated on plasma membrane
Toll Like Receptor TLR6:TLR2 Cascade
NLR signaling pathways
GeneRatio
0.05
0.10
0.15
0.20
0.25
p-value
0.01
0.02
0.03
Blue signature
Brown signature
Turquoise signature
C
Eigengene expression
0.5
0.0
-0.5
HC
nSS
pSS
Blue
Brown
Turquoise
Yellow
D
LFS
ESSDAI
ESSPRI
Schirmer
sIgG
ESR
CRP
C3
C4
Signatures
Pearson correlation
1
0.5
0
-0.5
-1
E
sIgG
r=0.33
p<0.0001
ESR
r=0.34
p<0.0001
Schirmer
r=0.31
p=0.0001
C4
r=0.31
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C3
r=0.55
p<0.0001
Gene significance
Module membership
F
Groups
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Signatures
Brown
Turquoise
Yellow
z-score
3
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pSS2
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nSS8
pSS10
nSS6
pSS4
nSS3
pSS11
G
ESR (mm/hour)
IFN-score
sIgG (g/L)
SSA positive (%)
LFS (foci/4 mm²)
Schirmer (mm/5min)
Groups
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Patients
pSS
nSS

Figure 2. Identification and functional characterization of pSS-monocytes signatures reveals associations with markers of local and systemic inflammation. Weighted gene co-expression network analysis was performed to identify signatures of co-expressed genes and their respective functional annotation. Heatmap visualization of the 1500 DEGs across the 4 identified signatures (blue, brown, turquoise and yellow; rows) and the studied groups (HC, nSS and pSS; columns) (**A**). Reactome pathway enrichment analysis was used for functional annotation of the signatures, there was no result for the yellow signature. Dot-size depicts the fraction of the genes within the pathway that is enriched, color indicates the statistical significance of the enrichment (**B**). Eigengene expression (first principal component of the module expression level) for each signature is depicted for HC, nSS and pSS patients (**C**). Correlation matrix of the module eigengenes and the clinical traits is depicted. Signatures are shown in rows and the clinical traits in columns. Dot-size and color indicates the Pearson correlation coefficient, p-values are shown inside the dots; (**D**). Intramodular analysis identifies hub-genes related with markers of systemic and local inflammation. Scatter plots of gene significance for the selected clinical trait versus the module membership per signature are depicted. Each dot represents one specific gene within a signature. Hub-genes are defined by a module membership of >0.8 and a gene significance of >0.4. Signatures can be identified by the corresponding color of the graph (**E**). nSS and pSS patient were clustered using hierarchical clustering of the selected hub-genes using Euclidean distance and Ward's method. Hub-genes of each signature are shown in columns and the patient clusters are indicated in rows (**F**). Violin plots depicts the expression of selected clinical parameters across the established patient clusters (**G**). *, ** and **** represent $p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively.

four gene signatures in the nSS group were in general intermediate between the HC and pSS groups (**Figure 2C**).

Identified Gene Signatures Are Associated With Systemic Inflammatory Parameters

To better understand the biological relevance of the different signatures for the disease, we next assessed the correlation between clinical traits (**Table 1**) and the eigengene expression in each signature. Eigengene expression is an ideal parameter to correlate with the clinical traits, since it represents the first principal component of the co-expressed gene network and thus accounts for most of the variance in gene expression within the signature. Genes from the brown signature had the most correlations with the clinical traits, including the strongest positive correlation with ESR ($r = 0.51$, $p = 0.003$), and with ESSDAI ($r = 0.37$, $p = 0.042$). In addition, the lymphocytic focus score (LFS), which reflects salivary gland inflammation, moderately correlated with the turquoise and yellow signatures ($r = 0.43$, $p = 0.015$ and $r = 0.40$, $p = 0.024$, respectively), whereas only the yellow signature moderately correlated with Schirmer test, a measure for lacrimal gland function ($r = 0.44$, $p = 0.013$). Of note, moderate negative correlations were found with C4 levels for the brown and yellow signatures ($r = -0.51$, $p = 0.003$ and $r = -0.43$, $p = 0.016$, respectively) (**Figure 2D**).

Next, we performed an intramodular analysis to seek out the biological significance of each gene within the respective signature for the clinical features that correlated with the signature expression (**Figure 2D**). The genes within the brown, and turquoise signatures significantly correlated with sIgG, ESR, and complement C3 levels, parameters usually associated with systemic inflammation. Likewise, the genes within the yellow signature correlated with complement C4 levels and with Schirmer test, indicative of lacrimal gland dysfunction (**Figure 2E**). No correlations were observed for LFS and ESSDAI. Genes with a module membership (a higher number indicates stronger correlation of the gene with the other genes in the signature) higher than 0.8 and a gene significance (a higher number indicates stronger correlation with the selected clinical trait) higher than 0.4 were selected as hub-genes for each signature independently. Analysis of the brown signature revealed 43 hub-genes with gene significance for sIgG, and 68 hub-genes with gene significance for ESR. Out of these, 31 hub-genes were found with gene significance for both parameters (**Figure 2E and Supplementary Table 5**). Within the yellow signature, 16 hub-genes were identified with gene significance for Schirmer test and 13 were found with gene significance for C4 levels, of these 7 hub-genes were identified with gene significance for both the Schirmer test and C4 levels (**Figure 2E and Supplementary Table 6**). In the turquoise signature, analysis of gene significance for C3 complement levels yielded 107 hub-genes (**Figure 2E and Supplementary Table 7**). Thus, the molecular signatures of circulating monocytes from pSS patients seems to reflect the ongoing systemic inflammation as result of local damage.

Given the fact that hub-genes are highly associated with the clinical features of pSS, we next investigated whether the hub-gene expression could be used to identify patients with a similar inflammatory profile. Unsupervised hierarchical cluster of the selected hub-genes (**Supplementary Tables 5–7**) identified four distinct clusters of nSS and pSS patients. Cluster 1 mainly included patients diagnosed as pSS patients (5 out of 6), and all patients were characterized by an increased expression of the brown signature (**Figure 2F and Supplementary Figure 3A**). As a matter of fact, patients in cluster 1 showed the highest IFN-score compared to the patients included in other clusters (**Figure 2G**). Clustering analysis identified a group of patients included in cluster 2 with a pronounced downregulation of the yellow signature hub-genes, and a trend towards a lower LFS, ESR, sIgG and IFN score (**Figure 2G and Supplementary Figure 3A**). Patients in cluster 3 were characterized by an overexpression of the turquoise

signature and displayed an intermediate profile for ESR, IFN-score and frequency of anti-SSA antibodies, as compared with patients in cluster 1 and 2 (**Figure 2G and Supplementary Figure 3A**). Patients in cluster 4 did not display a specific signature profile (**Supplementary Figures 3A, B**). Furthermore, comparison of systemic autoimmune and inflammatory parameters between clusters showed that patients in cluster 1 have an increased frequency of anti-SSA antibodies and presented with higher ESR. Moreover, a trend towards a higher sIgG and lower C3 and C4 levels was observed in these patients compared to the patients in the other clusters (**Figure 2G and Supplementary Figure 3B**). Finally, patients in cluster 1 have higher frequency of non-classical monocytes compared to the patients in cluster 3 and 4 (**Supplementary Figure 3C**).

pSS-Monocyte Produce More TNF-α Upon Toll-Like Receptor Stimulation

Considering that the functional annotation of the turquoise signature revealed enrichment in genes involved in TLR-signaling, we next assessed whether the altered transcriptional profile of pSS-monocytes could impact monocyte activation. To confirm this, whole-blood from pSS patients and HC was stimulated with a broad panel of toll-like receptor ligands and the intracellular expression of TNF-α was evaluated by flow cytometry (**Supplementary Figure 4**). Interestingly, the intracellular TNF-α expression in monocytes of pSS patients, as determined by the median fluorescence intensity (MFI), was significantly increased upon activation with TLR4, TLR5, and TLR7/8 ligands compared to HC monocytes (**Figure 3A**). We did not observe differences in TNF-α expression between HC and pSS monocytes upon TLR2/1, TLR2/6, and TRL3 activation (**Figure 3A**).

Exposure of Healthy Control Monocytes to Serum of pSS Patients Mimics the pSS-Monocyte Gene Signature

As the molecular signatures of monocytes from pSS patients and the expression of their hub-genes were mainly associated with systemic inflammatory markers, we sought to confirm whether the transcriptomic changes found in pSS-monocytes could be induced in monocytes from healthy individuals by sera of pSS patients. To this end, we selected hallmark genes to represent each signature, and ultimately the transcriptomic profile of pSS monocytes, by selecting those hub-genes with the highest degree of correlation with each signature (high module membership), the highest biological relevance (high gene significance) and highest fold-change of expression differences between pSS and HC (**Supplementary Table 8**). Treatment with serum from pSS patients upregulated both hallmark genes of the brown signature (*MX1*, *IFITM1*), two

of the three turquoise hallmark genes (*TNFSF10*, *IRF2*), one of the two yellow signature hallmark genes (*IRF9*), and downregulated one of the three blue signature hallmark genes (*RPL5*) (**Figure 3B**). In addition, we tested whether the inflammatory mediators present in the serum of pSS patients could prime healthy monocytes for higher TNF-α production upon TLR activation. Due to limited amount of serum, we were only able to test this upon TLR4 activation. We observed that the changes induced by serum from pSS patients led to a significantly higher TNF-α production upon TLR4 stimulation (**Figure 3C**).

Type-I IFNs Importantly Mediate Effects of pSS Serum on Signature Hallmark Gene Expression

As type-I IFNs are considered to be crucial mediators of pSS pathogenesis (39), we tested whether the transcriptomic alterations were mediated by type I IFNs. Interferon-α/β receptor (IFNAR) blockade completely abrogated the pSS-serum effect on the brown gene signature, and also reverted the pSS-serum effects on *TNFSF10* and *IRF9* expression, hallmark genes of the turquoise and yellow signature, respectively. In addition, *RPL5* expression, hallmark of the blue signature, was upregulated upon IFNAR blockade, although not statistically significant. Even though the expression of *RPL15* and *EEF1B2*, hallmarks of the blue signature, were not significantly downregulated upon treatment with serum from pSS patients, IFNAR blockade significantly increased the expression of both genes. (**Figure 3D**). Thus, type-I IFNs importantly drive the observed effects of pSS serum on the identified signature hallmark genes in monocytes.

Discussion

We here identified a large number of transcriptomic differences in circulating monocytes between patients with pSS and nSS and HCs. In addition, we observed an enrichment in the non-classical monocyte subset in pSS patients, and identified gene signatures in pSS monocytes which indicated dysregulation in translation processes, IFN-signaling, and TLR signaling. Furthermore, we show that the hub-genes of the identified gene signatures correlate with systemic and local inflammatory markers, indicating that the transcriptional dysregulation of pSS monocytes reflects ongoing inflammation. Interestingly, we demonstrated that unsupervised hierarchical cluster of the hub-gene expression identified four distinct clusters of nSS and pSS patients with different inflammatory profiles. Finally, we confirmed that the transcriptomic aberrations of pSS monocytes can be induced, at least in part, by mediators present in the circulation of pSS

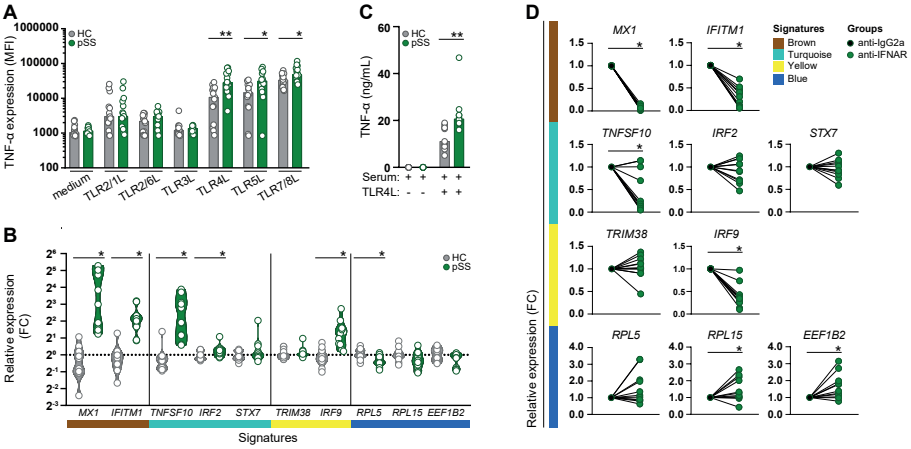

Figure 3. Monocytes from pSS patients produce more TNF-α upon TLR stimulation, and its transcriptomic signature is mimicked in HC monocytes by pSS serum and is partially prevented by IFNα/β receptor blockade. TNF-α cytokine production (given by the median fluorescence intensity, MFI) was assessed by flow-cytometry in CD14+ monocytes, after whole blood stimulation with TLR ligands (HC n= 15, pSS n= 15) (**A**). The effect of serum from HC or pSS patients (20% v/v; HC n= 12, pSS n= 11) treatment on monocytes from HC was assessed after 20h by qRT-PCR. Changes in the expression of the hallmarks of each signature were assessed (**B**). Monocytes from HC were pre-treated with serum from HC or pSS patients (20% v/v; HC n= 11, pSS n= 10) for 3h and then left untreated or stimulated with TLR4 ligand (0.1µg/mL of LPS) for a total of 20h. TNF-α secretion was measured by ELISA (**C**). Monocytes from HC were pre-treated for 1h with an isotype control (IgG2a) or an anti-IFNα/βR2 blocking antibody and subsequently treated with pSS serum (20% v/v; n=7) for 20h. Changes in the expression of the hallmark genes representing the pSS-monocyte signatures were assessed by qRT-PCR (**D**). * and ** represent $p < 0.05$, and $p < 0.01$, respectively.

patients, including type-I IFNs. Thus, the systemic inflammatory profile of pSS patients play an important role in shaping the monocyte transcriptomic profile and ultimately their function.

The current study shows that pSS monocytes are skewed to a pro-inflammatory profile, with elevated frequency of non-classical monocytes, consistent with previous reports (3, 17). This increased frequency might be related with the differentiation of classical monocytes into intermediate/non-classical monocytes sustained by the inflammatory environment, as previously described in patients with SLE and rheumatoid arthritis (40, 41). In addition, CD16+ monocytes more efficiently produce pro-inflammatory cytokines, including C-C Motif Chemokine Ligand 3 (CCL3), TNF-α and IL-6 (42, 43), known to be increased in pSS (44–46), thus contributing to the maintenance of the inflammatory environment. As such, these data suggest that the increased numbers of non-classical monocytes

enhance the inflammatory environment sustaining the differentiation of non-classical monocytes and therefore pSS immunopathology.

Using a gene co-expression network analysis, we have identified three new molecular gene signatures (blue, turquoise, yellow), which were not identified previously using differential expression analysis (25). In addition, we also identify differentially expressed genes directly related to the IFN-signature, as part of the brown signature, in line with previously reports (22, 25). Association of the brown signature with ESSDAI, complement consumption and with sIgG, a marker of B cell hyperactivity in pSS, indicates that the transcriptomic alterations induced by type I IFN contributes to enhance B cell activation and disease activity. In fact, IFN stimulation was shown to increase BAFF production by monocytes (22, 28), a key cytokine for B cell survival and activation (47), and CXCL13 production, crucial for ectopic lymphoid structures organization (48, 49), which can be found in pSS patients (50, 51). Moreover, non-classical monocytes from SLE patients, a type-I IFN disease, more efficiently promote B cell differentiation towards memory and plasma cells and IgG production (15). As such, the modulation of monocytes signature by type I IFN, is an important mechanism to promote B cell hyperactivity and local inflammation.

Functional annotation of the newly identified signatures revealed that the blue gene signature is primarily related with the cellular processes of translation, yet other relevant functions in innate immunity, including regulation of cytokine signaling, have been reported (52). Downregulation of ribosomal protein genes has been described in LPS-activated monocyte-derived dendritic cells and in peripheral blood mononuclear cells stimulated with type-I IFN (53) to act as a regulatory mechanism of cell activation. The turquoise gene signature importantly shows enrichment for TLR activation and signaling and is associated with the lymphocytic infiltration of the salivary glands (LFS). Thus, indicating that monocytes from pSS patients display an activated profile, in line with previous reports (20, 28), reinforcing the relevance of the altered monocyte function to the salivary glands' inflammation. Consistent with this, it was shown that CD16+ monocytes can differentiate into dendritic cells with a phenotype similar to those observed in salivary gland infiltrates in pSS patients (3). Functional annotation of the genes within the yellow signature, did not indicate any specific function, possibly due to the size of this signature, as it includes in total 128 protein coding genes, limiting the identification of share processed by these genes. Nonetheless, this signature was associated with LFS and Schirmer test, suggesting a potential link of these set of genes with immune cell infiltration, and damage of the exocrine

glands. As such, further research is needed to shed more light on the function of the yellow signature and confirm the role of the monocyte transcriptome in the regulation of local inflammation.

To the best of our knowledge, this is the first WGCNA analysis of RNA-sequencing data from monocytes of nSS and pSS patients which identifies hub-genes associated with systemic and local inflammatory markers. Although nSS patients may share severe objective dryness, and occasionally present with single systemic features similar to pSS patients, yet not compatible with generalized autoimmune disease, this group of patients is often poorly studied and difficult to characterize. Given the high-level of gene significance of the identified hub-genes with the clinical features of pSS we propose that their gene expression would allow the identification of patients, both nSS and pSS, with similar systemic or local inflammatory profile. Our in-depth characterization of the monocyte transcriptome demonstrates that the dysregulated mechanisms found in pSS monocytes are present in some nSS patients. As such, patient clustering based on different molecular signatures holds promise for a better characterization of sicca patients and implementation of novel therapeutic targets. We here provide proof of concept for further exploration of the transcriptome of nSS patients, as some patients share a molecular signature with pSS patients whereas the signs of local and peripheric autoimmunity are not yet present. Nevertheless, follow up studies of both nSS and pSS patients and longitudinal analysis in larger cohorts are needed to shed light on the role of the transcriptional signatures of circulating cells, and on its value for the implementation of new therapeutic options.

Transcriptomic analysis of pSS patients' monocytes suggests that these cells have an altered activation status, which was confirmed by the increased production of TNF-α by pSS monocytes. Moreover, increased NF-kB activation was previously observed in pSS-monocytes (20), which is in line with our findings, since NF-kB has a central role in the production of pro-inflammatory cytokines, including TNF-α (54). TNF-α is a potent pro-inflammatory cytokine known to be increased in tear fluid and saliva of pSS patients (55, 56), and has been strongly implicated in the apoptosis of salivary gland cells (57), causing secretory dysfunction (58, 59), contributing to the salivary gland destruction. This enhanced TNF-α production by monocytes of pSS patients could be related with the increased frequency of non-classical monocytes which we observed, as they are known to produce high amounts of TNF-α (16, 60). Although the results of anti-TNF-α treatment in pSS patients were discouraging, this cytokine does seem to play an important role in pSS immunopathology.

Furthermore, the tight association of the transcriptomic signatures from monocytes of pSS patients with the systemic inflammatory markers suggests a contribution of these mediators to reshape the monocyte transcriptome towards an inflammatory phenotype. Our results support this concept, demonstrating that serum from pSS patients modulates the transcriptome of healthy controls monocytes resembling the profile found in pSS patients. Moreover, the alterations induced by pSS-serum primed the monocytes to an increase production of TNF-α upon TLR stimulation, similar to what was observed in pSS patients after ex-vivo whole blood stimulation. Given the cross-regulation of TNF-α and type I IFN (61), and the fact that type-I IFN priming enhances TNF-α production upon stimulation (62) we hypothesize that the increased TNF-α production observed in pSS patients upon stimulation could be in part, a consequence of an altered transcriptomic profile induced by type I IFNs. In fact, IFNAR blockade partially abrogated the effect of pSS-serum in HC-monocytes, suggesting that the transcriptomic reshape of pSS monocytes consists of IFN-dependent and independent mechanisms. The IFN-dependent mechanisms were observed not only in the brown signature, which mainly reflects the IFN-signaling genes, but also in the hallmarks of the blue signature. The genes contained in this signature are involved in the regulation of gene and protein expression (63, 64) and are known to be downregulated upon stimulation as a regulatory mechanism of cell activation. As monocytes from pSS patients show a reduced expression of this signature, and IFNAR blockade restores it, our results suggest that the translation processes dysregulated in pSS are a consequence of monocyte activation by type-I IFNs. This may induce a loop of inflammation, which can activate other immune cells and favors pSS immunopathology.

A possible limitation of our study is represented by the sample size of the cohort used for RNA-sequencing and WGCNA analyses. Nevertheless, we were able to successfully replicate published findings (3, 20, 22, 25), and we used two independent cohorts of pSS patients and HC to experimentally validate our results. Future studies with integrated analysis of different multi-omics data could shed light on how the identified hub-genes and associated hub-signatures can affect pSS immunopathology.

Taken together, we show that monocytes from pSS patients have a transcriptome enriched in gene expression profiles associated with intermediate and non-classical monocytes subsets. As well, pSS monocytes show dysregulation in gene signatures involved in processes related with translation, IFN-signaling and TLR-

signaling. In addition, the correlation of hub-genes with the peripheral and local inflammatory mediators indicates that the transcriptional dysregulation of pSS monocytes results from their inflammatory environment. Finally, we confirm that the pSS monocyte transcriptome is partially induced by circulating mediators present in serum of pSS patients, and that IFNAR blockade in part prevents these alterations. In view of the important role of monocytes in the activation of innate and adaptive immunity, our data supports the concept that targeting the molecular mechanism underlying monocyte activation, including type-I IFNs holds promise for halting the immunopathology of Sjögren's Syndrome.

Data Availability

The RNA sequencing datasets generated for this study can be found in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE173670. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Ethics Statement

The studies involving human participants were reviewed and approved by the board of medical ethics committee of the University Medical Center Utrecht (METC no. 13-697). The patients/participants provided their written informed consent to participate in this study.

Author Contributions

AL, MH, SB, AK, TR, and JR were involved in conception and design of the study. AL, CB, MH, SB, AH, and AK were involved in data acquisition. AL, CB, MH, AP, AK, TR, and JR were involved in data analysis and interpretation. AL drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest Statement

TR was a principal investigator in the immune catalyst program of GlaxoSmithKline, which was an independent research program. He did not receive any financial support other than the research funding for the current project. Currently, TR is an employee of Abbvie where he holds stock. TR had no part in the design and interpretation of the study results after he started at Abbvie.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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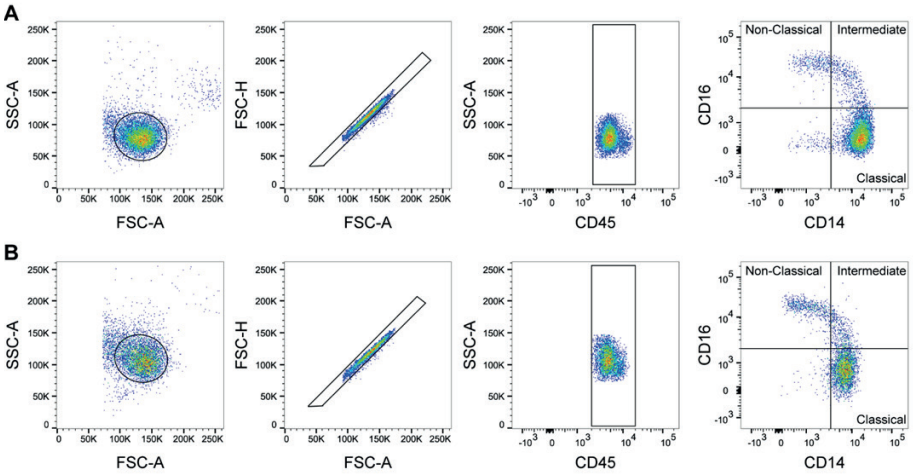
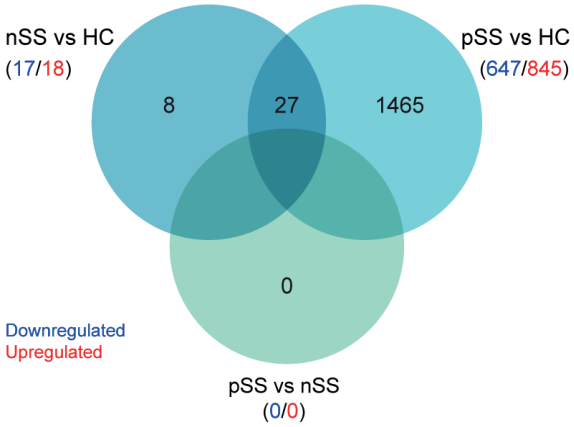
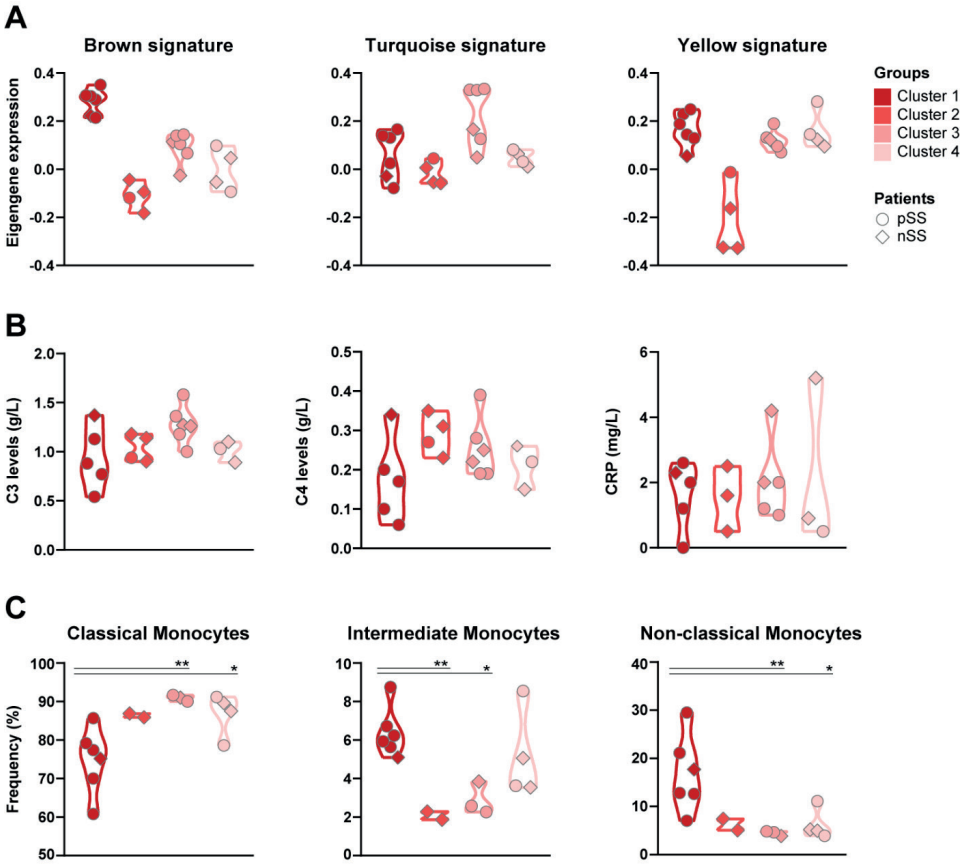
Supplementary material



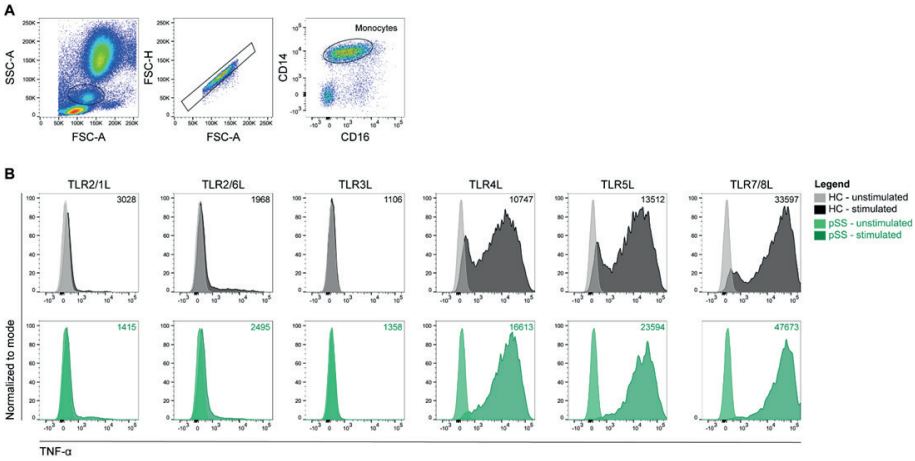
Supplementary Figure 1. Analysis strategy of monocyte subsets. Representative flow cytometry gating strategy analysis of the different monocyte subsets: classical (CD14+/CD16-), intermediate (CD14+/CD16+) and non-classical (CD14-/CD16+) monocytes in healthy controls (**A**) and primary Sjögren's syndrome patients (**B**).



Supplementary Figure 2. Monocytes from pSS patients are transcriptionally different from HC monocytes. RNA sequencing of peripheral blood isolated monocytes of non-Sjögren's sicca (nSS), primary Sjögren's syndrome (pSS) and healthy controls (HC) was performed and the differentially expressed genes (DEG) identified. Venn diagram shows the overlap of the differential expressed genes between the different comparisons with an FDR corrected p-value ≤ 0.05 and base mean >100. Downregulated or upregulated genes in each comparison are indicated in blue or in red, respectively.



Supplementary Figure 3. Hierarchical cluster based on hub-gene expression identifies cluster of nSS and pSS patients with similar signatures. Intramodular analysis identified hub-genes, defined by a module membership > 0.8 and a gene significance > 0.4, related with systemic and local inflammatory markers. Hierarchical clustering of nSS and pSS patient based on hub-genes expression identified clusters of patients with shared features. Violin plots depicts the eigengene expression (first principal component) of each signature (**A**), the C3, C4 complement levels, and C-reactive protein (CRP) (**B**) and the frequency of the monocyte subsets across the established clusters (**C**).



Supplementary Figure 4. Analysis strategy of intracellular TNF-α expression in CD14+ monocyte after TLR stimulation. Representative flow cytometry gating strategy analysis of monocyte ($CD14^+$) in healthy control (**A**) and intracellular TNF-α production after whole blood stimulation in monocytes of HC (black) and pSS patients (green). Unstimulated control and stimulation upon TLR ligands for 6h are depicted, as well as the median fluorescence intensity of the representative individuals (**B**).

Supplementary Table 1. List of antibodies used on the flow cytometry staining

Study	Target	Label	Manufacturer	Clone
Purity	CD45	PerCP	Biolegend	HI30
	CD14	FITC	Miltenyi	TÜK4
	CD16	PE	Dako	DJ130C
Whole blood	BDCA1	APC	Thermo Fisher Scientific	L161
	CD19	BV510	Biolegend	HIB19
	HLA-DR	BV605	BD Biosciences	G46-6
	CD14	BV785	BioLegend	M5E2
	cyTNF-α	BV421	BioLegend	MAb11

Supplementary Table 2. List of the TLR ligands used for whole blood stimulation

Target	Ligand	Manufacturer	Concentration
TLR2/1	Pam3CSK4	Invivogen	5µg/mL
TLR2/6	Pam2CSK4	Invivogen	1µg/mL
TLR3	Poly(I:C)	Invivogen	25µg/mL
TLR4	LPS	Invivogen	0.1µg/mL
TLR5	Flagellin	Invivogen	2µg/mL
TLR7/8	R848	Invivogen	2µg/mL

Supplementary Table 3. Sequences of primers used for RT-qPCR

Gene	Primer forward 5' – 3'	Primer reverse 5' – 3'
ACTB	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGATAGCAAC
B2M	GATGAGTATGCCTGCCGTGT	TGCGGCATCTTCAAACCTCC
MX1	GCATCCCACCCTCTATTACTG	CGCACCTTCTCCTCATACTG
IFITM1	CCAGCATCCGGACACCACAG	CCCCCAGCACAGCCACCTC
TNFSF10	TGCGTGCTGATCGTGATCTTC	GCTCGTTGGTAAAGTACACGTA
IRF2	TCCATACAGGAAAGCATCAACC	CATGGCGCATCTGAAATTCGC
STX7	GGCCCAGAGGATCTCTTCTAA	ACTGTTGCCTCAATTCAGGTG
TRIM38	GTTGAAGACGTATGCCAGGG	GCTTCTGCTCCGTACATCTGTC
IRF9	AGCCACAGGAAGTTACAGAC	TAGATGAAGGTGAGCAGCAG
RPL5	GAGGCTTGTCTATCCCTCACA	GTGCTTCCGATGTACTTCTGC
RPL15	CCCACCCGGCCTGATAAAG	CACGGCGAACACGAATCCT
EEF1B2	GGTGCTCAACGATTACCTGG	ATACCAACGTAGGGCATGACA

Supplementary Table 4. List of the differential expressed genes across the identified signatures

See: https://www.frontiersin.org/articles/10.3389/fimmu.2021.701656/full#supplementary-material

Supplementary Table 5. List of the hub-genes identified in the brown signature

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (sIgG)	Gene Significance (ESR)
DDX60	0.927	0.957	0.434	0.428
TRIM22	0.813	0.956	0.418	0.459
MX2	0.927	0.949	0.441	0.509
HERC6	0.889	0.941	0.443	0.415
RSAD2	1.029	0.940	0.421	0.424
HERC5	1.064	0.937	0.415	0.436
SERPING1	0.898	0.918	0.461	0.565
PML	0.434	0.916	0.402	0.512
GBP1	0.984	0.904	0.538	0.576
CUL1	0.479	0.885	0.506	0.584
IFITM1	0.676	0.882	0.461	0.539
TYMP	0.531	0.877	0.462	0.646
CD2AP	0.412	0.875	0.545	0.516
APOL6	0.721	0.868	0.402	0.449
STAT1	0.801	0.865	0.526	0.497
SLFN5	0.997	0.862	0.498	0.535
FKBP15	0.401	0.858	0.491	0.491
GBP4	0.803	0.855	0.531	0.486
PLAC8	0.552	0.844	0.452	0.502
FAM8A1	0.427	0.842	0.572	0.526
ADAR	0.450	0.840	0.427	0.432
VRK2	0.463	0.832	0.521	0.496
IFI27	0.434	0.832	0.414	0.635
RP4-620F22.2	0.865	0.823	0.475	0.607
UBE2L6	0.423	0.823	0.401	0.493
RTCB	0.249	0.813	0.456	0.526
PARP11	0.423	0.812	0.570	0.463
KLHDC7B	0.621	0.810	0.531	0.686
SP140	0.814	0.806	0.468	0.407
PI4K2B	0.454	0.804	0.529	0.490
GBP5	0.888	0.800	0.489	0.586
RP11-609D21.3	0.925	0.969	-	0.410
DHX58	0.681	0.959	-	0.451
EPSTI1	0.860	0.956	-	0.476

Supplementary Table 5. Continued

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (sIgG)	Gene Significance (ESR)
IFI44L	0.899	0.948	-	0.410
CMPK2	0.885	0.945	-	0.430
AP001610.5	0.894	0.943	-	0.403
MX1	0.889	0.939	-	0.403
PARP12	0.649	0.935	-	0.473
PARP14	0.806	0.930	-	0.464
IRF7	0.722	0.928	-	0.532
SIGLEC1	0.929	0.928	-	0.425
ZBP1	0.923	0.917	-	0.529
NEXN	0.785	0.916	-	0.403
IFI35	0.517	0.910	-	0.499
EIF4B	-0.374	0.908	-	0.500
IFI6	0.792	0.904	-	0.451
ISG15	0.708	0.893	-	0.502
LY6E	0.613	0.888	-	0.504
DDX60L	0.667	0.884	-	0.421
ATP10A	0.716	0.874	-	0.440
EIF3L	-0.413	0.874	-	0.423
EIF2AK2	0.598	0.868	-	0.415
MOV10	0.515	0.867	-	0.420
HELZ2	0.609	0.866	-	0.469
CXCL10	0.858	0.860	-	0.459
SPATS2L	0.844	0.859	-	0.478
AGRN	0.674	0.851	-	0.471
ATP13A1	0.303	0.851	-	0.422
CXCR2P1	0.690	0.838	-	0.425
ISG20	0.770	0.836	-	0.634
QARS	-0.293	0.831	-	0.421
HSH2D	0.699	0.827	-	0.473
TCF4	0.514	0.826	-	0.490
FXR1	-0.236	0.820	-	0.463
OASL	0.639	0.816	-	0.515
AC009950.2	0.551	0.815	-	0.409
RP11-983P16.4	-0.314	0.802	-	0.476

Supplementary Table 5. Continued

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (sIgG)	Gene Significance (ESR)
IFIT3	1.106	0.950	0.404	-
PARP9	0.945	0.950	0.417	-
IFIT2	1.095	0.936	0.403	-
SAMD9L	1.015	0.926	0.432	-
SAMD9	0.866	0.895	0.466	-
BATF2	0.906	0.883	0.451	-
RP1-71H24.1	0.862	0.859	0.488	-
OAS1	0.883	0.847	0.471	-
TRIM5	0.613	0.831	0.404	-
SSB	0.314	0.822	0.404	-
DTX3L	0.796	0.816	0.449	-
PPM1K	0.574	0.814	0.415	-

Supplementary Table 6. List of the hub-genes identified in the yellow signature

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (Schirmer)	Gene Significance (C4 levels)
FAM188A	0.410	0.936	0.462	0.488
DDOST	-0.213	0.921	0.582	0.407
KIAA0895L	0.510	0.908	0.441	0.438
TRIM38	0.255	0.906	0.423	0.492
RP11-6N17.10	0.313	0.856	0.424	0.438
MBOAT1	0.275	0.841	0.511	0.515
POLK	0.329	0.840	0.471	0.440
WDR55	0.262	0.906	-	0.463
RP11-488L18.4	0.409	0.894	-	0.412
GRIPAP1	0.348	0.878	-	0.472
IRF9	0.658	0.873	-	0.437
ZNF224	0.305	0.866	-	0.416
BLZF1	0.617	0.858	-	0.468
PRPF4B	0.256	0.853	-	0.408
APBB3	0.366	0.852	-	0.412
SP140L	0.275	0.852	-	0.554
DYM	-0.238	0.851	-	0.449
ATMIN	-0.166	0.849	-	0.478
NABP1	0.503	0.831	-	0.425
ATHL1	0.453	0.827	-	0.422
N4BP2L2	0.301	0.895	0.590	-
PPWD1	0.338	0.873	0.435	-
RSRP1	0.416	0.852	0.435	-
CAPRIN2	0.429	0.848	0.536	-
CLEC12B	0.641	0.844	0.538	-
TWF2	-0.191	0.832	0.449	-
RGL2	0.282	0.823	0.534	-
EBLN3	0.241	0.818	0.515	-
NOL8	0.275	0.817	0.432	-

Supplementary Table 7. List of the hub-genes identified in the turquoise signature

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (C3 levels)
HERC4	0.463	0.929	0.546
STK38	0.477	0.929	0.506
GIMAP4	0.618	0.923	0.466
SEC22B	0.499	0.923	0.497
SLC2A1	-0.936	0.921	0.472
GIMAP8	0.568	0.921	0.562
DDX23	0.387	0.909	0.566
IRF2	0.520	0.909	0.447
STX7	0.619	0.909	0.552
SACM1L	0.441	0.908	0.583
SNX1	0.378	0.908	0.464
SEC23IP	0.415	0.907	0.466
METTL13	0.470	0.906	0.428
RNASEL	0.599	0.905	0.474
COPB2	0.455	0.902	0.516
NLRC4	0.610	0.901	0.530
VDAC2	-0.420	0.900	0.478
ZNF146	0.392	0.898	0.409
CHMP7	0.417	0.897	0.487
CLCN3	0.408	0.896	0.459
HMGCL	0.467	0.895	0.437
HOMEZ	0.635	0.895	0.411
DCAF12	0.496	0.895	0.553
PREPL	0.591	0.893	0.606
ARF1	-0.225	0.892	0.457
PCMTD1	0.383	0.891	0.619
MPHOSPH8	0.408	0.889	0.538
YIPF4	0.598	0.888	0.549
TRAFD1	0.423	0.883	0.467
RGS18	0.607	0.883	0.494
VTA1	0.364	0.882	0.409
MAPKAPK2	-0.380	0.877	0.646
PEX11B	0.448	0.875	0.561
NADK	0.392	0.875	0.420

Supplementary Table 7. Continued

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (C3 levels)
GIMAP1	0.516	0.874	0.504
KLHL6	0.548	0.874	0.457
TRIP11	0.450	0.873	0.531
CCDC97	0.350	0.873	0.546
XPO1	0.311	0.873	0.509
SCIMP	0.480	0.871	0.401
ZC3H13	0.457	0.869	0.447
GLE1	0.305	0.868	0.460
TMEM184C	0.437	0.868	0.416
IGSF6	0.404	0.868	0.415
SUPT20H	0.399	0.867	0.514
CARD8	0.394	0.867	0.607
WDR45B	-0.287	0.864	0.437
PARP4	0.476	0.864	0.514
BRPF1	-0.575	0.861	0.534
EDEM3	0.481	0.859	0.423
CBR4	0.480	0.859	0.527
USO1	0.287	0.859	0.521
PGBD2	0.601	0.859	0.489
ADNP2	-0.476	0.858	0.562
GIMAP7	0.458	0.857	0.448
NINJ1	-0.433	0.855	0.450
DNTTIP2	-0.493	0.854	0.417
BBS7	0.651	0.853	0.524
CNOT6	0.323	0.853	0.477
EMC2	0.498	0.853	0.484
XPC	0.381	0.848	0.444
CTTNBP2NL	0.443	0.848	0.556
ALYREF	-0.266	0.846	0.506
RP11-400F19.6	0.410	0.845	0.482
RNF168	-0.377	0.845	0.441
SLC25A33	-0.693	0.844	0.517
MNDA	0.505	0.844	0.447
TTI1	0.327	0.844	0.482

Supplementary Table 7. Continued

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (C3 levels)
NCBP1	0.337	0.844	0.434
HMGA1	-0.450	0.844	0.525
TLR6	0.495	0.843	0.514
TTC33	0.570	0.842	0.494
RNF11	-0.310	0.839	0.479
RP11-361D15.2	-0.318	0.839	0.432
TRMT1L	0.506	0.838	0.469
IPO8	0.386	0.837	0.412
RBM14	0.410	0.837	0.472
NR1D1	-0.878	0.834	0.423
RBMXL1	-0.293	0.834	0.407
SLC35B3	0.463	0.832	0.571
TMEM60	0.564	0.830	0.408
MAPK14	0.303	0.827	0.425
PI4K2A	-0.679	0.827	0.526
PAFAH2	0.450	0.827	0.446
P2RY13	0.497	0.826	0.539
GOLGA5	0.263	0.826	0.516
TTC9C	0.502	0.825	0.407
MAP2K3	-0.318	0.824	0.533
PLRG1	0.256	0.824	0.494
NFE2	0.593	0.822	0.413
RP11-332M2.1	-0.307	0.822	0.499
EPB41L4A-AS1	-0.507	0.821	0.419
VPS37B	-0.299	0.821	0.410
SLC30A5	0.310	0.820	0.410
PIGV	0.583	0.820	0.457
LMBRD2	0.556	0.820	0.517
SCAMP4	-0.276	0.818	0.473
FPR3	1.026	0.817	0.408
CARD8-AS1	0.500	0.816	0.477
MAPRE1	-0.231	0.815	0.446
C1orf112	0.635	0.813	0.488
TRAF3IP3	0.328	0.811	0.441

Supplementary Table 7. Continued

Gene	Fold change pSS vs HC	Module Membership	Gene Significance (C3 levels)
PQLC1	-0.285	0.810	0.487
TMEM189	-0.351	0.807	0.552
ID2	-0.574	0.804	0.511
BET1	0.533	0.804	0.419
SLC37A2	0.380	0.802	0.471

Supplementary Table 8. List of selected genes to represent the pSS-monocyte transcriptomic signature

Gene	Module	Module Membership	Fold change pSS vs HC	Gene significance
MX1	Brown	**0.9391**	**0.889**	sIgG (0.3608) ESR (**0.4029**)
IFITM1	Brown	**0.8816**	**0.676**	sIgG (**0.4613**) ESR (**0.5392**)
TNFSF10	Turquoise	**0.8855**	**0.717**	C3 (0.3959)
IRF2	Turquoise	**0.9086**	**0.520**	C3 (**0.4247**)
STX7	Turquoise	**0.9085**	**0.619**	C3 (**0.5521**)
TRIM38	Yellow	**0.9063**	0.255	C4 (**0.4916**) Schirmer (**0.4227**)
IRF9	Yellow	**0.8731**	**0.658**	C4 (**0.4369**) Schirmer (0.3401)
RPL5	Blue	**0.9483**	**-0.462**	-
RPL15	Blue	**0.9717**	**-0.421**	-
EEF1B2	Blue	**0.9362**	**-0.448**	-

Selection of the hallmark genes to represent each signature, and ultimately the transcriptomic profile of pSS monocytes, were based on the following criteria: 1) highest degree of correlation with each signature (high module membership); 2) highest fold-change of expression between pSS and HC; 3) gene significance > 0.4 in any of the clinical features. Parameters which meet the selection criteria are depicted in bold.

Chapter 7

General Discussion

Summary of the main findings

Despite extensive molecular, histological and clinical studies, the underlying cause of primary Sjögren's syndrome (pSS) and its pathogenesis remains largely unknown (1). There is growing interest in the identification of well-characterized subgroups of patients, with common molecular signatures and cellular immune functions to improve the understanding of the mechanisms driving pSS and response to treatment (2). Increasing evidences underline the central role of innate immune cells such as type 2 conventional dendritic cells (cDC2s) and monocytes in pSS, in the initiation, progression and maintenance of autoimmune responses (3, 4). Hence, by investigating epigenetic and transcriptomic profiles, the results presented in this thesis demonstrate the functional and molecular alterations of cDC2s and monocytes as well as their contribution to maintain an inflammatory response in pSS.

As epigenetic regulators are key factors of heterogeneous disease phenotypes, we show that the circulating small non-coding RNAs (ncRNAs) expression pattern of pSS patients can reflect systemic inflammatory features and distinguish subgroups of patients (**chapter 2**). In addition, our functional studies demonstrate that ncRNAs, such as microRNA (miRNA)-130a, affects cDC2 activation and pro-inflammatory cytokine production (**chapter 3**), and long-non coding RNA (lncRNA) *HCP5* regulates pivotal cDC2 functions, affecting both innate and adaptive immunity (**chapter 4**). Their dysregulation in pSS suggests an important contribution in the regulation of inflammation and cell activation in this disease. The transcriptomic studies of cDC2s and monocytes (**chapter 5** and **6**) reveal novel molecular and functional mechanisms, by which these cells orchestrate immune cell activation and salivary gland dysfunction in pSS.

As such, the insights presented in this thesis disclose novel opportunities to understand and halt inflammation and interrupt the vicious perpetuating cycle that characterizes pSS immunopathology, and may yield novel therapeutic opportunities.

Non-coding RNAS in pSS: from unknown function to essential instructors of immune activation

The interplay of epigenetic mechanisms is essential for efficient gene expression regulation, and over the last decade, a growing body of studies has highlighted their relevance in autoimmune diseases (5). As dynamic events influenced by the environment, epigenetics can represent the bridge between the environmental

and genetic factors, which have been identified as risk factors for autoimmune diseases (6). Although their link is widely documented, the requirement to better understand the influence of these modifications in different cells and disorders remains. As such, in the **chapters 2** to **4**, we show that different epigenetic regulators including circulating and cellular miRNAs as well as lncRNAs may have pivotal roles in the regulation of immune activation in pSS.

In the era of precision medicine, the success of clinical trials, strongly relies on specific targeting but also on robust and versatile analytical technologies to ensure proper stratification and treatment of patients. Since the discovery that miRNAs are detected in several body fluids, such as serum, plasma and tears, their potential use as biomarkers became very attractive. Several studies attempted to establish miRNAs as biomarkers for cancer (7) and for immune-mediated inflammatory disorders (8, 9). However, there is a lack of consistency between many miRNA signatures that can be attributed to different technical measurements but more importantly to individual variability e.g., gender, age, prior treatments and blood cell composition (10). Thus, this dynamic feature of serum miRNAs, which is a hindrance for their application as disease specific biomarkers, can represent an advantage if used to monitor immunological activity in patients. For instance, miR-146a on its own, has minimal disease specificity (11), however it is indicative of NF-κB transcription factor activation and thus may be an important marker of immune activation (12).

In **chapter 2**, using a discovery-validation approach, we show that circulating small ncRNAs, at the group level could not distinguish pSS patients, from healthy controls (HC) or non-Sjögren's sicca (nSS) patients. However, based on the expression of a limited number of miRNAs pSS patients were clustered accordingly to their inflammatory status. In part, this can be explained by the fact that in circulation, immune cells are the major source of miRNA, thus changes in the immune profile will be reflected in the circulating miRNA levels (13). In fact, the pSS subgroup with more pronounced leukopenia and immune activation, was identified by their reduced miRNA expression, possibly associated with leukocyte migration to sites of inflammation.

A better definition of the pathological and biological profile in the different subgroups of patients is certainly required, yet the results of **chapter 2** highlight the need of exploiting relatively large cohorts of pSS patients in light of their heterogeneous immunological spectrum. It can be speculated that the longitudinal measurement of circulating miRNA could be a relevant tool

to monitor disease variations as they might reflect changes in immunological disease features. Nevertheless, determining the contribution of each tissue to the circulating miRNA pool in vivo, and how this might be modified in response to physiological changes or disease remains a major challenge.

To better understand the functional role of miRNAs in pSS, and their regulation of immune cell functions, in **chapter 3**, we evaluated the miRNome of cDC2s, in view of the key role of these cells as orchestrators of inflammatory responses. Combination of high-throughput epigenomic and proteomic approaches led us to the identification of a dysregulated mechanism in pSS patients, by which miRNA-130a regulates pro-inflammatory cytokine production by cDC2s. Based on the findings of **chapter 3**, it is possible to assume that the inflammatory environment seen in pSS patients, modulates the miRNA profile of cDC2s, (downregulating e.g., miR-130a), which affects the activation status of these cells. In addition, by showing that this mechanism can contribute to enhance both immune activation and tissue destruction in pSS, we could envision that this unveiled pathway can be a possible therapeutic target in the future. Nevertheless, further clarification is needed to understand whether blocking of mitogen- and stress-activated protein kinase-1 (MSK1), the miR-130a target in cDC2s from pSS patients, ameliorates its activated status.

In **chapter 4**, we show that lncRNA *HCP5* is an important regulator of several key adaptive immune functions in cDC2s, affecting both arms of the immune response. These results support that lncRNAs can regulate a multitude of cell functions as they have multiple mechanisms of action (14). Although the functional characterization of lncRNAs still remains challenging, in **chapter 4** we show that *HCP5* in cDC2s is an important mechanism that in pSS can sustains type-I IFN response and induce cell recruitment and activation. The increased expression of this lncRNA in the exocrine glands and in circulating cDC2s of pSS patients, suggests that *HCP5* is an important mediator of cDC2s function and immune activation in this disease. However, given the dual mechanism of *HCP5* to sustain B cell survival and to modulate T cell response, fine tuning the regulation of this epigenetic mediator is essential to restore immune cell tolerance. Hence, advances in the knowledge of epigenetic regulation in immune cells will underly their important role in immune-mediated diseases and provide new insights for the development of therapeutic approaches.

Lessons from circulating immune cells: the link to the salivary gland tissue?

Research of human immune-mediated inflammatory diseases, such as pSS, is restricted by the difficulties of access and collection of the relevant inflamed tissues. Exocrine glands sampling to study the immunological mechanisms is an invasive and complex method which causes pain and discomfort to patients (15). Although necessary for the clinical diagnosis, their use in translational research is limited by the sample size. To overcome this difficulty, and since pSS is a systemic disease, the vast majority of the studies which aim to understand the underlying immune mechanisms of inflammatory processes are based on circulating immune cells. Recently, novel high-sensitive throughput technologies, including single-cell platforms have elucidated how immune cells in blood relate to those in tissue sites demonstrating that the immune cell composition of peripheral blood is largely influenced by the tissue environment (16). As such, based on the findings from **chapters 3** to **5** showing aberrations in molecular and functional features of cDC2s it is tempting to speculate a pivotal role of cDC2s in the inflamed salivary glands of pSS patients (**Figure 1**).

In line with the studies presented in **chapter 5**, we propose a mechanism by which circulating cDC2s can be attracted to the tissues, dependent on the CX3CR1-CX3CL1 axis. Fractalkine levels (CX3CL1) are increased in the salivary gland of pSS patients, not only as a result of production by salivary gland epithelial cells but also B cell and follicular dendritic cell (17). This interaction can mediate the recruitment and adhesion of CX3CR1-expressing cDC2s leading to their accumulation in inflamed kidneys (18). Interestingly, in vivo CX3CR1-ablation, delayed $CD11c^+$ DCs migration from peripheral blood to lymph nodes and impaired antigen-specific T-cell response and NK cell activation in a viral infection model (19). As such, the CX3CR1-CX3CL1 axis might significantly regulate cDC2 migration in pSS patients, representing a mechanism by which salivary gland epithelial cells attract immune cells to initiate an inflammatory immune response (**Figure 1A**).

The increased uptake of apoptotic cells observed in **chapter 5** could induce a break of tolerance mechanism by which $CD4^+$ T cells become activated in pSS, contributing to B cell hyperactivation and autoantibody production. Apoptosis of glandular tissue is enhanced in pSS patients and supported by a variety of reports of experimental mouse models of pSS (including NOD-derived strains, C57BL/6.NOD-Aec1Aec2, NFS/sld) (20, 21). Apoptosis of the epithelial cells leads to the translocation of autoantigens characteristic of Sjögren's syndrome,

including Sjögren's-syndrome-related antigen A (SSA) protein, into apoptotic particles (22). The increased uptake capacity of cDC2s combined with efficient cross-presentation enables the activation of autoreactive $CD4^+$ T, $CD8^+$ T, and B cells as well as production of autoantibodies (**Figure 1B**). Interestingly, immunization of mice with short SSA-derived peptides recapitulates pSS manifestations, including salivary gland infiltration of $CD4^+$ T, $CD8^+$ T, and B cells, production of anti-SSA autoantibodies and salivary dysfunction (23).

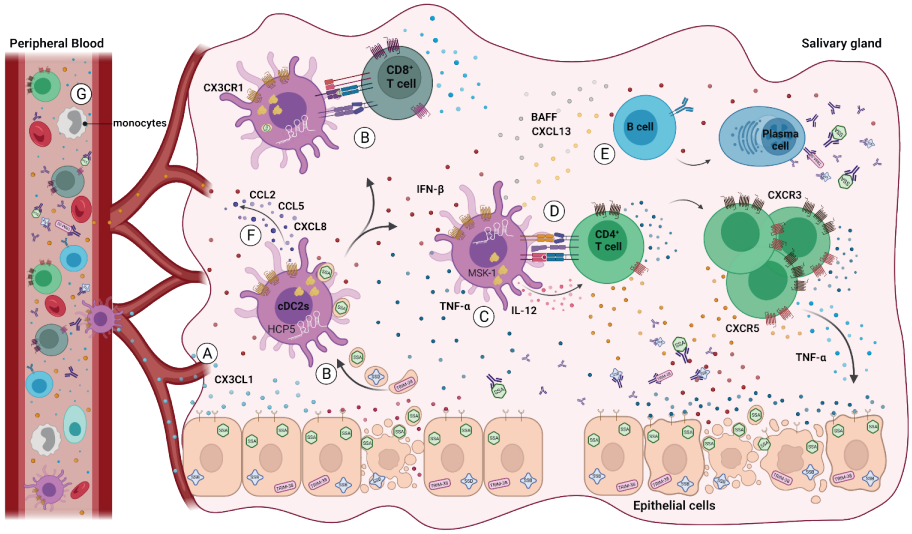


Figure 1. Central role of cDC2s in the initiation and maintenance of Sjögren's syndrome immunopathology. Proposed model for the contribution of type 2 conventional dendritic cells (cDC2s) to the immunopathology of Sjögren's syndrome. Danger triggers such as stress, trauma or infection induce tissue damage signals that lead to the accumulation of apoptotic debris and the production of inflammatory mediators, such as CX3CL1 and type-I interferons (IFNs). (**A**) CX3CR1-mediated recruitment of cDC2s (**chapter 5**) contributes to their presence in the exocrine glands. (**B**) IFN-primed cDC2s more efficiently uptake apoptotic debris and via class-I processing and presentation pathway activates $CD8^+$ T cells (**chapter 5**). (**C**) Activated cDC2s with reduced microRNA (miRNA)-130a and increased mitogen- and stress-activated protein kinase (MSK)-1 expression produce interleukin (IL)-12 and tumour necrosis factor (TNF)-α to sustain $CD4^+$ T cell and tissue destruction (**chapter 3**). (**D**) cDC2s also induce the proliferation and expression of CXCR3 and CXCR5 on proliferating $CD4^+$ T cells to increase their retention within the tissues (**chapter 5**). (**E**) cDC2s through a mechanism regulated by lncRNA *HCP5,* produce BAFF, CXCL13 and IFNβ promoting B cell survival as well as (**F**) CCL2, CCL5 and CXCL8 to mediate immune cell recruitment (**chapter 4**). (**G**) The inflammatory mediators produced in the tissues drain to the peripheral blood and can modulate the transcriptomic profile of circulating cells including monocytes and cDC2s (**chapter 6 and 5**).

The presence of cDC2s in exocrine glands of pSS patients is confirmed by the existence of a cDC2 gene signature in the inflamed salivary glands (24, 25). The insights unravelled in **chapter 3**, indicate that the decreased miR-130a expression and increased expression of its target MSK1, relevant for IL-12 and TNFα production, is an important mechanism to sustain $CD4^+$ T cell activation (**Figure 1C**). In addition, the results described in **chapter 5**, further support this hypothesis and brings forward the understanding of cDC2s functions in pSS. Peripheral blood cDC2s from pSS patients are primed with an increased capacity to induce $CD4^+$ T cells proliferation and to reshape their chemokines profile. Thus, we propose that in pSS, the increased infiltration of $CD4^+$ T cells in the salivary glands can be explained, in part, by the interaction with cDC2s (**Figure 1D**), in line with the results of tissue cell interaction (25). However, whether $CD4^+$ T cells undergo expansion within the gland, or whether it occurs elsewhere, e.g., in the lymphoid organs, with subsequent migration still remains an open question. Yet, we could envision that both mechanisms co-exist in pSS patients, indicated by the presence of proliferating lymphocytes in the salivary glands of pSS patients (26) and the detection of increased levels of chemokine (C-X-C motif) ligand (CXCL)9, CXCL10, CXCL11 (CXCR3 ligands) and CXCL13 (CXCR5) crucial for T cell migration (27, 28).

Furthermore, the findings reported in **chapter 4**, support the concept that cDC2s can promote B cell survival through a mechanism dependent on the lncRNA *HCP5*. Increased *HCP5* expression in cDC2s from pSS patients can contribute to increase B cell activating factor (BAFF) production by these cells to maintain B cell survival, activation and differentiation in the salivary glands (**Figure 1E**). In line with this hypothesis, transgenic mice expressing BAFF exhibited pSS-like manifestations, including enlarged and inflamed salivary glands and reduced saliva flow rates (29). Similarly, BAFF overexpression using systemic gene delivery, increases B cell hyperactivation, infiltration, proliferation, and germinal centre B cells formation in exocrine glands (30).

Hence, the results presented in this thesis provide new insights into the molecular and functional role of cDC2s in pSS and can possibly unveil the inflammatory microenvironment ongoing in the exocrine glands of pSS patients. Nevertheless, future studies are necessary to unravel the complexity of the cDC2s interactions in the tissues and to integrate the diverse molecular, cellular and tissue layers.

Cutting-edge technology to study tissue-immune cell interactions

A deeper understanding of the tissue spatial environment and cell heterogeneity in pSS is highly relevant to better characterize the disease mechanisms and highlight novel therapeutic targets.

Given the presence of cDC2s, activated T and B cells in the salivary glands of pSS patients, detailed spatial information would reveal whether these cells have an active interaction in tissue, as proposed based on the results of **chapter 4** and **5**. Spatial omics technologies are rapidly evolving and extending beyond transcriptomics into metabolomics and proteomics (31, 32). By combining single cell gene expression profiles, proteomics and in situ spatial location these techniques permit to explore the spatial patterns of distinct cell subsets and characterize gene and protein dynamics at the subcellular level (33).

The development of 3D tissue models represents a useful tool to study tissue-immune cell interactions under specific controlled conditions. Recently, efforts have been made to established in vitro models of pSS exocrine glands, including pSS patients-derived organoids from salivary gland and lacrimal glands which resemble the characteristics of normal tissue (34, 35). However, mainly due to the low numbers of salivary gland stem cells found in pSS patients and their reduced ability to differentiate, few progress has been achieved (35). To overcome the challenges of these models, we hypothesize that a promising tissue model of pSS disease could derive from spheroids cultures, which can be formed either from salivary gland cell lines or from cells isolated from biopsies of pSS patients (36). This model could be relevant to study the CX3CR1- mediated mechanism proposed for cDC2s migration to the salivary glands described in **chapter 5**. On the other hand, this model could also be valuable to understand the contribution of stromal cells to modulate the phenotype of monocyte as proposed in **chapter 6**. Finally, it could also represent an advantageous instrument to identify immune mediators and the producing cells responsible for salivary gland destruction as well as to test novel compounds.

Cultures from partially dissociated salivary biopsies derived from pSS patients can represent a more complex model to mimic the salivary tissue in a dish. This model can retain the key features of the native biopsy microenvironment as well as the different stromal cell populations and immune cells, including lymphoid and myeloid cells of each patient (37). As such, this model offers a valuable opportunity to study the behavior of cDC2s including antigen uptake,

as well as $CD4^+$, $CD8^+$ T and B cell activation (**chapter 5** and **chapter 4**) in the tissue microenvironment. The influence of the inflammatory/regulatory mediators produced by the stromal cells, and the signals provided by the lymphocytes present in the tissue will affect the response of cDC2s allowing a better approximation of the in vivo events. In addition, this model also enables to study the consequences that the rescue of miRNA-130a and lncRNA *HCP5* expression (**chapter 3** and **chapter 4**), in tissue derived cells, can have in the control of immune activation and epithelial cell function.

These models can also be used to push forward the goal of personalized medicine in autoimmune diseases. As response to therapy could be heterogeneous, these models can also offer the possibility to test the therapeutic efficacy in hindering immune cell activation and restoring epithelial cell function in a personalized manner.

These emerging disease models (38) and other models including the organ-on-chip models (39) can represent a sustainable alternative and/or complement to in vivo animal models (40, 41). Nevertheless, a major challenge in the development of 3D cultures relates with the capacity of generate and maintain biologically relevant models which are able to recapitulate microenvironmental factors and resemble in vivo tissue and disease pathology.

Type-I interferons: the usual suspect or guilty by association?

Immunological perspective

Although the key role of IFN signalling in the pathogenesis of pSS has been largely studied, the precise pathological mechanisms triggered by the different IFNs are far from being clear. Type-I IFNs are characterized by a wide variety of antiviral and anti-neoplastic properties (42, 43), and by several immunomodulatory effects, such as the induction of BAFF, immunoglobulin switching, increased antigen presentation, T-cell-mediated, and natural killer cell (NK) cytotoxicity (44, 45).

The results described in **chapter 4**, show that IFNα activation of cDC2s enhances *HCP5* expression, and in turn, *HCP5* positively regulates IFNβ production and IFN-inducible genes. As in pSS patients both *HCP5* and IFN-inducible genes expression are increased, this feedback loop can represent a novel mechanism to auto sustain IFN production. As such, these results provide evidences that in pSS the type-I IFNs production can also be regulated by cDC2s through a positive feedback mechanism.

In **chapter 5**, we show that IFNα priming modulates important cDC2s functions, such as antigen uptake and possible processing, affecting T cell presentation and activation. Based on these findings, we speculate that the presence of type-I IFN in pSS patients, reshapes cDC2s to increase autoantigen uptake, resulted from salivary gland apoptosis, inducing auto-reactive $CD4^+$ T cells activation. In turn, activated $CD4^+$ T cells produce pro-inflammatory cytokines, such as IFNγ and TNFα, and induce B cell activation and differentiation, which results in hyperactive B cells and autoantibodies production.

On the other hand, the effect of type-I IFNs in cross-presentation and antigen storage provide a potent $CD8^+$ T cell activation which can contribute to exacerbate the immune inflammation in the salivary glands. Hence, we propose that in pSS, the exacerbated production of type-I IFNs by pDCs (46), drives cDC2s to acquired inflammatory characteristics resulting not only in aberrant $CD4^+$, but also $CD8^+$ T cell activation, in line with the results of **chapter 5**. Consistent with this hypothesis, a subset of cDC2s, known as inflammatory cDC2s (inf-cDC2s) and dependent on interferon-α/β receptor (IFNAR)-signalling display a superior capacity to induce $CD4^+$ T cell polarization and simultaneously to cross-present antigen to $CD8^+$ T cells (47). Moreover, the fact that inf-cDC2s are present within the inflammatory sites as well as in multiple mice models of infection and inflammation (47) reinforces the role of type-I IFNs in modulating cDC2s functions.

Besides the effect on cDC2s, in **chapter 6** we demonstrate that type-I IFNs not only induce the expression of IFN-inducible genes but also reshape the monocyte transcriptome towards an inflammatory phenotype. These transcriptomic aberrations found in monocytes of pSS patients are tightly associated with systemic inflammatory markers and shed light on the association between type-I IFN signature found in monocytes and clinical disease activity of pSS patients (48). Our results demonstrate that monocytes from pSS patients have an altered activation status confirmed by enhanced TNFα production. This cytokine is increased in pSS patients and is implicated in the apoptosis of salivary gland cells, which can lead to secretory dysfunction and glandular destruction (49). The results of IFNAR blockade (**chapter 6**) which abrogate the majority of the effects of pSS-serum in HC-monocytes, supports the role of type-I IFNs in modulating monocyte transcriptome contributing to perpetuate the inflammatory environment.

Recently high-throughput profiling technologies able to unveil the molecular mechanisms, combined with traditional clinical evaluation, identified a shared

"interferon" cluster among patients with different autoimmune diseases including pSS (50). Thus, it is likely possible that the findings described in **chapters 4** to **6**, driven by type-I IFN can also be present in other systemic autoimmune diseases. In this context, there is growing interest in establish well-characterized sub groups of patients, based on the premise that patients that share molecular features may evolve and behave in similar manners and therefore might respond similarly to specific drugs. As such, it is expected that molecular classification provides valuable foundation and motivation to develop tailored therapies to significantly decrease off target effects.

Clinical perspective

The results presented in **chapter 5** and **6**, corroborate the central role of type-I IFNs in driving inflammation and provides additional rational to target this pathway in pSS. The development of therapies aimed to modulate IFN signalling are in progress and tested in several clinical trials including RSLV-132 (NCT03247686), anti-ILT7 receptor (NCT02780674, NCT03817424) and JAK inhibitors (NCT03100942, NCT04496960). However, the preliminary results are not entirely conclusive and often contradictory (39).

In line, the results of type-I IFN modulation, in pSS patients, by broad therapeutic approaches such as classical DMARDs are also, in part, conflicting. For instance, treatment with hydroxychloroquine an immunomodulatory drug, was effective to reduce IFN scores and IFN-inducible genes, but it failed to improve clinical response (51). By contrast, combination of leflunomide and hydroxychloroquine (LEF/HCQ) showed clinical efficacy in pSS patients, but did not affect IFN-signature (52).

Despite these observations could partly reflect the difficulty of capturing clinical disease activity, it also suggests that disease activity is not tightly associated with modulation of IFN-signature. Nevertheless, these association have been described in pSS (48), a detailed analysis indicates that IFN-signature is mainly associated with inflammatory rather than clinical components of the disease activity score (48, 53). As such, it is possible to speculate that the modulatory role of IFNs in immune cells functions is not directly related with clinical features, and in part can explain the lack of clinical improvement after IFNs targeting. Other possible explanation might be that the initiation, but not the persistence, of certain pSS associated symptoms are immunologically driven. In accordance, IFNα treatment of chronic Hepatitis C Virus (HCV) infection, induces in some cases a persistent fatigue, which does not resolve once the immune activation is no longer present (54).

We may also consider that the IFN-induced mechanisms might remain imprinted in circulating and tissue infiltrating cells, likely requiring longer time to be normalized after IFN neutralization. In line with this hypothesis, pSS clinical responders to LEF/HCQ treatment showed a reduction of circulating IFN-induced proteins including myxovirus resistance protein 1 (MxA), CXCL10 and galectin-9, but not in the IFN-signature (52). A longitudinal and simultaneous assessment of the actual IFN levels and the IFN-signature would provide new insights on how these measurements are related and their kinetics during treatment. Nonetheless, detection of type-I IFNs in peripheral blood remains a challenge, due to their low levels in circulation, likely reflecting their high biological potency. Currently, the transcriptional analysis of IFN-induced genes is the most used method to demonstrate IFN-activity (55), despite the recent development of an ultra-sensitive technology with 5,000-fold increase in sensitivity allowing the detection of IFNα at attomolar (femtogram per millilitre) concentrations (56). As such, future studies assessing the accurate IFN levels during treatment could shed light on their association with clinical activity.

Opportunities of molecular classification for non-Sjögren's Sicca patients

The studies presented in **chapter 2**, **chapter 5** and **chapter 6**, demonstrate that despite the differential clinical diagnosis, pSS and nSS patients are immunologically related. These results highlight the need for a better patient classification not only to improve patients care but also to gain insights into the mechanisms driving immune activation and development of pSS immunopathology.

nSS patients have similar levels of dryness compared with pSS patients, but show limited or absent signs of systemic and local autoimmunity. This group of patients does not meet the classification-criteria for Sjögren's syndrome, is often poorly studied and clinically difficult to characterize. The in-depth transcriptomic analysis of cDC2s in **chapter 5** and monocyte in **chapter 6** revealed that the altered molecular signature found in pSS patients is, at least in part, shared by a subset of nSS patients, indicating that some of these patients do have immune dysregulation. In accordance, the overall transcriptional profile of plasmacytoid dendritic cells from nSS patients, represented an intermediate phenotype between HC and pSS, although in some cases largely overlap with that of pSS patients (46). Differences between pSS and nSS patients in processes including IFN-signalling and non-sense mediated decay could indicate that nSS patients still maintain some regulatory mechanisms to ensure cellular homeostasis (57).

The presence of these regulatory mechanism could explain why nSS patients occasionally present with a moderate molecular inflammatory profile but do not exhibit generalized autoimmune disease.

As nSS may share severe objective dryness, and occasionally present with single systemic features similar to pSS patients, we hypothesize that the heterogenous clinical presentation of this patients, may partially be driven by differences in underlying dysregulated biological pathways. As such, better stratification of sicca patients based on different molecular signatures and follow-up investigation of the regulation of the immune profile of nSS patients would improve the characterization of disease driven mechanisms and implementation of novel therapeutic targets.

Novel therapeutic avenues to halt Sjögren's syndrome

The discovery and clarification of underlying pathogenetic pathways in pSS fueled several efforts towards the discovery of novel therapeutic modalities targeting immune pathways (58), and recently positive clinical outcomes were reported (59). Further characterization of the molecular and functional networks involved in this disease is crucial and can led to novel personalized treatments and elucidate why some patients are refractory to treatment. Based on the results described in this thesis it is tempting to speculate that targeting of these pathways could potential disclose novel therapeutic opportunities for pSS patients.

Concerted efforts contributed to the development of miRNA-based drug candidates into clinical trials, ranging from kidney diseases to cardiac abnormalities, and from different types of cancer to infectious disease (60, 61). Considering the results from **chapter 3** and **chapter 4,** specific delivery of miR-130a mimics and lncRNA *HCP5* silencing molecules in cDC2s could represent an option to reshape their phenotype and function. By modulating among other, IL-12 production, BAFF and costimulatory molecule expression, this approach could reduce T cell activation and B cell survival.

Currently, several RNA-based therapeutics directly targeting genes or miRNA are approved or in phase II or III clinical development (62). As for lncRNA-based therapeutics, they are still a step behind to enter the clinic, possibly due to the relative recent comprehension of their functional roles. Nevertheless, the use of miR-130a analogue, or *HCP5* inhibitor, to restore cDC2 function in pSS, seems attractive since it would allow the reestablishment of target-specific pathways

without fully abrogating cell function. The ability to fine-tuning gene expression dependent on coordinated effects of a set of regulatory machinery represents an advantage to mitigate off target and prolonged effects. As all the mechanisms necessary for miRNAs or lncRNAs processing and the downstream target selection are available, their targeting represents an encouraging strategy. Moreover, the use of ncRNAs which can target multiple genes within one pathway may potentially boost therapeutic effects compared with approaches that influence only a single target gene and thus cause a broader, yet specific response (62).

Despite the numerous options that ncRNAs targeting represent, the delivery of these agents to specific cell types or tissues represents a key challenge of this approach, since ncRNAs can be broadly expressed. Efficient in vivo delivery systems have been studied and recently liposomes have emerged as interesting vehicles, since they can be designed to provide robust tissue or cell specific target delivery. In addition, the use of mathematical models in combination with functional validation will further contribute to decipher the highly complex regulatory networks minimizing the risk of unwanted side effects (63, 64). Overall, based on the results present in this thesis and on the new advances in the field, it is tempting to envision targeting ncRNAs to reprogram cDC2s activation in pSS as a reasonable and evidence-based strategy with potential therapeutic success.

The CX3CR1-CX3CL1 mediated cDC2s migration to the exocrine glands of pSS patients, proposed in **chapter 5**, represents another promising targetable pathway in pSS. In line with this hypothesis, blockade of CX3CL1, with a monoclonal antibody (E6011) was effective in patients with active rheumatoid arthritis with inadequate response to methotrexate (NCT02960438) (65). Likewise, the use of a selective small-molecule inhibitor of CX3CR1 (AZD8797) (66), was effective in suppressing apoptosis, necrosis and inflammatory responses in an acute rat model of spinal cord injury (67). As such, these observations are promising to halt cDC2s-driven inflammation in pSS by preventing their migration to the inflammatory sites.

From the point of view of the results presented in **chapter 5** and **chapter 6**, the blockade of the biological activity of type-I IFNs to reduce immune cell activation seems promising in pSS too. However, fully abrogation of this pathway at long term might be harmful for patients. In fact, patients with systemic lupus erythematosus (SLE) treated with anti-IFNAR1/anifrolumab (NCT02446912/

NCT02106897/NCT02446899) had significantly higher occurrences of *herpes zoster* and respiratory tract infections, compared with the placebo group. Thus, the understanding of the precise pathological mechanisms triggered by IFNs, as shown in **chapter 5** and **chapter 6** can represent an alternative approach to prevent immune cell activation in pSS while limiting the unwanted off target effects induced by total blocking of IFN pathways. As such, based on our results and others (68), a combined modulation of the inducers (*HCP5*/pDCs) or downstream effector functions (antigen uptake/BAFF production) of type-I IFNs might represent a better option to improve patients' clinical response.

Antigen presentation is an important cDC2 mechanism to drive T cell activation, and the findings described in **chapter 5** show that dysregulation of this pathway in pSS patients contributes to increase $CD4^+$ and $CD8^+$ T cell activation. These complex processes are fundamental for immune surveillance and their disruption needs to be properly regulated (69). As such, we proposed that a dual approach that includes targeting of the initial activation steps (such as IFNs or apoptotic antigen uptake) of cDC2s and simultaneously prevents T cell activation could be a better alternative to inhibition of antigen presentation.

In this respect, inhibition of cathepsin S, which is crucially involved in major histocompatibility complex (MHC)-II processing and $CD4^+$ T cell stimulation, by RO5459072, was tested in pSS patients (NCT02701985), however it was not beneficial to improve systemic disease activity. One would assume that this unexpected result can be associated with the presence of antigen experienced memory and effector T cells, that remain unleashed and are more dependent on cytokine activation, such as IL-7 and IL-15 and less dependent on antigen presentation (70-72). On the other hand, the results described in **chapter 5**, demonstrate that MHC-I processing pathway and $CD8^+$ T cell activation should also be considered. $CD8^+$ T cell activation can directly mediate the destruction of salivary glands epithelial cells sustaining the presence of autoantigens and immune complexes and in turn promote immune cell activation.

Other alternative approach to halt adaptive immunity in pSS could be associated with the rewiring of cDC2s to a more tolerogenic phenotype. Overall, the pro-inflammatory properties of cDC2s described in **chapter 3** (increased IL-12 and TNFα), **chapter 4** (functional consequences of *HCP5* upregulation) and **chapter 5** (increased antigen uptake and presentation) provide rational for the development of tolerogenic cDC2s to halt aberrant T and B cell activation in pSS.

Considering the similarity of the phenotype of tolerogenic DCs (tolDCs) generated in vitro from monocytes of pSS patients with those from HC, it is encouraging to envision this approach as a possible therapeutic. TolDCs derived from monocytes of pSS patients were able to in vitro suppress naïve T cell activation in the presence of fully matured DC loaded with pSS-related autoantigens (73). Various strategies can be used to generate ex vivo tolDCs, but the extensive manipulation and the high manufacturing costs due to tailor-made preparation led to the development of new approaches based on in vivo antigen-delivery. These methods can act directly on naturally occurring tolDCs or can polarize DCs toward a tolerogenic phenotype through the antigen co-delivered with immunomodulatory factors (74). Based on our results, we foresee that reduction of antigen-specific T cell response could be achieved by combined delivery of tolerogenic agents, such as miRNA-130a mimic, lncRNA *HCP5* inhibitor and self-antigens with specific antigen delivery (with monoclonal antibodies or specific biomaterials). Delivery to cDC2s from pSS patients would be facilitated by their increase uptake capacity showed in **chapter 5**. In line with this hypothesis, in vivo delivery of liposomes containing NF-kB inhibitors to antigen presenting cells suppresses the cellular responsiveness to NF-kB and induce antigen-specific $FoxP3^+$ regulatory T cells in an animal model of arthritis (75). Furthermore, in a phase 2a clinical study in patients with celiac disease, challenge with nanoparticles encapsulating gluten reduced antigen-specific T cell response and circulating activated memory T cells (76). An increasing interest in moving towards in vivo targeting strategies has proven to be very effective in a range of mouse models including multiple sclerosis, diabetes and allergies, but are still missing in pSS models. These studies will be an important contribution to decipher the molecular mechanisms involved in tolDCs-induced tolerance (77).

Although targeting of the immune system is a relevant approach in pSS, considering the (auto)immune manifestations and systemic nature of the disease, the direct target of epithelial cells also seems to be important and necessary to stimulate epithelial cell regeneration (78). Multifaceted approaches consisting of both anti-inflammatory and pro-epithelial function, e.g., immunotherapy combined with cell therapy, might provide crucial insights to revert epithelial cell function.

The studies presented in this thesis provide valuable novel insight at different epigenetic, transcriptional and function levels, which may yield new strategies for treatment of pSS patients. Although, these results lack longitudinal research

or in vivo validation to clarify the ideal targeting timeline of each mechanism, their potential for future clinical translation and therapeutic application seems very promising.

Overcoming immune adaptability to improve treatment efficacy

The therapeutic management of pSS has not changed substantially in recent decades (79), despite the advances in the understanding of the biological pathways, cell types and molecules underlying pSS. The development of biological therapies led to a great therapeutic advance to specific and direct target single or classes of molecules, genes and proteins. Although their availability opened up a large number of possibilities for the management of pSS (80), the findings of randomized clinical trials performed in patients with pSS are, in part, disappointing (81-88).

Several reasons can be appointed to explain the disappointing clinical trial outcomes, including treatment duration, patient characteristics, and single target approaches. As pSS is a chronic autoimmune disease, it is possible that the treatment duration of the clinical trials is not sufficient to capture a significant improvement in clinical parameters. In fact, the effects of hydroxychloroquine can take up to six months to be observed (51). On the other hand, the inclusion of patients with low systemic disease can be a hindrance as it is hard to see clinical improvement, such as reduction of disease activity measured by the EULAR Sjögren's syndrome disease activity index (ESSDAI), once they are not highly elevated at baseline. Moreover, the timing of therapy application is an important factor to take into consideration, in order to increase the chances of reversing the functional organ derangement, given the general early loss of salivary gland function in pSS (78).

With respect to therapeutic approaches that are directed to single targets, e.g., specific cell subsets or mediators to reduce immune cell activation in pSS patients, their clinical response remained quite disappointing despite the extensive efforts (58). The variety and complexity of the inflammatory pathways, which is also demonstrated in **chapters 4** to **6**, can represent the major drawback to single target therapy efficacy. Hence the versatility of the immune system which can be demonstrated by the ability of several immune mediators to compensate and overcome immune cell dysregulation should be taken into account when developing novel therapeutic regimens.

Multiple studies were carried out to understand the role of B cells and to specifically tackle this cell subset (89). These developments led to treatments that either directly deplete B cells (anti-CD20/rituximab NCT00363350) or indirectly affect their development by targeting B-cell dependent cytokines e.g., anti-BAFF/belimumab (NCT01160666/NCT01008982), anti-IL-6 receptor/tocilizumab (NCT01782235) and anti-lymphotoxin-β receptor/baminercept (NCT01552681) (90). Although these therapies are effective, at least partly, in reducing B cell activation, these clinical trials did not show convincing efficacy for pSS patients. This absence of clinical benefit could be related to prominent activation of other immune cells including (autoreactive) T cells, NK cells, ILCs and DCs, which may continue to drive inflammation. Additionally, compensatory mechanisms such as increased BAFF production by myeloid cells can help B cells to overcome drug effects. In line with this hypothesis, a new biologic (anti-BAFF receptor/ianalumab; NCT02962895) with a dual mode of action combining direct lysis of B cells and blockade of BAFF-mediated B-cell maturation, proliferation, and survival, was effective to reduce disease activity in pSS patients (91).

Recently, the results of different clinical trials that simultaneously trigger multiple pathological pathways showed clinical efficacy and are paving the way to consider combinatory therapies in pSS treatment. Given the wide impact of the bi-directional interaction between T and B cells, for germinal center responses, isotype class switching and for long-term effective T cell and B cell memory (92), their simultaneous inhibition constitute an important therapeutic approach. Combined treatment of leflunomide, which mainly inhibits T cell proliferation and activation, and hydroxychloroquine, which suppresses TLR7/9-driven activation of B cells and pDCs showed unprecedent clinical efficacy, as measured by ESSDAI and significant improvements in other clinical parameters, including dryness and fatigue (52).

In line with blockade of bi-directional interactions, a therapeutic approach blocking CD40/CD40L axis with anti-CD40/iscalimab (NCT04541589) was also clinically effective to improve ESSDAI scores (93). Nonetheless, treatment with anti-CD80/86/abatacept (NCT02067910/NCT02915159), which also blocks the interaction of T cells with B cells, did not show clinical efficacy assessed by ESSDAI in pSS patients compared with placebo (84, 94). It can be speculated that the differential response of these two therapies, is not dependent on blocking T and B cell interaction, as both drugs have this potential, but on their capacity to broadly modulate other cell-cell interactions. As CD40 is constitutively expressed on dendritic cells, macrophages, salivary gland ductal epithelial

cells and endothelial cells (95, 96), iscalimab treatment can also disrupt stromal-mediated immune activation, antibody-mediated and cellular immune processes. Thus, therapeutic strategies, which combined distinct modes of action and targeting different cells or pathways seem to represent the most promising treatment options for pSS patients.

Hence, it can be postulated that the specific targeting of immune processes in pSS should consider the interaction between the different immune functions and the existence of many compensatory and complementary inflammatory mechanisms. By understanding the role of the different immune cell subsets in the disease we will be a step closer to efficiently halt immune activation in pSS. The results described in this thesis show that cDC2s can orchestrate and maintain both T and B cell activation (**chapters 3** to **5**), and that the inflammatory mediators can alter the transcriptome of monocytes (**chapter 6**), stressing their role in pSS. Our results point to the need to intervene in the most optimal way to tackle both the chain of activating events and the effector functions of T and B cells. Altogether these results, support a stepwise approach, to damp immune cell hyperactivation (pDCs, T and B cells) and then restore a state of immune tolerance (cDC2s and monocytes) to prevent reactivation of the disease process. Combination of different treatments including DMARDs, biologicals and synthetic inhibitors to exploit their synergistic and complementary mechanisms may lead to important changes in the future management of pSS and other autoimmune diseases. These tailored therapeutic approaches combined with optimized patient stratification seem promising to reduce the chances of developing side effects.

Challenges and limitations

Among the challenges of studying cDC2s in pSS, their low numbers in circulation hampers and limits certain functional experiments in samples from patients, representing the main hindrance for experimental validation. In line with findings described in **chapter 3** and **4**, validation with rescue experiments to restore the normal expression of miRNA-130a and lncRNA *HCP5* in cDC2s from pSS patients would be relevant to confirm their role in pSS. However, these experiments require relatively high blood volumes that are not possible to obtained from patients.

The identification of cDC2s in the exocrine glands also represents a major challenge, considering that these cells express different sets of markers depending on the tissue or the state of immune response. The studies described

in **chapters 3** to **5** would benefit from confirmation of the presence of cDC2s in the salivary glands of pSS patients documenting their altered epigenomic features and their proximity with T and B cells. However, the lack of a proper method to accurately identify these cells or the use of a limited set of markers, generally used in conventional imaging approaches, hinders their proper identification. Nevertheless, the recently improved imaging technologies and transcriptomic data might help researchers to establish reliable markers and methods to accurately define and investigate cDC2s subsets in pSS.

Recent advances in single-cell technologies have contribute to a rapid acceleration in the discovery of new cDC2 subpopulations leading to the identification of two subsets, the DC2 and DC3 with distinct phenotypic and functional characteristics (97). Changes within cDC2s and within DC3 subsets were described in SLE and SSc patients (97), but were not assessed in pSS patients or mouse models of pSS yet. A better characterization of these subsets in pSS could improve the understanding of the finding described in **chapter 4** and might provide rationale for new therapeutic strategies targeting specific cDC2s subsets.

The studies presented in this thesis are based on results observed in circulation and their functional consequences rely on in vitro studies, which do not take into account the tissue microenvironment. Thus, validation of these findings in more complex culture systems, or in vivo mouse models would be valuable to confirm and extend the primary findings, and to understand the immune system interaction in pSS. Previous research based on mouse models of pSS uncovered many essential factors involved in the disease, including the role of autoreactive T cells, B cell hyperactivity, autoantibodies production and apoptosis of glandular epithelial cells (21). As such, the use of mouse models could be beneficial to test whether in vivo the application of the compounds proposed based on our findings could be relevant for pSS patients. Although there is controversy to exactly extrapolate the results from murine models into a clinical setting, these models are essential to elucidate underrated aspects of immune cell interactions. To overcome these differences, the use of 3D culture models could represent an important future step for pSS research as they can be developed to mirror multiple disease aspects, support different cell subsets and be used for drug screening.

Concluding remarks and future considerations

The complexity of pSS disease has become increasingly clear, and current technological development has raised different perspectives and visions into the global and pathway-specific immune landscape of pSS. Altogether, the results presented in this thesis provide novel insights into the mechanisms driving immune cell activation and contribute to strengthen the knowledge of how biological pathways, cell types and molecules interact, and interplay in the development and perpetuation of pSS. At the moment, still with limited clinical application, significant progresses have been made to comprehend the role of cDC2s and monocytes in pSS pathophysiology. The direct involvement of these cells on priming immune responses, producing inflammatory mediators, and potentially inducing tissue destruction, identify them as promising candidates for therapeutic intervention in pSS and other related immune mediated autoimmune diseases. Our studies reinforce the concept that patients with pSS as well as nSS display a large variability of clinical and biological phenotypes and thereby the use of a single therapy, is unlikely to give positive results in all patients.

As we move toward patient-orientated precision medicine, it will be equally important to move toward patient-orientated clinical trial designs to test new therapeutic interventions. Novel trial design should take into account distinct subgroups of pSS patients, established based on the drug targeting mechanisms, rather than on random allocation. In this way it will be possible to have a better understanding on the clinical efficacy, by specifically targeting the derailed mechanisms while decreasing the chances of developing side effects. Innovative biological technologies like single-cell proteomic, and transcriptomic analyses applied to peripheral blood, and exocrine glands will open novel possibilities to improve patients' characterization and treatment. Finally, optimal therapies approaches should be based on drugs that modify more than one disease-associated pathway in a fine-tuned way, in which the immune system is modified enough to restrict autoimmunity but, simultaneously, remains functional active to effectively respond to infections and cancer.

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Appendix

English Summary

Background

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease characterized by lymphocytic infiltration of exocrine glands, particularly, salivary and lacrimal glands, associated with glandular destruction and dysfunction. As a result, the clinical consequences are typically keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). The immunological hallmarks of pSS include B cell hyperactivity, the presence of anti-Ro/SSA and anti-La/SSB autoantibodies and interferon (IFN) signature. The immune mechanisms behind the self-directed damage of exocrine gland tissue are still not fully understood, however the composition of the tissue lesions seen in pSS patients suggests an altered crosstalk between the innate, adaptive immunity and salivary epithelial cells. The role of T and B cells in pSS has been long-standing evidence, since they represent the major infiltrating population into the salivary glands and are drivers of pro-inflammatory cytokine and autoantibody production. Type 2 conventional dendritic cells (cDC2s) and monocytes are important activators of these cells, either by direct interaction or through the production of inflammatory mediators, but their contribution to pSS immunopathology has been poorly studied.

Recently, noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), have emerged as important regulators of gene expression with relevance in immune cell activation. Their role in inflammation and immune responses is highlighted in the maturation, proliferation, differentiation and activation of immune cells. In addition to their cellular posttranscriptional role, these epigenetic regulators, in particular miRNAs, can be present in the extracellular environment and thus mediate cell–cell signalling. Considering the key role of noncoding RNAs, cDC2s and monocytes in orchestrating inflammatory responses, understanding their role in pSS immunopathology is relevant and may disclose novel opportunities to halt immune dysregulation in pSS.

Aim of this thesis

The aim of the studies presented in this thesis was to investigate systemic and cellular epigenetic regulators in pSS, their contribution to cDC2s function and to study the key mediators and molecular mechanisms that drive cDC2s and monocyte activation in pSS.

Summary of the findings

Considering the clinical and immunological heterogeneity of pSS patients and the importance of circulating small non-coding RNAs (snc)RNAs, including miRNAs in the regulation of immune functions, in **chapter 2**, we performed serum sncRNAs profiling and correlate their levels with systemic and local inflammatory features. We identified and validated, in circulation, the increased expression of U6-snRNA and miR-661 in non-Sjögren's sicca patients compared to healthy controls (HC), and pSS patients. Although, circulating sncRNAs were not differently expressed in pSS patients compared to HC, their heterogeneous expression was associated with local immune infiltration and circulating inflammatory parameters. We identified three clusters of pSS patients with distinct sncRNA expression reflecting distinct levels of B cell hyperactivity and IFN signature. Thus, we show that despite not being differentially expressed in circulation, the sncRNA expression pattern, can be used to distinguish pSS patients based on their systemic inflammatory features.

Given the important role in regulation of cell activation by miRNAs and the potential of cDC2s to orchestrate immune activation, in **chapter 3**, we investigated the miRNA profile of circulating cDC2s from pSS patients and HC. The expression of the miR-708 and miR-130a was consistently decreased in cDC2s from pSS patients in two independent cohorts. The expression of both miRNAs was downregulated upon stimulation, likely as a consequence of cell activation. The mitogen- and stress-activated protein kinase-1 (MSK1), an upstream mediator of cytokine production, was identified as a novel endogenous target of miR-130a in cDC2s. MSK1 expression in cDC2s as well as IL-12 and TNF-α-producing cDC2s were increased in pSS patients. MSK1-inhibition reduced cDC2 activation and production of IL-12, TNF-α, and IL-6. Thus, we show that in cDC2s of pSS patients miRNAs are dysregulated and that these alterations contribute to increase cDC2s activation and pro-inflammatory cytokine production, which in turns contributes to maintain the epigenetics imbalance.

Recently, lncRNA histocompatibility leukocyte antigen complex P5 (*HCP5*) was associated with innate and adaptive immune response and a single-nucleotide polymorphism was identified as susceptibility locus for pSS. Thus, in **chapter 4**, we sought to analyze the role of *HCP5* in the regulation of cDC2s function. In cDC2s, *HCP5* expression is upregulated by immune activation and is dependent on the transcription factors SP1, NF-kB and STAT1. *HCP5* robustly regulates type-I IFN signalling as well as IFN-β and chemokines production to sustain

immune cell recruitment. We showed the impact of *HCP5* in driving B and $CD4^+$ T cell activation by cDC2s and found reduced B cell survival, in line with reduced *TNFSF13B* expression. As for $CD4^+$ T cells, *HCP5* silencing in cDC2 enhanced T follicular and Th1/17 cytokine production associated with modulation of PD-L1 expression and $CD14^+$ DC3 skewing. *HCP5* was overexpressed in the salivary glands of pSS patients and its expression was associated with immune infiltration and cDC2s. In circulation, *HCP5* is upregulated in cDC2s, pDCs and B cells of pSS patients possibly driven by immune activation. Thus, we show that lncRNA *HCP5* is an important orchestrator of cDC2 function and immune activation, impacting both innate and adaptive immunity. Its dysregulation in pSS suggests that *HCP5* could play an important role in regulating inflammation and cell activation in this disease.

In light of the important role of cDC2s to initiate inflammatory responses and activate T cells, in **chapter 5**, we exploited the transcriptomic profile of cDC2s to unravel the regulation of immunological pathways and functions in pSS patients. The transcriptome of circulating cDC2s from pSS patients revealed dysregulation in cell trafficking, activation, interferon signalling, and class-I mediated antigen processing and presentation. Phenotypic validation showed increased CX3CR1 expression and decreased expression of integrin beta-2 and plexin-B2 on pSS-cDC2s, corroborating the transcriptomic profile. Functional validation confirmed that cDC2s from pSS patients process antigens less efficiently, which can lead to a prolonged antigen storage and supply to MHC-I for $CD8^+$ T cell cross-presentation. In addition, cDC2s from pSS patients have an increased antigen uptake compared to cDC2s from HC, including self-antigens derived from salivary gland epithelial cells. These changes in antigen processes were linked to anti-SSA autoantibodies and the presence of type-I IFNs, and validated by the significantly enhanced antigen uptake of cDC2s from HC after IFN-α priming. In line with increased antigen uptake, cDC2s from pSS patients increased the proliferation of $CD4^+$ T cells with a tissue homing phenotype and increased TNF-α levels, suggestive of efficient migration to the inflammatory sites. Thus, we demonstrate novel molecular and functional evidences for the role of cDC2s in orchestrating immune response in pSS, which may yield novel avenues for treatment.

The contribution of monocytes in pSS is well established not only in the initial phase of the immune response but also in the maintenance of chronic inflammation. Yet, the molecular mechanisms and inflammatory mediators underlying their aberrant function have been poorly studied. In **Chapter 6**,

we set out to investigate the transcriptional profile of circulating monocytes of pSS patients to unravel the mechanisms linked to cell activation. Monocytes from pSS patients exhibited a transcriptome enriched in gene expression profile associated with intermediate and non-classical monocytes subsets. In addition, gene signature analysis revealed transcriptional dysregulation of processes related with translation, IFN-signaling and TLR-signaling, which were associated with clinical features of patients with pSS. We functionally demonstrated, using HC monocytes, that mediators present in serum of pSS patients, partially mimicked the transcriptomic signatures of pSS monocytes. In addition, we also identified IFN-dependent and -independent mechanisms as drivers of transcriptional alterations since IFN-α/β receptor blocking prevented these alterations, to a certain extent. Thus, we demonstrate that circulating inflammatory mediators, including type-I IFNs, underly transcriptional alterations in monocytes from patients with pSS, contributing to their activation.

Concluding remarks

The results presented in this thesis demonstrate that epigenetic regulators such as circulating miRNAs may aid to identify subclusters of pSS patients as they seem to reflect their systemic inflammatory profile. In addition, our findings also reveal different levels of epigenetic, transcriptomic and functional dysregulation in cDC2s and monocytes from pSS patients which significantly increases the understanding of their contribution to immune cell activation and salivary gland dysfunction.

Given the crucial role of cDC2s and monocytes in the initiation and maintenance of immune activation under normal physiological conditions, but more importantly in immune-mediated inflammatory diseases, such as pSS, the insights presented here disclose novel opportunities to halt inflammation, which may yield novel therapeutic avenues. Nonetheless, future research targeting the pathways (e.g., IFN and antigen processes) and regulators (e.g., miRNA-130a and lncRNA-*HCP5*) identified in this thesis will contribute to extend the understanding of their precise role in pSS immunopathology.

Nederlandse samenvatting

Achtergrond

Het Primaire Sjögren syndroom (pSS) is een chronische auto-immuun ziekte die gekarakteriseerd wordt door infiltratie van de exocriene klieren door lymfocyten, in het bijzonder de speeksel en traan klieren. Dit gaat gepaard met afbraak en een slechter functioneren van deze klieren dat zorgt voor de typische klinische manifestaties als keratoconjunctivitis sicca (droge ogen) en Xerostomia (droge mond). Immunologische kenmerken van pSS zijn o.a. B cel hyperactiviteit, de aanwezigheid van autoantilichamen gericht tegen Ro/SSA en La/SSB en een verhoogde activiteit van interferonen, gemeten dmv de zogenaamde interferon signatuur. De onderliggende ontstekings mechanismen die zorgen voor de destructie van de exocriene klieren zijn nog niet volledig opgehelderd maar de samenstelling van het aangetaste weefsel in pSS patiënten doet een verandering in de communicatie tussen afweer cellen en de epitheel cellen van de klieren vermoeden. Er is al langer bekend dat T en B cellen hierbij een belangrijke rol spelen. Zij maken de hoofdmoot uit van de cellen die de klieren binnendringen en ze produceren ontstekingsbevorderende cytokines en autoantilichamen. Echter over de cellen die T en B cellen aanjagen is nauwelijks iets bekend. Type 2 conventionele dendritische cellen (cDC2s) en monocyten staan bekend als belangrijke aanzetters van deze cellen door direct contact, en door de productie van ontstekingsbevorderende mediatoren. Echter, de precieze rol van deze cellen in de ontsteking en weefseldestructie van pSS patienten is niet of nog maar nauwelijks onderzocht. Het onderzoeken van de rol van deze cellen waren daarom belangrijke doelen van dit proefschrift.

In onze cellen, inclusief onze afweer cellen, dient het RNA als code voor het aanmaken van eiwitten, onder andere ontstekingsbevorderende eiwitten. Recentelijk zijn RNA moleculen die niet coderen voor eiwitten, zoals micro RNAs (miRNAs) en lange niet-coderende RNAs (lncRNAs), in beeld gekomen als belangrijke regulatoren van cel functies. Deze RNAs zijn cruciaal voor het reguleren van gen expressie met grote relevantie voor activatie van immuun cellen. Hun bijdrage in ontsteking en immuun reactie wordt duidelijk door hun rol bij de rijping, proliferatie, differentiatie en activatie van immuun cellen. Naast hun rol in immuun cellen (intracellulair) spelen deze zogenaamde epigenetische regulatoren, in het bijzonder miRNAs, ook een rol bij de communicatie tussen cellen doordat ze aanwezig zijn in de lichaamsvloeistoffen (extracellulair). Gezien de belangrijke rol van cDC2s en monocyten, en van niet coderende RNAs, bij ontstekingsreacties is het zeer relevant om hun rol in de ontsteking en

weefselschade van pSS op te helderen. Dit kan nieuwe inzichten opleveren om de verstoorde immuun reactie in pSS tot staan te brengen.

Doel van dit proefschrift

Het doel van de experimenten beschreven in dit proefschrift was om systemische en cellulaire epigenetische regulatoren in pSS te onderzoeken en hun bijdrage aan het functioneren van cDC2 cellen en monocyten, en om de belangrijkste spelers en moleculaire mechanismen die cDC2s en monocyten aanzetten tot activatie in pSS te bestuderen.

Samenvatting van de bevindingen

Gezien de klinische en immunologische diversiteit van pSS patiënten en het belang van circulerende kleine niet-coderende RNAs (sncRNAs), waaronder miRNAs, in de regulatie van het immuunsysteem hebben we in **hoofdstuk 2** onderzocht welke sncRNAs in serum voorkomen en of hun hoeveelheid te correleren was aan systemische en/of lokale ontstekingskenmerken. We ontdekten een toegenomen hoeveelheid van U6-snRNA en miR-661 in het bloed van non-Sjögren's sicca patiënten ten opzichte van gezonde controles en pSS patiënten. Alhoewel het voorkomen van snRNAs in het bloed niet verschilde tussen pSS patiënten en gezonde controles hing het voorkomen wel samen met lokale ontstekingsprocessen en in het bloed te meten ontstekingsparameters. We konden drie clusters van pSS patiënten onderscheiden met een verschil in sncRNA expressie die overeenkwamen met een verschillend niveau van B cel hyperactiviteit en de zogenaamde IFN signatuur. We konden aantonen dat het expressie patroon van circulerende sncRNAs, hoewel niet verschillend, toch gebruikt kan worden om een onderscheid te maken in pSS patiënten op basis van hun ontstekingskenmerken in het bloed.

Gezien de belangrijke rol die miRNAs spelen bij de regulatie van activatie van cellen, en de potentie van cDC2 cellen om de immuun activatie te beïnvloeden, hebben we in **hoofdstuk 3** het miRNA profiel van circulerende cDC2s van pSS patiënten en gezonde controles onderzocht. De expressie van miR-708 en miR-130a was consequent verlaagd in cDC2s van pSS patiënten in twee verschillende patiënt cohorten. De expressie van beide miRNAs werd verlaagd na stimulatie, mogelijk als gevolg van cel activatie. MSK1 werd ontdekt als een nieuw doelwit van miR-130a in cDC2s. In pSS patiënten was de expressie van MSK1 in cDC2s verhoogd, evenals de hoeveelheid IL-12- en TNFα-producerende cDC2s. We konden tevens aantonen dat in cDC2s van pSS patiënten de miRNAs ontregeld zijn en dat de veranderingen bijdragen aan een verhoogde cDC2 activiteit en

ontstekingsbevorderende cytokine productie, die op hun beurt weer bijdragen aan de epigenetische onbalans.

Recentelijk is ontdekt dat het lncRNA histocompatibilty leukocyte antigen complex (*HCP5*) geassocieerd is met de immuun reacties in pSS patienten en dat genetisch variatie in het gen voor *HCP5* geïdentificeerd is als gevoeligheidslocus voor pSS. Daarom hebben we in **hoofdstuk 4** getracht om de rol van *HCP5* in de regulatie van het functioneren van cDC2s te analyseren. In cDC2s zorgt immuun activatie voor de toename van *HCP5* en is deze toename afhankelijk van de transcriptie factoren SP1, NF-kB en STAT1. *HCP5* zorgt voor een robuuste regulatie van type-I IFN signalering evenals IFNβ en chemokinen productie om zodoende de activatie van immuun cellen en het aantrekken naar de ontstekingshaarden in stand te houden. We toonden aan dat de hoeveelheid HCP5 gevolgen heeft voor de activatie van B en $CD4^+$ T cellen door cDC2 cellen en vonden verminderde overleving van B-cellen en dat dat in lijn was met een gereduceerde expressie van *TNSF13B*. Het terugdringen van HCP5 in cDC2 cellen zorgde in $CD4^+$ T cellen voor een toegenomen productie van ontstekingsbevorderende T folliculaire en Th1/17 cytokinen Dit was tevens geassocieerd met de verandering in PD-L1 expressie en het uitrijpen naar $CD14^+$DC3 cellen. Daarbij is *HCP5* verhoogd in de speekselklieren van pSS patiënten en is het geassocieerd met lokale ontsteking en cDC2 markers. De expressie van *HCP5* is tevens verhoogd in cDC2s, pDCs en B cellen uit het bloed van pSS patiënten. We tonen dus aan dat lncRNA HCP5 een belangrijke speler is bij het activeren van cDC2 cellen en immuun reacties. De veranderde rol van HCP5 in pSS kan een aanwijzing zijn dat het een belangrijke rol speelt in de regulatie van ontsteking in deze ziekte.

Omdat cDC2s een belangrijke rol spelen bij de initiatie van een ontstekingsreactie en de activatie van T cellen hebben we in **hoofdstuk 5** gebruik gemaakt van het transcriptie profiel van cDC2 cellen om uit te zoeken hoe de regulatie en functie van immunologische processen bij deze cellen in pSS patiënten is. Het transcriptoom van cDC2 cellen in het bloed van pSS patiënten toonde een verandering in cel transport, activatie, interferon signalering, en klasse-I gemedieerde antigeen processing en presentatie. Daarbij werd een toegenomen expressie van CX3CR1 en een afgenomen expressie van integrin beta-2 en plexin-B2 op pSS-cDC2 cellen waargenomen. Functionele studies bevestigde dat cDC2s van pSS patiënten minder efficiënt antigenen kunnen verwerken, wat weer kan leiden tot een verlengde antigeen opslag en aanvoer aan MHC-I moleculen. Dit kan activatie van $CD8^+$ cytotoxische T cellen bevorderen. Daarbij komt dat

cDC2s van pSS patiënten een toegenomen opname hebben van antigenen in vergelijking met cDC2s van gezonde controles, waaronder lichaamseigen antigenen van klier epitheel cellen. Deze veranderingen in antigeen processing konden gekoppeld worden aan verhoogde anti-SSA autoantilichamen en de aanwezigheid van een interferon signatuur. Deze processen werden bevestigd door een significante toename van de antigeen opname van cDC2s van gezonde controles na eerst te zijn behandeld met het IFN-α. In overeenstemming met een toegenomen antigeen opname waren cDC2s van pSS patiënten in staat om $CD4^+$ T cellen, met een voorkeur voor weefsels en een toegenomen TNF-α niveau, meer te laten prolifereren, wat kan duiden op een efficiëntere migratie naar plaatsen van ontsteking. Daarmee geven we nieuw moleculair en functioneel bewijs van de rol van cDC2s en de beïnvloeding van de immuun reactie wat mogelijk kan leiden tot nieuwe wegen van behandeling.

De bijdrage van monocyten in pSS is algemeen aanvaard, niet alleen bij de primaire immuun reactie maar ook bij het in stand houden van een chronische ontsteking. Toch zijn de moleculaire mechanismen en ontstekingsmediatoren die hieraan ten grondslag liggen nog maar weinig bestudeerd. In **hoofdstuk 6** hebben we het transcriptionele profiel van circulerende monocyten van pSS patiënten bestudeerd om er achter te komen welke mechanismen gerelateerd kunnen worden aan cel activatie. Monocyten van pSS patiënten hebben een transcriptoom dat verrijkt is in gen expressie profielen geassocieerd met intermediare en niet-klassieke monocyten. Daarbij toonde bestudering van de gen signatuur analyse een ontregeling aan van processen betrokken bij eiwittranslatie, de IFN-signatuur en TLR-signalering. Deze processen waren geassocieerd met klinische eigenschappen van pSS patiënten. Gebruikmakend van monocyten van gezonde controles konden we aantonen dat componenten aanwezig in het serum van pSS patiënten deels hetzelfde transcriptie patroon, zoals gevonden in monocyten van pSS patënten, konden bewerkstellen. Verder vonden we ook IFN-afhankelijke en –onafhankelijke mechanismen als drijvende kracht van transcriptionele veranderingen omdat het blokkeren van de IFN-α/β receptor deze veranderingen deels kon voorkomen. Zo konden we aantonen dat ontstekingsmediatoren in het bloed, waaronder de type-I interferonen, ten grondslag liggen aan veranderingen in monocyten van pSS patiënten die bijdragen aan hun activatie.

Concluderende opmerkingen

De resultaten beschreven in dit proefschrift tonen aan dat epigenetische regulatoren zoals miRNA een bijdrage kunnen leveren bij de identificatie

van sub-clusters van pSS patiënten omdat ze hun ontstekingsprofiel lijken te reflecteren. Daarbij tonen onze resultaten ook verschillende niveaus van epigenetische, transcriptionele en functionele ontregeling in cDC2s en monocyten van pSS patiënten aan. Deze, nieuwe kennis doen ons begrip van de bijdrage van deze factoren aan activatie van immuun cellen en het niet goed functioneren van speeksel klieren significant toenemen.

cDC2s en monocyten spelen een cruciale rol bij het initiëren en handhaven van immuun activatie onder fysiologische condities, maar belangrijker ook in immuun-gemedieerde ontstekingsziekten zoals pSS. Het inzicht vergaard in dit proefschrift opent nieuwe wegen om ontstekingen een halt toe te roepen en daarmee nieuwe therapeutische behandelingen mogelijk te maken.

Toekomstig onderzoek gericht op de processen (b.v. type I IFN en antigeen processing) en epigenetische regulatoren (b.v. miRNA-130a en lncRNA-*HCP5*) ontdekt in dit proefschrift zullen bijdragen aan de kennis van hun precieze rol in de immuno-pathologie in pSS.

Resumo em português

Introdução

A síndrome de Sjögren primária (SS) é uma doença autoimune sistémica crónica caracterizada pela infiltração linfocítica das glândulas exócrinas, em particular das glândulas salivares e lacrimais, associada à sua destruição e disfunção. Como resultado, estes doentes apresentam ceratoconjuntivite seca (olhos secos) e xerostomia (boca seca). As características imunológicas da SS incluem hiperatividade dos linfócitos B, a presença de autoanticorpos anti-Ro/SSA e anti-La/SSB, e a expressão excessiva de genes dependentes de interferão (perfil de IFN). Os mecanismos imunológicos subjacentes à autodestruição das células das glândulas exócrinas ainda não são totalmente compreendidos. No entanto, a composição celular das lesões teciduais dos doentes com SS sugere uma interação anormal entre as células da imunidade inata, adaptativa e as células epiteliais salivares. O papel dos linfócitos T e B na SS encontra-se bem caracterizado, uma vez que estas células representam a maioria das células que infiltram as glândulas salivares e são responsáveis pela produção de citocinas pró-inflamatórias e autoanticorpos. As células dendríticas convencionais do tipo 2 (cDC2s) e os monócitos são mediadores importantes na ativação dos linfócitos T e B, quer seja por interação direta, ou pela produção de mediadores inflamatórios, contudo, a sua contribuição para a imunopatologia da SS tem sido pouco estudada.

Recentemente, as moléculas de RNAs não codificantes, incluindo os microRNAs (miRNAs) e os RNAs longos não codificantes (lncRNAs), surgiram como importantes reguladores da expressão genética com relevância na ativação de células imunes, podendo controlar processos como a maturação, proliferação, diferenciação e ativação das células imunes durante o processo inflamatório. Além de responsáveis por modificações pós-translacionais da própria células, estes reguladores epigenéticos, em particular os miRNAs, podem também mediar a sinalização célula-célula, por estarem presentes no ambiente extracelular. Considerando que as moléculas de RNAs não codificantes, as cDC2s e os monócitos têm um papel fundamental na organização da resposta inflamatória, é relevante entender a sua contribuição na imunopatologia da SS. Deste modo, estes resultados poderão identificar novas oportunidades para combater a desregulação imune observada na SS.

Objetivo da tese

O objetivo dos estudos apresentados nesta tese foi investigar o papel de diferentes reguladores epigenéticos presentes em circulação e a sua contribuição para a função das cDC2s na SS. Foi também foco destes estudos avaliar os mecanismos moleculares e potenciais mediadores que regulam a ativação das cDC2s e dos monócitos na SS.

Resumo dos resultados

Considerando a heterogeneidade clínica e imunológica dos doentes com SS e a importância em circulação das pequenas moléculas de RNAs não codificantes (snc)RNAs, incluindo os miRNAs, na regulação das funções imunes, no **capítulo 2**, estabeleceu-se o perfil sérico das sncRNAs e correlacionou-se com as características inflamatórias locais e sistémicas. A expressão de U6-snRNA e miR-661, em circulação, encontra-se aumentada em doentes com síndrome sicca (secura ocular e/ou salivar na ausência de doença autoimune) em comparação com indivíduos saudáveis (HC) e doentes com SS, e foi posteriormente validada noutro grupo independente de doentes. Embora a expressão das sncRNAs em circulação dos doentes com SS seja comparável com os HC, a sua expressão associa-se com parâmetros de infiltração imune das glândulas salivares e com parâmetros inflamatórios sistémicos. Com base no padrão de expressão das sncRNA, foi possível identificar três subgrupos de doentes com SS com diferentes graus de hiperatividade dos linfócitos B e perfil de IFN. Assim, estes resultados demonstram que, apesar da expressão das sncRNA em circulação não ser diferente dos HC, o seu padrão de expressão pode ser utilizado para distinguir doentes com SS com diferentes características inflamatórias sistémicas.

Dado o importante papel dos miRNAs na regulação da ativação celular e o potencial das cDC2s para coordenar a ativação do sistema imune, no **capítulo 3**, analisou-se o perfil de miRNA das cDC2s em circulação de doentes com SS e HC. A diminuição da expressão do miR-708 e miR-130a nas cDC2s dos doentes com SS foi consistente em duas populações independentes de doentes e HC. A expressão de ambos os miRNAs diminui após estimulação, provavelmente em consequência de uma regulação negativa da ativação celular. A proteína quinase-1 ativada por mitógeno e stresse (MSK1), que é um mediador da produção de citocinas, foi identificada como um novo alvo endógeno do miR-130a nas cDC2s. A expressão da proteína MSK1 em cDC2s dos doentes com SS encontra-se aumentada, bem como a frequência de cDC2s que produzem IL-12 e TNF-α. A inibição da proteína MSK1 reduz a ativação das cDC2 e a produção

de IL-12, TNF-α e IL-6. Assim, conclui-se que os miRNAs estão desregulados nas cDC2s dos doentes com SS e que estas alterações contribuem para aumentar a sua ativação e a produção de citocinas pró-inflamatórias, o que por sua vez contribui para manter o desequilíbrio epigenético.

Recentemente, além de ser associado com a resposta imune inata e adaptativa, um polimorfismo de nucleotídeo único com elevado risco de suscetibilidade para SS foi identificado no lncRNA complexo P5 do antigénio de histocompatibilidade leucocitário (*HCP5*). Assim, no **capítulo 4**, procurou-se estudar o papel do *HCP5* na regulação da função das cDC2s. Nas cDC2s, a expressão do *HCP5* é regulada positivamente após ativação e é dependente dos fatores de transcrição SP1, NF-kB e STAT1. O *HCP5* regula ainda de forma positiva a sinalização da via do IFN do tipo I, bem como a produção de IFN-β e quimiocinas de forma a promover o recrutamento de células imunes. Para além disso, a expressão do *HCP5* nas cDC2s afeta a função dos linfócitos B e T $CD4^+$, tendo-se observado uma redução da sobrevivência de células B, em linha com a redução da expressão de *TNFSF13B*. Quanto aos linfócitos T $CD4^+$, o silenciamento do *HCP5* nas cDC2s aumenta a produção de citocinas relacionadas com o fenótipo T folicular e Th1/17. Este aumento parece estar associado com a modulação da expressão da proteína ligando 1 da morte celular programada (PD-L1) e com a alteração do fenótipo das cDC2s para um fenótipo semelhante às $CD14^+$ DC3. A expressão do *HCP5* encontra-se aumentada e associada à infiltração de células imune e cDC2s nas glândulas salivares dos doentes com SS. Em circulação, observa-se uma expressão aumentada do *HCP5* nas cDC2s, nas células dendríticas plasmacitóides e nos linfócitos B dos doentes com SS, possivelmente devido à presença de ativadores do sistema imune. Neste sentido, é possível concluir que o lncRNA *HCP5* é um importante regulador da função das cDC2 e consequentemente da ativação do sistema imune com impacto, quer na imunidade inata, quer na imunidade adaptativa. A desregulação do *HCP5* em doentes com SS sugere que este lncRNAs poderá desempenhar um papel importante na regulação da inflamação e ativação celular nesta doença.

Tendo em conta o importante papel das cDC2s no desencadear da resposta inflamatória e na ativação dos linfócitos T, no **capítulo 5**, estabeleceu-se o perfil transcriptómico das cDC2s de forma a desvendar os mecanismos por detrás das funções imunológicas destas células em doentes com SS. A análise do transcriptoma das cDC2s revelou que processos como a migração e ativação celular, sinalização pela via do IFN e o processamento e apresentação de antigénios mediados pela via do complexo principal de histocompatibilidade

de classe I (MHC-I) estão desregulados em doentes com SS. A validação fenotípica destes resultados demostra um aumento da expressão de CX3CR1 e uma diminuição da expressão da integrina beta-2 e da plexina-B2 nas cDC2s dos doentes com SS, corroborando o perfil transcriptómico. Por outro lado, a validação funcional confirmou que as cDC2s dos doentes SS são menos eficientes a processar antigénios, o que pode levar a um armazenamento prolongado e apresentação via MHC-I aos linfócitos T $CD8^+$. Além disso, as cDC2s dos doentes com SS têm uma maior capacidade de capturar antigénios, incluindo os autoantigénios derivados das células epiteliais das glândulas salivares, em comparação com as cDC2s dos HC. Estes resultados associam-se à presença de autoanticorpos anti-SSA e de IFN do tipo I e foram confirmados após exposição das cDC2s de HC a IFN-α. As células expostas a IFN-α captaram uma maior quantidade de antigénios em comparação com as células não tratadas. Em linha com estes resultados, as cDC2s dos doentes com SS são mais eficientes a induzir a proliferação dos linfócitos T $CD4^+$, com um padrão fenotípico típico de migração para os tecidos inflamados, e a produção de TNF-α. Deste modo, estes resultados evidenciam, pela primeira vez, características moleculares e funcionais sobre o papel das cDC2s na resposta inflamatória em SS, abrindo novas possibilidades de tratamento para esta doença.

O papel dos monócitos na SS tem sido substancialmente estudado, não apenas na fase inicial da resposta imune, mas também na forma como contribuem para manutenção da inflamação crónica. No entanto, os mecanismos moleculares e os mediadores inflamatórios subjacentes à sua função têm sido pouco estudados. No **capítulo 6**, investigou-se o perfil transcriptómico dos monócitos em circulação dos doentes com SS para entender os mecanismos associados à ativação celular. Observou-se que o perfil de expressão génica dos monócitos dos doentes com SS está mais associado aos subtipos de monócitos intermediários e não clássicos. Para além disso, a análise do perfil transcriptómico revelou também uma desregulação nos processos celulares relacionados com a síntese de proteínas, a sinalização da via do IFN e dos recetores do tipo *toll*. Funcionalmente demonstrou-se que os mediadores presentes no soro dos doentes com SS induzem alterações transcriptómicas em monócitos de HC, semelhantes às encontradas nos monócitos dos doentes. Verificou-se ainda que o bloqueio do recetor do IFN-α/β impede parcialmente estas alterações, sugerindo a presença de mecanismos dependentes e independentes do IFN. Deste modo, conclui-se que os mediadores inflamatórios sistémicos, incluindo em parte o IFN do tipo I, promovem alterações transcriptómicas nos monócitos dos doentes com SS, que contribuem para sua ativação.

Considerações finais

Os resultados apresentados nesta tese demonstram que reguladores epigenéticos, como os miRNAs presentes em circulação, podem ser uteis na identificação de subgrupos de doentes com SS, uma vez que se parecem relacionar com diferentes perfis de inflamação sistémica. Além disso, os nossos estudos revelaram diferentes níveis de desregulação epigenética, transcriptómica e funcional nas cDC2s e nos monócitos dos doentes com SS, contribuindo assim significativamente para a compreensão do papel destas células na ativação do sistema imune e consequente destruição das glândulas salivares na SS.

Dado o papel crucial das cDC2s e dos monócitos na iniciação e manutenção da resposta imune, não só em condições normais, mas também em doenças inflamatórias, como a SS, os resultados apresentados nesta tese revelam novas oportunidades para regular a inflamação, o que poderá resultar em novas opções terapêuticas. No entanto, estudos adicionais focados, por exemplo, nos processos de apresentação de antigénio, e nos mediadores inflamatórios (por exemplo, IFN, miRNA-130a e lncRNA-*HCP5*) são necessários para ampliar a compreensão do seu papel na imunopatologia da SS.

"Caminhante, são as tuas pegadas
o caminho e nada mais;
caminhante, não há caminho,
faz-se caminho ao andar"

"Walker, are your footprints
the path and nothing else;
walker, there is no path,
the path is made by walking"

Proverbios y cantares; poema XXIX, Antonio Machado

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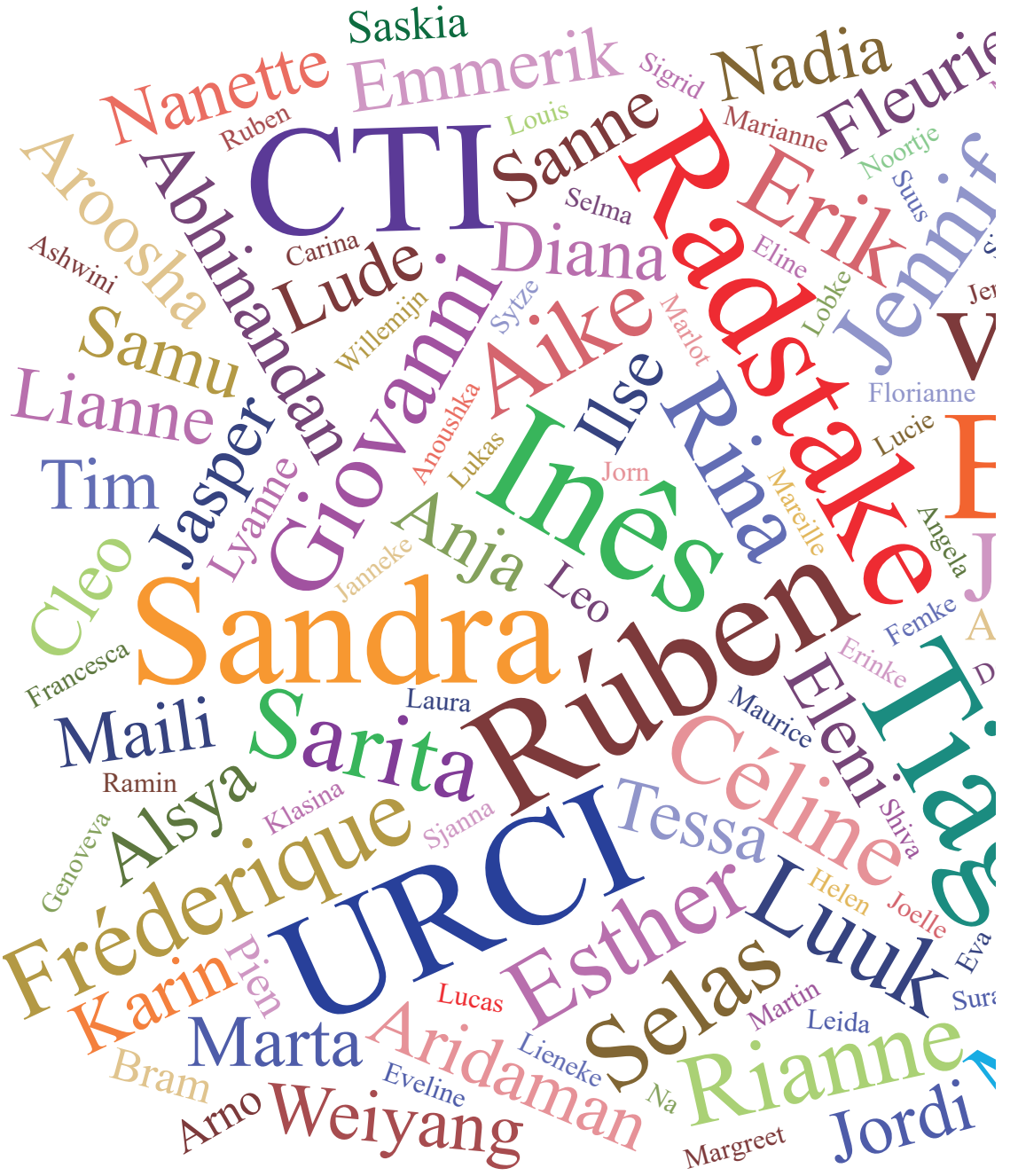
"The most worth-while thing is to try to put happiness into the lives of others."

B.P. Baden-Powell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Amber
Marzia
Pawel
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Lotte
Aniek
Sofie
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Nila
Marleen
Jorre
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Yvonne
Els
Bea
Marthe
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Maud
Kiki
Anne Marie

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Curriculum Vitae

Ana Lopes was born on 28 December 1989 in Guarda, Portugal. She grew up in Manteigas, a village in the heart of the Serra da Estrela, the highest mountain in Portugal mainland. In 2011 she obtained her bachelor's degree in Biomedical Laboratory Sciences at College of Health Technology of Coimbra – Polytechnic Institute of Coimbra. In 2012, she initiated an internship in Artur Paiva Lab at the Blood and Transplantation Center of Coimbra - Portuguese Institute of Blood and Transplantation. There she developed as a curious scientist, egger to learn and truly immunology fascinated, acquiring a wide experience in the field of allergology, immunology and rheumatology. In 2013 she initiated her master studies at Aveiro University and one year later she moved to the Netherlands to pursue her goal of developing as an independent researcher. She obtained her master's degree in Biochemistry - specialization in Clinical Biochemistry, in 2015, with the thesis "Expression of CXCR3 in different T cells subsets in rheumatoid arthritis", conducted under supervision of Dr. Artur Paiva and co-supervision of Dr. Maria do Rosário Domingues at Aveiro University. In 2016 she initiated her PhD studies in the Center for Translational Immunology and Department of Rheumatology & Clinical Immunology at the University Medical Center Utrecht under the supervision of Dr. Joel van Roon and Prof. Dr. Timothy Radstake. In 2017 she was awarded with a personal PhD fellowship by the Portuguese national funding agency for science, research and technology – Fundação para a Ciência e a Tecnologia. During her PhD studies they investigated different levels of epigenetic, transcriptomic and functional dysregulation in circulating type 2 conventional dendritic cells and monocytes from patients with Sjogren's Syndrome and non-Sjögren's sicca. In October 2022, Ana started as Postdoc in Sebastiaan van Heesch Lab at Princess Maxima Center to study the role of novel tumor-specific peptides for immunotherapeutic applications in childhood cancer.

List of publications

This thesis

1. **Lopes AP**, Bekker CPJ, Hillen MR, Wichers CGK, Hinrichs AC, Blokland SLM, Pandit A, Kruize AA, Rossato M, Radstake TRDJ, van Roon JAG. Long non-coding RNA HCP5 is a key regulator of cDC2s function: implications for Sjögren's Syndrome. *in preparation*

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3. **Lopes AP**, Bekker CPJ, Hillen MR, Blokland SLM, Hinrichs AC, Pandit A, Kruize AA, Radstake TRDJ, van Roon JAG. The Transcriptomic Profile of Monocytes from Patients With Sjögren's Syndrome Is Associated With Inflammatory Parameters and Is Mimicked by Circulating Mediators. *Front Immunol. 2021.* DOI: 10.3389/fimmu.2021.701656

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4. Affandi AJ, Carvalheiro T, Ottria A, de Haan JJ, Brans MAD, Brandt MM, Tieland RG, **Lopes AP**, Malvar Fernández B, Bekker CPJ, van der Linden M, Zimmermann M, Giovannone B, Wichers CGK, Garcia S, de Kok M, Stifano G, Xu YJ, Kowalska MA, Waasdorp M, Cheng C, Gibbs S, de Jager SCA, van Roon JAG, Radstake TRDJ, Marut W. CXCL4 drives fibrosis by promoting several key cellular and molecular processes. *Cell Rep. 2022*. DOI: 10.1016/j.celrep.2021.110189

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A

"Procurai deixar o mundo um pouco melhor de que o encontrastes"

"Try to leave the world a little better than you found it"

B.P. Baden-Powell