The role of CCR9-expressing T cell subsets in the immunopathology of primary Sjögren's syndrome

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The role of CCR9-expressing T cell subsets in the immunopathology of primary Sjögren's syndrome

De rol van diverse CCR9-positieve T-cel populaties
in de immunopathologie van het
primair syndroom van Sjögren

(met een samenvatting in het Nederlands)

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Thesis outline

Chapter 1

Introduction

Primary Sjogren's syndrome: clinical perspective

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disease characterized by lymphocytic infiltration of exocrine glands.(1) Over 95% of pSS patients present with dryness of eyes and mouth, and additionally patients often experience debilitating fatigue, arthralgia and myalgia.(2) Apart from these complaints 15-80% of pSS patients also develop extraglandular manifestations, e.g. pulmonary, renal or cutaneous disease involvement.(3–6) In order to capture disease activity in this heterogenous disease a disease activity index consisting of 12 domains has been formulated to capture systemic, and organ-specific features.(7) Each domain consists of several levels, each with an attributed weight (reflected by a specific amount of points). This European Alliance of Associations for Rheumatology (EULAR) Sjogren's syndrome disease activity index (ESSDAI) has been validated to measure disease activity in pSS patients (ESSDAI<5 low disease activity, 5≤ESSDAI≤13 moderate disease activity, and ESSDAI≥14 high disease activity), and capture clinical response (ESSDAI improvement of 3 or more points) in clinical trials.(8) Also certain lymphomas are considered extraglandular manifestations of pSS, which develop in 5-10% of the pSS patients.(9,10) Particularly B cell-derived mucosa-associated lymphoid tissue (MALT) lymphomas of the salivary glands are seen, with pSS patients having a 10-40 fold higher risk of developing a lymphoma compared to the general population.(11,12)

Although pSS can debut at any age, most patients are diagnosed between 30-60 years of age.(13) As for several other autoimmune diseases, there is a clear female predominance of pSS: the male to female ratio is approximately 1:9.(14) The prevalence of pSS ranges between 0.01% and 0.60%, making it the second most common rheumatic autoimmune disease after rheumatoid arthritis (RA).(15–19)

Due to a wide array of possible presenting clinical features (patient heterogeneity), recognizing pSS in an early stage may be difficult, with both patient's delay and doctor's delay.(20,21) Based on clinical expertise, and after excluding other explanations for the patient's complaints and symptoms, the diagnosis Sjögren's syndrome might be most fitting, after which confirmation of the clinical diagnosis through autoimmune serology and/or histology will take place. When no other autoimmune disease is present it will be considered as primary Sjögren's syndrome, and in case another autoimmune disease, such as RA or systemic sclerosis (SSc) is present, it is considered secondary Sjögren's syndrome (sSS).

However, to ensure clear characterization of patients willing to participate in research, classification criteria have been developed. The most recent ACR-EULAR criteria have been in use since 2016.(22) Patients can be classified as pSS patients if a total of 4 or more points are scored. In practice this means that either focal infiltration of a (minor) salivary gland (≥1 focus of >50 lymphocytes/4 mm^2 tissue), or autoantibodies (anti-SSA or anti-SSB) need to be present, as these items each contribute 3 points to the classification score, whereas objectified oral and ocular dryness contribute 1 point per test (unstimulated salivary flow ≤0.1 ml/minute, ocular staining score ≥5, and Schirmer ≤5mm/5 minutes).(22) The classification criteria also leave space for the definition of non-Sjögren sicca (nSS) patients. Patients who suffer from the classical triad of dryness, fatigue, and myalgia, without any other known cause, but who do not have inflammatory or autoimmune features (no focal infiltration and no autoantibodies in the circulation).

Despite many efforts to treat pSS, advances in pSS treatment remain unsatisfying. Treatment strategies effective in other immunological conditions often lack a clear effect in pSS. For example rituximab and etanercept, both effective in RA, are not effective in pSS in terms of the ESSDAI response criteria.(8,23) Hence, there is an unmet need to find an effective treatment, and to be able to predict on an individual basis who will respond to a specific treatment. Currently, the potential implementation of novel promising biologicals like anti-CD40 monoclonal antibody and CTLA4-Ig fusion protein are also challenged by high costs.(24,25) Many new biologicals are expected to cost an estimated €10.000 per year per patient.(26) In addition, safety remains to be demonstrated, and these medicines may not be user-friendly, e.g. in their administration.

Hence, increased understanding of the underlying immunopathological processes is crucial. This will allow better classification, prediction and optimized treatment. The studies in this thesis aim to add to increased understanding of immunopathology and biomarkers to monitor disease.

Immunopathology of pSS

Primary Sjogren's syndrome is a multifactorial disease

Like in most other autoimmune diseases the pathophysiology of pSS is complex and not fully understood. pSS is considered a multifactorial disease, in which environmental factors and epigenetic mechanisms are thought to cause inflammation in individuals with a genetic predisposition.(27,28) However, the exact processes leading to and sustaining pSS are unknown. Although it's hard to pinpoint the starting point of disease, many pSS disease models indicate activation of mucosal epithelial cells, which could be by e.g. exposure to viruses, or other environmental factors.(28–30) The involvement of multiple genetic components of both the innate and adaptive immune system is also suggested by Genome Wide Association Studies (GWAS) in Sjögren's syndrome. Few loci have been identified passing the genome-wide significant (GWS) threshold of $P = 5 \times 10^{-8}$, or are considered

strongly suggestive regions of association, depending on the studied population.(28) The strongest associations with pSS are seen for HLA, with polymorphisms of HLA-DQ and HLA-DR (HLA/MHC class II), which indicates involvement of CD4 T cells that recognize antigen presented to them by MHC class II molecules.(31,32) More recently a polymorphism of *MICA*, or MHC class I chain-related gene A, was confirmed to be associated with pSS.(33) MIC proteins are selectively expressed within epithelial and mucosal tissues and are recognized by CD8 T cells and NK cells that express NKG2D.(34,35) Additionally, several polymorphisms in non-HLA-related loci have been identified with an association to pSS.(32,36) These genetic loci are involved in the interferon (IFN) pathway and T cell activation (*IRF5*, *STAT4*, *IL12A*, *OAS1*, *KLRG1*, *SH2D2A*, and *NFAT5*); dysregulation of the NFκB pathway (*TNIP1*, and *TNFAIP3*); activation of B cells (*BLK*); activation of plasma cells (*PRDM1* or *BLIMP-1*); and formation of lymphocytic foci and (ectopic) follicle formation (*CXCR5*).
Together these results suggest that the immunopathological processes in pSS involve multiple cell types from the immune system. Since both immune cells (e.g. antigen presenting cells), and tissue cells can express MHC class I and II molecules, this indicates that immunopathology may result from the interplay of different types of immune cells and tissue cells, and involve both the innate and the adaptive immune system.

Lymphoid organization in primary Sjögren's syndrome: hallmark of the disease

The interplay of different cell types, innate, and adaptive immunity is seen in the histopathologic hallmark characteristic of pSS: lymphocytic infiltration and lymphoid organization of exocrine glands. In individuals who are genetically susceptible to developing pSS, a cascade seems to be triggered in which epithelial cells and innate cells such as dendritic cells and macrophages activate T and B cells from the adaptive immune system, and conversely infiltrating lymphocytes influence epithelial and innate cells by producing inflammatory mediators.(37,38) Epithelial cells in pSS were shown to express an array of molecules related to, e.g. T cell activation (MHC class I and II); T cell co-stimulation (e.g. via molecules such as CD80 and CD86); B cell survival (B cell activating factor (BAFF)); and cytokines and chemokines related to the expansion and organization of infiltrates (among others CXCL10, CXCL13).(39–47)

Biopsies of salivary gland tissue of pSS patients show that the formed lymphocytic aggregates mainly consist of adaptive immune cells: CD4 T cells, B cells and plasma cells.(14,48) However, also elevated numbers of cell types such as CD8 T cells, NK cells and NK T cells have been demonstrated in pSS salivary glands.(49–52) Innate(-like) cells, as dendritic cells, macrophages, mucosal-associated invariant T (MAIT) cells and innate lymphoid cells (ILCs) appear to be present in smaller proportions.(48,53–55) In approximately 25-30% of pSS patients the lymphocytic infiltrates in the exocrine glands develop into ectopic lymphoid structures (ELS).(56) In ELS T and B cells are segregated in discrete areas, high endothelial venules (HEVs) are developed, and networks of stromal follicular dendritic (FDC) cells differentiate.(56–58) These FDC networks are populated by germinal center (GC) B cells, and support ectopic GC response and perifollicular accumulation of GC-derived plasma cells.(59,60) Both the presence of GC-like structures and a high number of lymphocytic infiltrates (≥3 foci/4 mm2) in the salivary glands are associated with lymphoma development.(1,61)

Drivers of B cell hyperactivity in primary Sjögren's syndrome

B cell hyperactivity is another hallmark feature of pSS. In pSS this is reflected by the production of autoantibodies (ANA, rheumatoid factor (RF), anti-Ro60/SSA, anti-Ro52/SSA, and anti-La/SSB), increased circulating concentrations of these autoantibodies, and total serum IgG.(1) In addition, elevated numbers of IgM/IgG+ plasma cells in the salivary glands, as well as increased RNA expression of IgM, IgG1, IgG2, and IgG3, are associated with increased lymphocytic focus scores, T follicular helper cells, and systemic measures of B cell hyperactivity such as elevated serum IgG levels.(62) B cell hyperactivity is strongly associated with an increased risk for developing lymphoma.(63)

Understanding the drivers behind lymphoid activation, B cell hyperactivation, and lymphocytic aggregate organization into ELS is crucial as they appear to be pivotal components of pSS immunopathology which might lead to targets for treatment. Although it is not clear what drives B cell activation, a number of T cell subsets found in the salivary gland infiltrates can contribute to B cell hyperactivity.

Tfh and Tfh-like cells orchestrating lymphoid activity and B cell hyperactivity

At present it is not clear what drives T cell activation at inflammatory sites in pSS patients. Tissue cells such as epithelial cells, or innate cells such as monocytes, macrophages, and dendritic cells might initiate inflammation upon innate triggering, e.g. through toll-like receptor 7 and 9 triggering upon encountering environmental agents such as viruses. This could initiate T cell activation. On the other hand the same environmental triggers might directly induce activation of resident effector/memory T cells and offset inflammation.(38,64,65) Also specific ligands activating a particular subset of T cells with innate recognition, i.e. MAIT cells (discussed below), could initiate inflammation.

Different types of activated CD4 T cells in the salivary glands can activate and maintain ELS function, but also potently stimulate B cell activity in patients who do not develop ELS, but have many lymphocytes organized in T and B cell aggregates.(56,66)

T follicular helper (Tfh) cells are C-X-C chemokine receptor 5-expressing (CXCR5+) CD4 T cells that were shown to be key drivers of B cell hyperactivity in many inflammatory conditions, including pSS.(67–70) Tfh cells are canonically defined as memory CD4 T cells, with expression of transcription factor Bcl6, surface expression of CXCR5, ICOS, and PD-1, and secretion of CXCL13, and IL-21.(71–73)

The phenotype clearly indicates the function of this cell subset. Bcl6 is the master transcription factor regulating Tfh differentiation, i.e. cells fail to differentiate to Tfh cells in the absence of Bcl6.(74–76) Additionally Bcl6 regulates B cell differentiation and formation of GCs.(77–79) Interestingly, IL-21 can induce the expression of PRDM1 to facilitate plasma cell commitment, the expression of Bcl6 which drives GC formation, and the expression of AICDA inducing B cell class switching.(80) CXCR5 is a chemokine receptor that is expressed by T cells, B cells and DCs. CXCR5 interacts with CXCL13, which is secreted in B cell follicles, by stromal cells, Tfh cells, and FDCs.(81) In salivary glands of pSS patients CXCR5, CXCR5-expressing CD4 T cells, and the chemokine CXCL13 are overexpressed.(58,60,62,68,82–86) Increased expression of CXCL13 is associated with increased lymphoid aggregates (foci) in salivary gland tissue and increased organization into ectopic lymphoid structures, and is subsequently associated with increased B cell hyperactivity and lymphoma development.(48,58,60,87) Tfh (T cell)-expressed ICOS, or CD278, is a protein which binds to B cells through its ligand ICOSL (expressed on activated B cells), after which high levels of IL-21 are released and further differentiation of both T cells and B cells is induced.(81,88,89) PD-1, or CD279, is expressed on activated T cells, B cells and myeloid cells and binds to its ligands PD-L1 and PD-L2.(90,91) PD-L1/2 expression is increased on GC B cells, and whereas lack of PD-L1/2 or PD-1 leads to more GC cell death, less Tfh cell produced cytokines, and fewer long-lived plasma cells, the remaining plasma cells have a greater antigen affinity, suggesting that for class switching and affinity maturation of B cells in GCs PD-L1-expressing B cells need interaction with PD-1-expressing T cells.(92) Overall, the balance between ICOS/ICOSL and PD-1/PD-L1/2 on B and T cells interacting in the light zone of germinal centers is crucial for immune tolerance and immune response.(93,94) Elevated frequencies of circulating PD-1/ICOS/CXCR5-expressing Tfh cells have been reported in pSS patients and were associated with increased B cell activity and autoimmunity in these patients.(67,95–99) Also, with epigenetic quantification, PD-1/ICOS/CXCR5-expressing cells were found to correlate to the lymphocytic focus score in pSS patients, CXCL13 expression and B cell hyperactivity.(62)

After the first descriptions of Tfh cells in the early years 2000 several Tfh-like cell subsets were described that lack CXCR5-expression, but might have similar lymphocytic organizing, and B cell activating potential in pSS: peripheral T helper cells (Tph), and C-C motif chemokine receptor 9-expressing (CCR9+) Tfh-like cells.

T peripheral helper (Tph) cells are described as a Tfh-like cell subset defined as CXCR5-PD-1^{hi} memory CD4 T cells.(100) Unlike Tfh cells Tph cells do not express Bcl6, but similarly to Tfh cells they can co-express ICOS, and secrete high levels of CXCL13 and IL-21.(100–102) The first description of Tph cells was in RA patients, but also in other autoimmune diseases, such as systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (JIA), elevated numbers of Tph cells were found locally and/or systemically.(100,102,103) In pSS patients the number of circulating Tph cells was found to be increased compared to controls, and a correlation was seen between CXCR5-PD-1^{hi} (not memory defined) cells and plasmablasts, and rheumatoid factor positivity.(96,104,105)

CCR9+ Tfh-like cells are potent B cell activating cells, which produce high amounts of e.g. IL-21.(106,107) CCR9-expressing T cells were first studied in the gastrointestinal tract. After DC priming under influence of all-trans retinoic acid cells often co-express CCR9 and integrin α4β7, and migrate to sites that express cytokine CCL25, such as the gut mucosa.(108,109) Although CCR9+ cells are important for maintaining mucosal immune homeostasis, they also are linked to mucosal inflammation, e.g. in inflammatory bowel disease.(110,111) For example in patients with Crohn's disease elevated numbers of CCR9+ T cells were found both in the circulation and in the inflamed tissue.(112,113) Also ligand CCL25 was upregulated in the inflamed small bowel, whereas normal (control) tissue did not express CCL25.(112) Also in RA synovium elevated levels of CCR9+ cells and CCL25 were found, and inhibition of CCR9 improved disease features in a collagen induced arthritis model.(114,115) The first observation that CCR9+ CD4 T cells might play a role in pSS was found when non-obese diabetic (NOD) mice developed Sjögren-like symptoms, and IL-21-expressing CCR9+CXCR5- CD4 T cells infiltrated mucosa-associated tissues of accessory organs of the digestive tract, such as the salivary glands and pancreas, and mediated immunopathology in these organs.(106) pSS patients were shown to have a higher frequency of CCR9+ Tfh-like cells in their circulation, and these cells frequently demonstrate an effector phenotype, e.g. with elevated PD-1/ICOS expression, high CD127 (IL-7Rα) expression, and IFN-γ production.(107) In addition, in salivary glands of pSS patients numbers of CCR9+ CD4 T cells are elevated, and CCR9-attracting cytokine CCL25 is also overexpressed, whereas CCL25 is not detectable in healthy human salivary gland tissue.(107,116) CCL25 levels in secretomes of salivary gland tissue were associated with SSA positivity, B cell hyperactivity (serum IgG levels), and levels of IL-21 and soluble IL-7R.(86)

Apart from Tfh cells, Tph cells, and CCR9+ Tfh-like cells, other cell subsets, in particular ones that are mucosa-associated, are also indicated to contribute to immunopathology in pSS. One specific T cell subset with innate-like properties, called mucosal-associated invariant T cells was further studied in this thesis.

Mucosal-associated invariant T cells and CD8 T cells in pSS immunopathology

Mucosal-associated invariant T (MAIT) cells are innate-like T cells that become activated by binding to glycolipid molecules that are presented by the MHC class I-like molecule MR1.(117,118) Such molecules involve those derived from vitamin B2 (riboflavin) that can be cleaved by bacteria to induce antigens that elicit activation of MAIT cells.(117,118) MAIT cells are abundant in e.g. peripheral blood, liver, gastro-intestinal tract, and mesenteric lymph nodes.(119) MAIT cells are defined by expression of CD3 and TCRVα7.2 in combination with CD161 and/or IL-18Rα.(120–122) Furthermore high levels of CCR6, CXCR6, CD127 (IL-7Rα), ABCB1 and NKG2D were found.(120) Data on CCR9 expression by MAIT cells are inconsistent, and site-specific.(120,123) High CCR9 expression is particularly found in colonic MAIT cells, even though there was no difference in CCR9 expression between colonic CD8 MAIT cells and non-MAIT CD8 T cells in the gut.(123) Most MAIT cells in humans are CD8+ (approximately 70-80%), followed by CD4-CD8- MAIT cells (±15%).(120,124–126). In mucosal tissues an enrichment of CD4-CD8- cells of up to 50% of MAIT cells may be seen, however these cells seem functionally similar to CD8+ MAIT cells.(120,127,128) MAIT cells play an important role in mucosal immunity, rapidly producing cytokines, such as IFN-γ, TNF-α, IL-17, perforin, and granzyme B, upon TCR stimulation or cytokine stimulation. (120,122,129–132) In the presence of IL-18, IL-15, and type I interferons (IFNs) MAIT cells can recognize antigens from viruses upon viral infections, and subsequently boost the adaptive immune response.(118,133) In oral mucosa MAIT cells produce more IL-17 than circulating MAIT cells.(128) Enrichment of MAIT cells was found in synovial fluid of patients with psoriatic arthritis (PsA), and RA.(134) In pSS patients elevated MAIT cell numbers were found in the salivary glands, and local MAIT cells produced more IL-17 after stimulation with IL-7 or IL-23.(53,135) In patients with RA, SLE or pSS decreased circulating MAIT cell numbers were found as compared to controls.(53,135,136) The potential role of MAIT cells as instigators of autoimmune disease is ongoing, but e.g. in a lupus-like model MAIT cells activated B cells to produce autoantibodies through CD40-CD40L interaction.(119)

The example of CD8 MAIT cells that are able to activate B cells in lupus-like disease, could indicate similar potential in pSS. This fits to the idea that CD8 T cells are not merely "cytotoxic killer cells", and a more sophisticated view on CD8 T cells and their potential to orchestrate inflammation might be necessary. For example CXCR5+ CD8 T cells were found to display Tfh features, and CD103+CD69+ CD8 T resident memory (Trm) cells were found in elevated numbers in previously inflamed joints in RA, and through production of CCL5 have been shown to regulate inflammation in experimental models of arthritis. (137,138) In Sjögren-like disease knockdown of CD8α showed diffuse infiltration without acinar atrophy, duct damage, or leukocyte infiltration, and similar results were found after therapeutic depletion of CD8 from salivary glands, resulting e.g. in improvement of saliva production.(138,139) Also, circulating activated CD8 T cells correlate with a multi-omics-based pSS disease signature, and HLA-DR+ CD8 T cells correlate to disease activity (scored by ESSDAI).(140,141). Together this suggests that CD8 T cells and specifically CD8 MAIT cells might play a significant role in immunopathology of pSS.

IL-7/IL-7R axis: key driver in pSS immunopathology

In pSS patients and in Sjögren-like disease in mice IL-7 has been found in increased levels in the salivary glands, saliva, and serum, and correlates to salivary output, lymphocytic infiltration, and autoantibody positivity.(142–145) Also IL-7R-expressing cells were found in elevated numbers in salivary gland tissue of pSS patients compared to nSS controls, and correlated to the number of T cells, B cells, and IgM/IgG+ plasma cells.(143) IL-7 is expressed by stromal cells in primary lymphoid organs, and by fibroblasts, endothelial cells, macrophages, and dendritic cells at the site of inflammation.(145,146) IL-7 induces skewing of T cells towards pro-inflammatory Th1 or Th17 phenotype, and induces Th1-associated chemokines, such as CXCL10, which is abundantly present in pSS salivary glands and serum.(46,142,147) Th1 activity has been shown to predominate locally in pSS patients.(142,144,148) Furthermore, IL-7 plays a critical role in the formation of GCs and ELS by induction of e.g. lymphotoxin (LT)α, LTβ, TNF-α, and CCL21, and promoting survival of lymphoid tissue inducer (LTi) cells.(145,149,150)

An interesting resemblance between many of the above-mentioned cell types is the expression of IL-7Rα (CD127), and the strong response of these effector cells to the cytokine IL-7. Important with respect to regulation of B cell hyperactivity in pSS, deficiency in IL-7Rα affects T cell lymphopoiesis, and is associated with reduced T cell stimulated B cell activity in humans.(151) In addition, blockade of IL-7R was associated with a reduction of leukocyte infiltration in the salivary glands, and increase of saliva production in experimental Sjögren models.(152) Apart from clear IL-7-driven T cell-dependent immune responses recently it has been suggested that IL-7 can also contribute to inflammation by directly activating innate cells such as macrophages that have increased IL-7R expression.(153) Although previously it has been reported that a small proportion of monocytes/macrophages (7%) in salivary glands from pSS patients express IL-7R, it is unclear to what extent IL-7R-expressing innate cells or innate-like cells, such as ILCs and MAIT cells, contribute to pSS immunopathology.(143) In this thesis some new aspects of IL-7-driven activation of CD4 T cells and MAIT cells were studied.

Figure 1 provides a schematic overview of the in this chapter described immunopathological processes in pSS salivary gland tissue, of which the reflection in peripheral blood will be studied in this thesis.

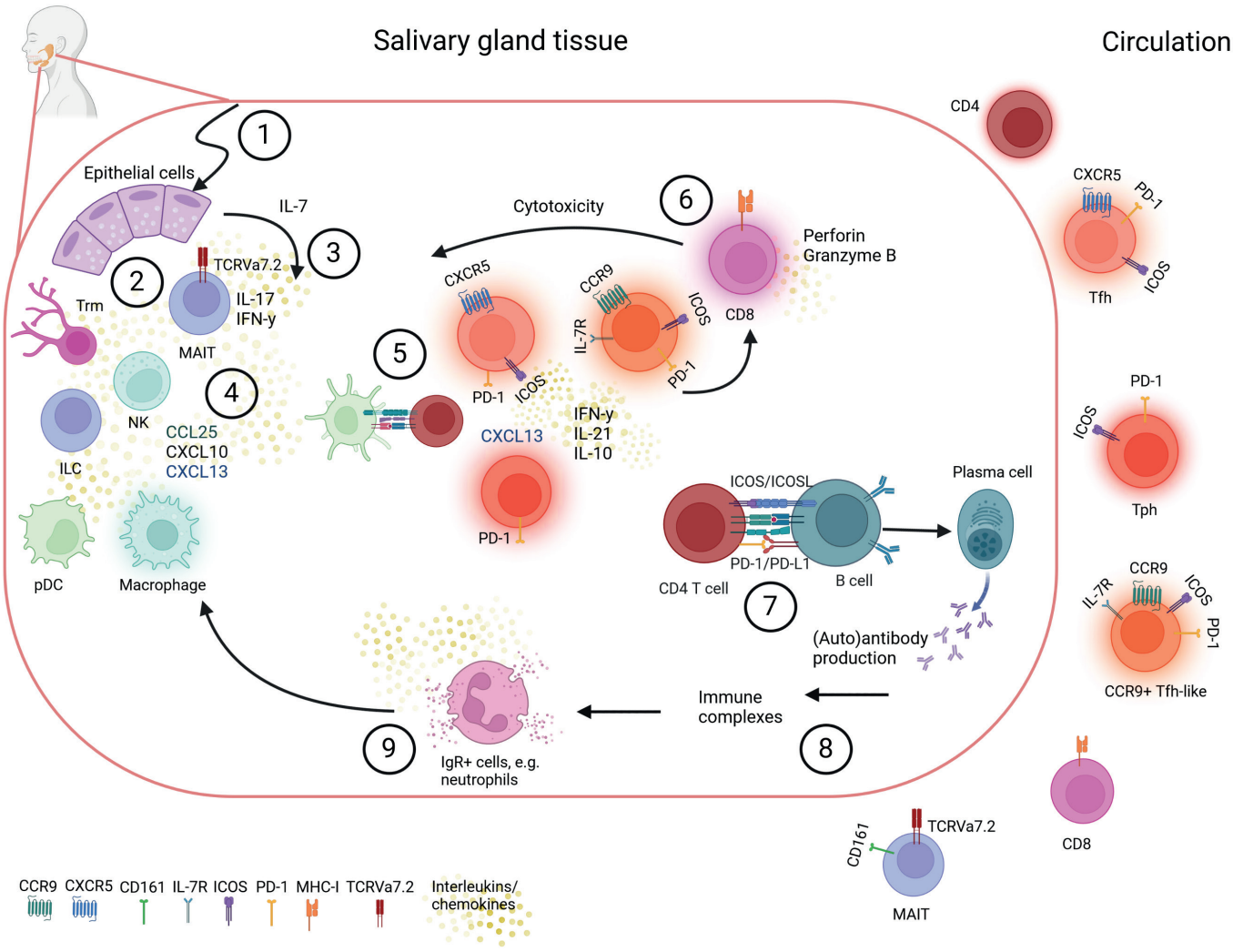

Figure 1 | Immunopathological processes in pSS salivary gland tissue and their circulating counterparts. *In brief* (1) Exposure to a trigger (infectious, environmental) may disrupt epithelial tissue homeostasis in mucosa-associated tissue of pSS patients. Tissue cells are activated and/or damaged. (2) Alternatively, this trigger can directly activate innate(-like) cells and tissue resident immune cells. Furthermore, activated tissue cells stimulate these cells. (3) Production of cytokines like IL-7 by activated tissue and innate(-like) cells, stimulating other immune cells, and creating a proinflammatory environment. (4) Production of chemokines by activated tissue and innate(-like) cells, causing chemotaxis of specific cells towards the tissue. (5) Activated antigen presenting cells (APCs) interact with T cells primed in the proinflammatory environment, further activating T cell response. (6) The activation of T cells by APCs allows cross presentation of antigens to CD8 T cells, which in turn mediate cytotoxicity. (7) Activated T cells interact and stimulate B cells. B cell differentiation leads to plasma cell development and production of (auto)antibodies. (8) The increased production of (auto)antibodies leads to formation of immune complexes (e.g. by binding to autoantigens) that induce complement activation and reduced C3 and C4 levels due to complement consumption. (9) Cells expressing immunoglobulin-receptors (IgR), such as neutrophils and macrophages, become activated by immune complexes and release cytokines and chemokines, causing local damage, driving chemotaxis, and creating further local inflammation. *Figure created with BioRender.com*

Search for new minimal invasive techniques to study biomarkers of immunopathology and to monitor disease

In recent years many new techniques have been developed to study immunopathology. Yet, research on pSS, and other systemic autoimmune diseases, also still aims to develop new techniques to study and monitor disease with as minimal invasive procedures as possible. Currently, the use of invasive techniques such as venipunctures, and salivary gland biopsies (from minor/labial salivary glands, or from the parotid gland) are regular practice in pSS diagnostics and research. With a single venous blood donation many facets of pathophysiology can be studied, e.g. with new techniques for transcriptomics, genomics, and proteomics that strife to optimize the number of determinable parameters in small sample volumes. Also surplus material acquired through biopsy performed (only) for diagnostic reasons, may be used for research purposes, e.g. to study molecular and cellular derangements by using several technological platforms such as bulk and single cell RNA sequencing, epigenetic cell counting, and proteomics of salivary gland secretomes (culture supernatants) in pSS.(86) Assessments of biomarkers in tear fluid and saliva obtained through routine diagnostic tests as Schirmer's test and (un)stimulated whole saliva collection are attractive alternatives as they are considered less invasive techniques. However, a clear drawback of these tools is that for pSS patients with more severe sicca symptoms, the amount of produced tear fluid and saliva will be limited if not unavailable, making it harder to use these fluids for research and diagnostic purposes.

Non-invasive research techniques to study immunopathology are also attractive and therefore increasingly studied welcome alternatives in diagnosing, stratifying and monitoring disease (activity). New promising techniques that are currently studied include imaging technology, such as salivary gland ultrasonography, MRI, and PET-CT, although these methods are limited in their resolution of molecular responses.(154,155) Also, gut and oral microbiome analysis is increasingly studied in pSS, until now not having resulted in clinical meaningful tools yet.(156)

One type of exocrine gland that could be easily sampled and non-invasively studied in pSS is the sebaceous gland (which produces skin-moisturizing sebum). In pSS sebaceous glands and sebum have barely been studied, but e.g. in Parkinson's disease sebum metabolomics recently proved a useful tool in identifying potential biomarkers for disease.(157) Hence, sebum metabolomics might be an interesting tool to study immunopathology in pSS.

Outline of this thesis

In this thesis ***the role of CCR9-expressing T cell subsets in the immunopathology of primary Sjogren's syndrome*** is studied. The role of multiple CCR9-expressing T cells, including CD4 and CD8 T cells, and innate-like MAIT cells are studied in pSS using several novel techniques to better understand their molecular, phenotypic, and functional features, and their potential role in immunopathology of pSS. Furthermore, a new minimal invasive method using sebum collected from the skin with small tapes, is evaluated for its potential to monitor immunopathology and to discover novel biomarkers in pSS. For this purpose sebum is analyzed using high-throughput metabolomics technology.

In **chapter 2** transcriptomics is used to compare CD4 CCR9+ Tfh-like cells to CXCR5+ T cells, and CXCR5-CCR9- T cells from pSS patients and controls. Computational analysis following RNA sequencing is used to identify coherent gene modules, and enriched pathways within these modules. Subsequently, differentially expressed genes of interest are replicated in additional cohorts, using phenotypic and functional analyses.

The results from the studies in chapter 2 prompted the study of a new hypothesis for the potential role of CCR9+ CD8 T cells in pSS, another CCR9-expressing immune cell (**chapter 3**). For this purpose, firstly, epigenetic cell counting evaluates the presence of CD8 T cells in labial salivary gland tissue and their association with inflammation, and in particular Tfh cells, to appreciate any potential relationship with lymphocyte organization and ectopic follicular responses. Then, peripheral blood mononuclear cells are analyzed using flow cytometry to study CCR9 expression on CD8 T cells in pSS patients and controls. **Chapter 3** concludes with the study of functional properties of (CCR9+) CD8 T cells by analyzing the release of the chemokine CCL5 by CCR9+ CD8 T cells and CCR9- CD8 T cells in pSS patients and controls.

CCR9+ Tfh-like cells and T peripheral helper (Tph) cells have both been described as CD4 T cell subsets with features of T follicular helper (Tfh) cells, but both cell types lack CXCR5 expression. In **chapter 4** it is studied whether these cells are unique or whether there is overlap between CCR9+ Tfh-like cells and Tph cells. Additionally, the expression of markers PD-1 and ICOS, associated with B cell activation, is compared between effector (memory) CXCR5/CCR9-defined cell subsets, including CXCR5/CCR9 co-expressing CD4 T cells.

IL-7 previously has been described as a key cytokine that can drive inflammation and lymphoid organization in pSS. In this thesis it is discussed which cells IL-7 may target to induce immunopathology in autoimmune diseases such as pSS (**chapter 5**). In this respect innate-like cells are interesting candidates. Since innate-like mucosal-associated invariant T (MAIT) cells may play a pivotal role in the initiation of specific immune responses in

inflammatory conditions such as pSS, which are characterized by inflammation in mucosa-associated tissues, these cells are investigated in **chapter 6**. In **chapter 6** the aberrances in CCR9 expression of IL-7R-expressing innate-like MAIT cells are studied in pSS patients compared to healthy controls. In vitro the capacity of IL-7 to activate CCR9-expressing MAIT cells, and to induce IFN-γ and IL-21 production, is studied. Currently, the disease modifying anti-rheumatic drugs (DMARDs) leflunomide and hydroxychloroquine are tested in pSS patients for their therapeutic value. Hence, the capacity of these DMARDs to inhibit activation of MAIT cells is tested.

Understanding and being able to use information on molecular changes that underly immunopathology and disease activity is crucial. In this respect minimal invasive strategies would be welcomed. For this purpose saliva and tear fluid have been evaluated previously. So far, however, no consistent data or biomarkers could be implemented in clinical practice. One challenge that is faced when analyzing these samples is that tear fluid and saliva are more difficult to obtain or cannot be obtained in the patients with more outspoken dryness. In this thesis the potential use of sebum was studied. Sebum, produced by exocrine sebaceous glands, moisturizes the skin, and may provide another biological secretion for -omics analyses. In **chapter 7** we present a technique to collect sebum from the skin using sebutapes, and we performed exploratory work on the use of sebum metabolomics by untargeted mass spectrometry to determine disease biomarkers in a minimal invasive way.

Finally, the main findings of this thesis are summarized and discussed in **chapter 8**.

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Chapter 2

Transcriptome analysis of CCR9+ T helper cells from primary Sjögren's syndrome patients identifies CCL5 as a novel effector molecule

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Abstract

Introduction. CCR9+ Tfh-like pathogenic T helper (Th) cells are elevated in patients with primary Sjögren's syndrome (pSS) and indicated to play a role in pSS immunopathology. Here we delineate the CCR9+ Th cell-specific transcriptome to study the molecular dysregulation of these cells in pSS patients.

Methods. CCR9+, CXCR5+ and CCR9-CXCR5- Th cells from blood of 7 healthy controls (HC) and 7 pSS patients were FACS sorted and RNA sequencing was performed. Computational analysis was used to identify differentially expressed genes (DEGs), coherent gene expression networks and differentially regulated pathways. Target genes were replicated in additional cohorts.

Results. 5131 genes were differentially expressed between CCR9+ and CXCR5+ Th cells; 6493 and 4783 between CCR9+ and CCR9-CXCR5- and between CXCR5+ and CCR9-CXCR5-, respectively. In the CCR9+ Th cell subset 2777 DEGs were identified between HC and pSS patients, 1416 and 1077 in the CXCR5+ and CCR9-CXCR5- subsets, respectively. One gene network was selected based on eigengene expression differences between the Th cell subsets and pathways enriched for genes involved in migration and adhesion, cytokine and chemokine production. Selected DEGs of interest (HOPX, SOX4, ITGAE, ITGA1, NCR3, ABCB1, C3AR1, NT5E, CCR5 and CCL5) from this module were validated and found upregulated in blood CCR9+ Th cells, but were similarly expressed in HC and pSS patients. Increased frequencies of CCR9+ Th cells were shown to express higher levels of CCL5 than CXCR5+ and CCR9-CXCR5- Th cells, with the highest expression confined to effector CCR9+ Th cells. Antigenic triggering and stimulation with IL-7 of the Th cell subsets co-cultured with monocytes strongly induced CCL5 secretion in CCR9+ Th cell co-cultures. Additionally, effector CCR9+ Th cells rapidly released CCL5 and secreted the highest CCL5 levels upon stimulation.

Conclusion. Transcriptomic analysis of circulating CCR9+ Th cells reveals CCR9-specific pathways involved in effector T cell function equally expressed in pSS patients and HC. Given the increased numbers of CCR9+ Th cells in the blood and inflamed glands of pSS patients and presence of inflammatory stimuli to activate these cells this suggests that CCR9-specific functions, such as cell recruitment upon CCL5 secretion, could significantly contribute to immunopathology in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disorder characterized by lymphocytic infiltration of exocrine glands in association with dryness of eyes and mouth. The lymphocytic infiltrates mainly consist of CD4 T cells, CD8 T cells and B cells and activation of these cells has been linked to immunopathology in pSS.(1–4) A hallmark feature of pSS is B cell hyperactivity, reflected by autoantibody production, elevated serum IgG levels and increased risk of lymphoma development (in 5-10% of patients).(1–3) Both the presence of germinal center-like structures (GCs) and a high number of lymphocytic infiltrates in the salivary glands are associated with lymphoma development.(5)

T follicular helper (Tfh) cells are characterized by the expression of C-X-C Motif chemokine receptor 5 (CXCR5) and are potent B cell stimulating cells that can reside in GCs in lymph nodes.(6) Tfh cells are elevated in salivary glands and peripheral blood of pSS patients, correlating with autoantibody levels, disease severity and aberrant memory B cell and plasma cell subsets.(7–9) Recently, a novel "Tfh-like" cell subset expressing C-C chemokine receptor 9 (CCR9) instead of CXCR5 was described with similar characteristics as Tfh cells including interleukin (IL)-21 production and ICOS and Bcl6 expression.(10) CCR9+ Th cells are present in secondary lymphoid organs of both mice and humans.(11,12) In humans, these CCR9+ Th cells produce high levels of IFN-γ, IL-17, IL-10 and IL-4 and strongly induce B cell responses.(11,13,14) CCR9+Th cells specifically migrate to mucosal sites in response to their ligand, the C-C Motif chemokine ligand 25 (CCL25) and are important for mucosal immune homeostasis.(15,16) However, these cells are also indicated to have a function in mucosal inflammation, contributing to inflammatory bowel disease (IBD).(17,18) Increased numbers of CCR9-expressing cells have been found in the peripheral blood and inflamed intestinal tissue of Crohn's disease patients as well as elevated CCL25 production at the inflammatory site.(19,20) Inhibition of CCR9+ Th cells decreases intestinal inflammation in an ileitis mouse model. In Crohn's disease patients, inconsistent results were demonstrated, possibly due to poor pharmacokinetic properties of the small molecule inhibitors used for therapy.(21–23)

CCR9+ T cells were also shown to mediate immunopathology in mucosal tissues in accessory organs of the digestive tract in non-obese diabetic (NOD) mice, including the pancreas and salivary glands. The NOD mice spontaneously developed sialadenitis and had infiltration of IL-21-expressing CCR9+ Th cells in the salivary glands.(10) In mice, CCL25 gene expression is upregulated in the oral mucosa upon antigenic triggering and during wound healing.(24,25) In addition to this pivotal role for CCR9+ Th cells in experimental Sjögren-like disease, CCR9+ Th cells are enriched in the circulation of pSS patients and both CCR9+ cells and their ligand CCL25 are elevated in their salivary glands(10,26,27). CCL25 mRNA is not detectable

in healthy human salivary gland tissue, but is upregulated during oral inflammation.(28) Interestingly, the CCR9+ Th cell subset shares functional characteristics with the newly described pathogenic T 'peripheral helper' (Tph) cells (PD-1^{hi}CXCR5-) which drive B cell activation in rheumatoid arthritis patients.(29) In pSS patients these PD-1^{hi}CXCR5- Tph cells were identified in elevated numbers both in salivary glands with germinal centers and in peripheral blood, frequently co-expressing IL-21 and IFN-γ.(30) CXCR5- Tph cells were found to have low *CCR9* RNA expression.(29) Hence, circulating CCR9+ Th cells from pSS patients that express ICOS and PD-1, as was recently described(26), seem to represent a separate population. In addition, CCR9+ Th cells are IL-7R^{hi} and robustly respond to IL-7 in vitro.(26) Since the IL-7/IL-7R axis plays an important role in pSS immunopathology and in GC formation this supports a role for CCR9+ Th cells in pSS.(31–38)

To investigate which processes may drive CCR9+ Th cell pathogenicity, transcriptomic profiling of circulating CCR9+ Th cells was performed in healthy controls as compared to pSS patients. Here we demonstrate the transcriptome of CCR9-expressing pathogenic T helper cells from primary Sjögren's syndrome patients and healthy controls and identify CCL5 as a novel effector molecule of CCR9+ Th cells.

Methods

Patients and healthy controls

For cell sorting and subsequent RNA sequencing, 7 healthy controls (HC) and 7 pSS patients were included. For replication experiments separate cohorts were used. qPCR validation was performed in n=18 HC and n=9 pSS patients and for flow cytometry n=23 HC and n=22 pSS patients were included, respectively. For validation of CCL5 n=24 HC and n=16 pSS patients were included. All pSS patients were diagnosed by a rheumatologist and met the 2002 American-European Consensus Group (AECG) criteria and the ACR-EULAR criteria.(39,40) All healthy volunteers and pSS patients were included in the University Medical Center (UMC) Utrecht. The UMC Utrecht Medical Research Ethics Committee (METC) approved the study (reference 13/697) and all participants gave written informed consent. Demographic and clinical data are shown in Table 1.

	RNA sequencing		qPCR		Flow cytometry		CCL5 validation	
	HC (n=7)	pSS (n=7)	HC (n=18)	pSS (n=9)	HC (n=23)	pSS (n=22)	HC (n=7+13 FACS, n=4 culture)	pSS (n=13 FACS, n=3 culture)
Female gender, n (%)	7 (100)	7 (100)	16 (89)	9 (100)	18 (78)	20 (91)	24 (100)	15 (94)
Age, years	44±14	48±12	52±9	56±8	48±11	58±13	51 ± 11	57 ± 10
Anti-Ro/SSA positive, n (%)	-	4 (57)	-	8 (89)	-	17 (77)	-	11 (69)
Anti-La/SSB positive, n (%)	-	3 (43)	-	6 (67)	-	10 (45)	-	7 (44)
ANA positive, n (%)	-	5 (71)	-	8 (89)	-	17 (77)	-	10 (63)
Lymphocytic focus score (foci/4mm²)	-	3.2±2.5	-	1.2±1.1	-	2.7±1.7	-	2.5±2
IgA positive plasma cells (%)	-	43±29	-	59±26	-	47±25	-	57±24
Schirmer (mm/5min)	-	11±11	-	6±8	-	4±4	-	1±2
Serum IgG (g/L)	-	11.8±2.9	-	18.1±4.5	-	16.5±8.2	-	13.6±4.1
ESSDAI score (0-123)	-	7.4±5.7	-	5.0±3.3	-	6.1±4.9	-	4.6±3.2
ESSPRI score (0-10)	-	6.4±1.8	-	6.5±1.1	-	6.0±1.8	-	7.0±1.3
Immunosuppressants, n	-	5	-	1	-	5	-	5
Hydroxychloroquine, n	-	4	-	1	-	2	-	4
Other, n	-	1	-	0	-	3	-	4

Table 1 | Patients' characteristics. Mean ± SD are shown unless otherwise specified. pSS: primary Sjögren's syndrome; HC: healthy controls; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index.

Fluorescence-activated cell sorting (FACS)

Fresh peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from Li-heparin blood. PBMCs were stained with fluorochrome-conjugated antibodies against CD3, CD4, CXCR5 and CCR9 (Supplementary Table 1). CCR9+CXCR5- (CCR9+), CXCR5+CCR9- (CXCR5+) and CCR9-CXCR5- (double negative, DN) Th cells were sorted with fluorescence-activated cell sorting (FACS) using BD FACSARIAIII and harvested into tubes with RPMI 1640 containing 10% FCS and 1% penicillin/streptomycin (gating strategy, Figure 1A). Cells were lysed in RLTplus buffer (Qiagen) with 1% beta-mercaptoethanol.

RNA sequencing and computational analyses

RNA was extracted from the sorted Th cell subsets from HC and pSS patients using the AllPrep Universal Kit in QIACube (both Qiagen). Two CCR9+ Th cell samples from healthy controls had to be excluded from analyses since they failed the quality control.

RNA sequencing libraries were generated with the TruSeq RNA Library Prep Kit (Illumina) and sequenced on an Illumina HiSeq4000 generating approximately 20 million 150bp paired-ended reads. The sample qualities were assessed by FastQC. The sequencing reads were aligned to human genome (GRCh38 build 79) using STAR aligner.(41) For all the samples >90% of the reads were uniquely mapped to the human genome.

HTSeq-count was used to generate read counts. To obtain normalized read counts (NRC), which are log2 normalized, we performed the variance-stabilizing transformation (VST) on the raw read counts using R/Bioconductor package DESeq2.(42) To identify differentially expressed genes between Th subsets, paired analyses were performed using likelihood ratio test (LRT). Differential expression between HC and pSS was based on Wald test. Venny (http://bioinfogp.cnb.csic.es/tools/venny/) was used to generate Venn diagrams. Genes were considered differentially expressed with a nominal p-value <0.05. Differentially expressed genes (DEGs) from all comparisons were selected for further analyses. No filtering of genes was performed based on read counts, as all samples had similar gene count distributions. Multidimensional scaling (MDS) plot was generated using R function 'cmdscale'. Hierarchical clustering based on Euclidean distances was performed in R using the top 100 DEGs from all comparisons. Using weighted gene co-expression network analysis (WGCNA) we constructed fifteen gene co-expression networks (modules). WGCNA parameters: soft threshold power = 7, network type = unsigned, correlation method = Spearman, minimum module size = 30, cutHeight or maximum dissimilarity = 0.25. For each module pathway enrichment analysis was performed using ToppFun (https://toppgene.cchmc.org/enrichment.jsp), with FDR corrected p<0.05. The categories GO: molecular function, GO: biological process and pathway were considered.

Target genes for validation were selected from the modules of interest using criteria including expression level (normalized read counts >8), module membership >0.7, size of differential expression between Th subsets (FC >1.5) and between HC and pSS (FC >1.5 or <0.7).

Gene expression replication by qPCR

To validate RNA sequencing results, TaqMan assays were performed for selected target genes (*ITGA1* Hs00235006_m1, *C3AR1* Hs00269693_s1, *ABCB1* Hs00184500_m1, *CCL5* Hs00982282_m1, *SOX4* Hs04987498_s1 and *HOPX* Hs04188695_m1, and *B2M* Hs00187842_m1 , all from ThermoFisher, LifeTechnologies) using RNA extracted from FACS sorted CCR9+, CXCR5+ and CCR9-CXCR5- Th cell subsets from HC and pSS patients as described above. RNA was extracted using the AllPrep Universal Kit (Qiagen), according to manufacturer's instructions. RNA was quantified using NanoDrop and cDNA was constructed using Superscript (Invitrogen). cDNA was measured with the specific TaqMan assay on the Quantstudio 12k flex System using the TaqMan Fast Advanced master mix (LifeTechnologies). Relative mRNA expression was calculated according to the comparative threshold cycle, using *B2M* as endogenous control. The fold change (FC) was calculated using the mean of the CCR9-CXCR5- Th cell subset from the HC as a reference.

Gene expression replication by flow cytometry

For phenotypic validation of RNA sequencing results fresh PBMCs were stained with fixable viability dye (eBioscience) and fluorochrome-conjugated antibodies against CD3, CD4, CXCR5, CCR9, NCR3 (NKp30), CD8, CD56, CD73 (NT5E), CD45RO, CD27, CD103 (integrin αE), CD49a (integrin α1), ABCB1 (MDR-1, P-glycoprotein, CD243) and CCR5 (Supplementary Table1). ABCB1 and CCL5 were stained intracellularly using the Fixation-Permeabilization protocol from the manufacturer (eBioscience). CCL5 expression was quantified intracellularly upon culture for 4 hours with or without stimulation with PMA and ionomycin in the presence of Brefeldin A. This protein secretion inhibitor was added as in part of the analyses IFN-γ and TNF-α expression was assessed to identify CCL5 expression by IFN-γ/TNF-α -producing effector Th cells. Importantly, in contrast to IFN-γ and TNF-α, CCL5 is hardly affected by inhibitors of cytokine secretion such as Brefeldin A.(43) Thus T cell stimulation results in CCL5 release reflected by reduced intracellular expression and subsequently release was expressed as the difference (delta) of mean fluorescence intensity (MFI) from Th cells with and without T cell stimulation (PMA/ionomycin).

Culture

For analysis of CCL5 secretion, 2.10^4 CCR9+, CXCR5+ or CCR9-CXCR5- FACS sorted Th cells were cultured with 5.10^3 monocytes (MACS sorted with CD14 beads) for 3 days with 10 ng/mL IL-7 or 0.1 ng/mL superantigen (Staphylococcal enterotoxin B, SEB) and restimulated with PMA and ionomycin for 24h. CCL5 release in the supernatants was measured by Luminex technology as previously described.(44)

Statistical analysis

RNA sequencing data were analyzed as described above. For the validation experiments (flow cytometry, qPCR and CCL5 production) Student's t-test, paired parametric t-test, Mann-Whitney U test and Wilcoxon non-parametrical paired test were used where appropriate. Data were analyzed using FlowJo™ Software, Graphpad Prism 6 and IBM SPSS Statistics 26. Differences were considered statistically significant at $p \leq 0.05$.

Results

Transcriptome analysis identifies differentially expressed genes between Th cell subsets and between healthy controls and pSS patients

To validate our technical procedure of sorting CCR9+, CXCR5+ and CCR9-CXCR5- (double negative, DN) Th cells and RNA isolation procedure, we confirmed elevated *CCR9* mRNA expression in the CCR9+ Th subset and elevated *CXCR5* mRNA expression in the CXCR5+ subset (Figure 1A,B). The isolated Th subsets were robustly distinguished based on their transcriptomic profile as shown in the multidimensional scaling (MDS) plot in Figure 1C. 5131 genes were differentially expressed between CCR9+ and CXCR5+ Th cells; 6493 genes were differentially expressed between CCR9+ and DN and 4783 genes were differentially expressed between CXCR5+ and DN (Figure 1D). Transcriptomic profiles differed between HC and pSS, with the largest number of DEGs in the CCR9+ subset, followed by the CXCR5+ subset and the DN subset (2777, 1416 and 1077, respectively Figure 1E). Based on the top 100 of DEGs from all comparisons, the Th cell subsets clearly cluster together per subset using hierarchical clustering analysis and most of the samples from patients and controls also cluster separately (Figure 1F). These results indicated that gene expression of circulating CCR9+, CXCR5+ and DN Th cell subsets differ in their transcriptomes and that the transcriptomes of HC and pSS patients differ.

Network analysis reveals Th subset- and disease-associated modules enriched for differentially expressed pathways

Next we used weighted gene co-expression network analysis (WGCNA) to cluster DEGs into 15 different gene correlation modules each containing a set of genes exhibiting coherent expression patterns. Each module was color named. We further selected modules with the consensus expression pattern (eigengene expression) of interest, for example, modules that revealed gene expression which was strikingly different between the Th subsets or between pSS and HC. As a result, 9 modules were selected: 3 modules with elevated eigengene expression in CCR9+ Th cells (black, blue and yellow, Figure 2A) or in CXCR5+ Th cells (brown, lightcyan and midnightblue, Figure 2B), and modules with the most differential eigengene expression between HC and pSS (cyan, darkgreen and purple, Figure 2C). 6 modules were excluded based on lack of clearly distinct patterns between subsets or between HC and pSS (Supplementary Figure 1).

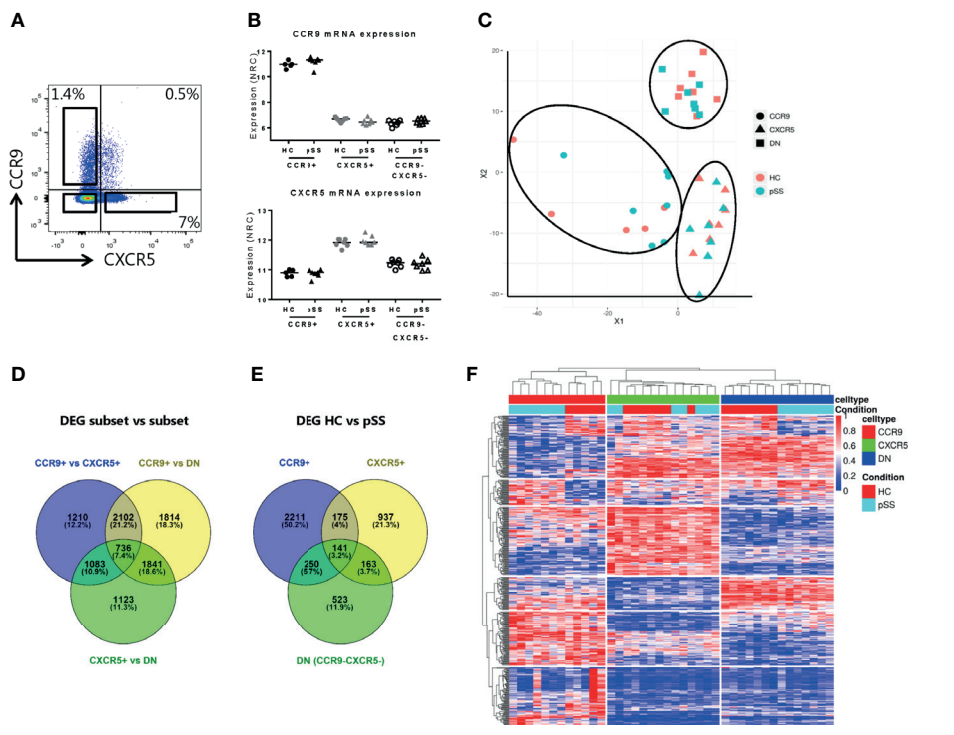

Figure 1 | Transcriptomic profiling robustly separates CCR9+, CXCR5+ and CCR9-CXCR5- Th cell subsets and identifies differentially expressed genes in pSS patients. RNA sequencing was performed on FACS sorted CCR9+, CXCR5+ and CCR9-CXCR5- (double negative, DN) T helper cells from PBMCs from healthy donors (HC, n=7, CCR9+ subset n=5) and primary Sjögren's syndrome patients (pSS, n=7). Representative flow cytometry plot of CCR9 and CXCR5 expression on CD4+ Th cells used for FACS sorting (A). Confirmation of elevated mRNA expression of CCR9 and CXCR5 in the sorted CCR9+ and CXCR5+ Th cells, respectively (B). NRC: normalized read counts, log2 normalized. Multidimensional scaling plot (MDS) shows all the differentially expressed genes (DEGs) between the Th cell subsets (C). Venn diagrams show number and percentages of DEGs between the CCR9+ vs CXCR5+ vs DN Th cell subsets (D) and between HC and pSS patients within the CCR9+, CXCR5+ and DN Th cell subsets (E). Heatmap representation of hierarchical clustering analysis using the top 100 DEGs of all comparisons shows distinct gene expression profiles between the subsets and between HC and pSS patients (F).

To functionally annotate each module, we performed pathway enrichment analysis. The modules distinguishing between CCR9+ and CXCR5+ Th cells mainly contained pathways involved in cell cycle (black and yellow), but also in effector T cell functions including migration and adhesion, proliferation, T cell activation and TCR signaling and cytokine and chemokine signaling (blue and brown). The modules showing the most distinct patterns between HC and pSS patients, mainly comprised of genes involved in transcription and translation, interferon signaling and metabolism (cyan, darkgreen and purple, respectively) (Figure 2D). In the midnightblue module no enrichment for pathways was found, therefore it is excluded from the graph.

Differentially expressed genes between CCR9+ versus CXCR5+ and CCR9-CXCR5- Th cells reveal genes involved in effector T cell function and transcription factors associated with Th1 differentiation

To narrow down to potential key genes from the RNA sequencing analysis as candidates for validation, a set of genes from the 9 modules was selected. The selection was focused on both the most robust differences between the Th subsets and between HC and pSS. Genes were selected based on several data-driven criteria including expression level (normalized read counts >8), module membership >0.7: indicating a strong correlation of the gene expression profile with the eigengene expression of the module(45), differential expression between the Th cell subsets with a fold change of >1.5 or <0.7, or between HC and pSS of >1.5 or <0.7, finally transcription (co)factors were selected (Figure 3A). Representative genes selected from the separate modules following this procedure are shown in Figure 3B.

Following these selection criteria we subsequently focused on genes from the blue module as this is the module that has the highest number of upregulated genes in CCR9+ Th cells and shows enrichment for the largest number of pathways crucial for effector Th cell function. 8 target genes out of 12 (Figure 3A) that were differentially expressed in HC vs pSS and have a known function based on literature evidence, were subsequently selected for validation. These included two transcription (co)factors *HOPX* and *SOX4*, and in addition *ITGAE*, *NT5E*, *C3AR1*, *CCL5*, *ITGA1* and *ABCB1*. Additionally from the 20 genes identified in the blue module that were not differentially expressed between HC vs pSS, *CCR5* and *NCR3* were selected given their effector function and potential role in pSS.(46–48) Next, we tested whether these selected genes could be replicated in an additional validation cohort using qPCR or flow cytometry. All of the selected DEGs upregulated in the CCR9+ Th subset as compared to the other subsets were validated by qPCR: *ITGA1*, *C3AR1*, *SOX4*, *HOPX* and *ABCB1*, (CCR9+ vs CXCR5+ and CCR9-CXCR5- all p<0.001, Figure 4A and B) or by flow cytometry: *ITGAE* (CD103), *NT5E* (CD73), *NCR3* (NKp30), *CCR5* and, *ABCB1* (CCR9+ vs CXCR5+ and CCR9-CXCR5- all p<0.05, Figure 4C and D, Supplementary Figure 2). However, the differential expression of these genes between CCR9+ Th cells from HC

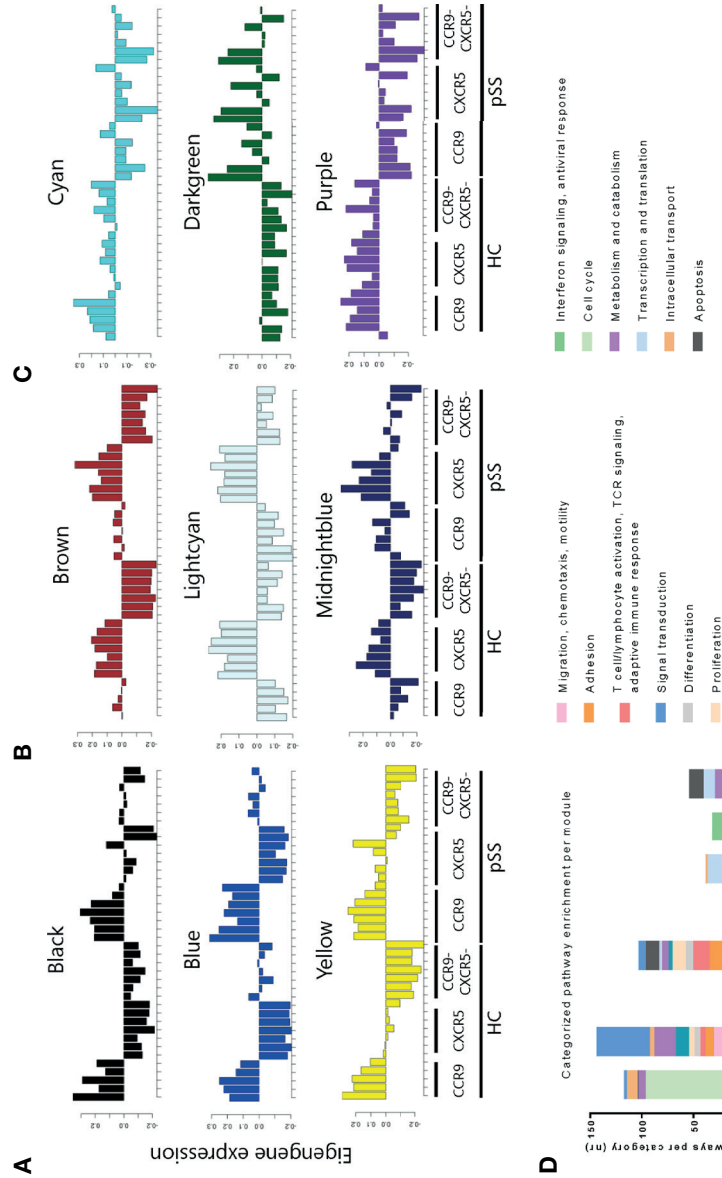

Figure 2 | Weighted gene co-expression network analysis (WGCNA) reveals networks of genes with coherent expression patterns involved in distinct Th cell functions. Modules with elevated eigengene expression in CCR9+ Th cells (A) or CXCR5+ Th cells (B), and modules with differential eigengene expression between HC and pSS patients (C) are shown. Each bar represents the eigengene expression of one sample in the indicated Th cell subset of a donor. Pathway enrichment analysis reveals different pathways within the modules. Pathways are categorized, and categories are indicated by the colors in the legend. The number of pathways per category is shown. The total number of pathways per module is indicated behind the module name on the X-axis (D).

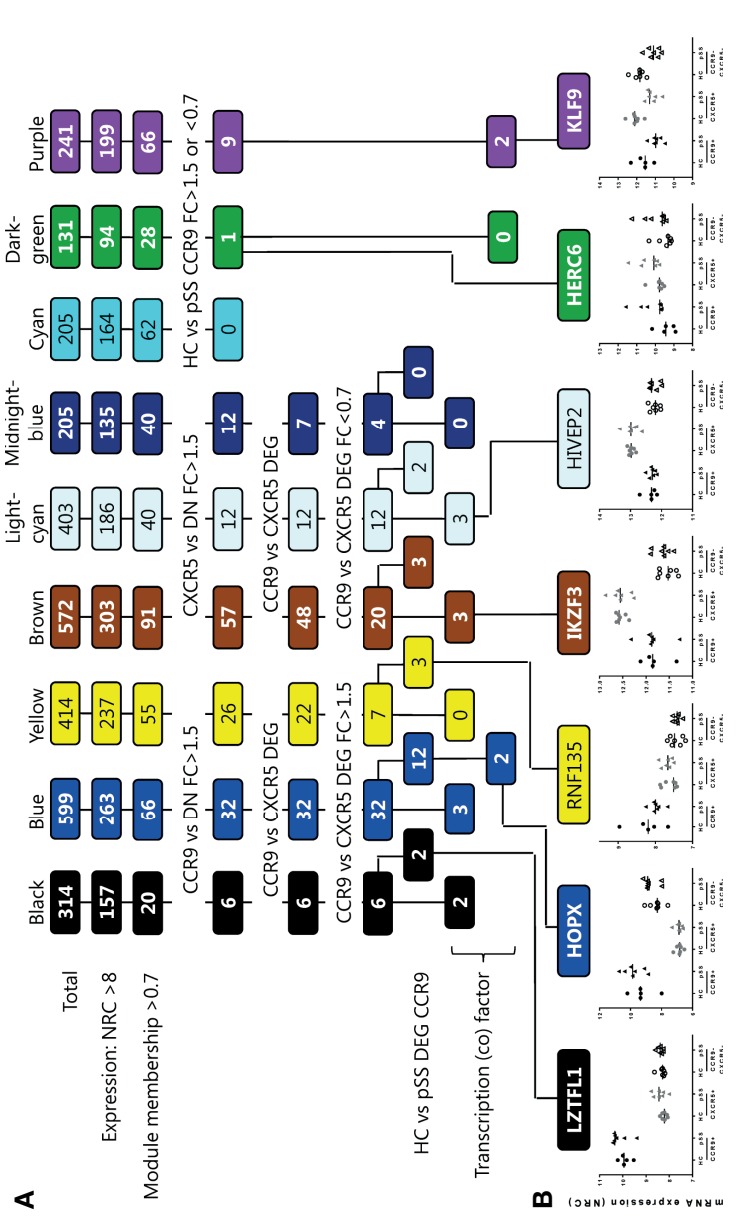

Figure 3 | Workflow for selection of target genes for validation per module. Genes from the modules are selected based on the criteria shown, including expression level (normalized read counts >8), module membership >0.7, differential expression between the Th cell subsets with a fold change of >1.5, or between HC and pSS of >1.5 or <0.7, and finally transcription (co)factors are selected. From the lower 4 rows, target genes that are also supported by literature evidence are selected for further analysis (A). Representative examples of genes selected based on the criteria and per module are shown (B).

versus pSS patients was not confirmed in the additional cohort, neither on mRNA nor protein level (Figure 4).

As for association with clinical parameters we only found a significant correlation of NT5E with the ESSDAI score (r=0.9274, p=0.0079, based on 7 pSS CCR9+ cell subsets). However, this was not validated in the flow cytometry cohort. Other selected target genes did not have significant correlations with ESSDAI score, LFS or serum IgG nor in RNA sequencing, nor in validation cohorts (data not shown). Also when subdividing in low vs moderate-high ESSDAI scores (ESSDAI<5 and ESSDAI≥5) no significant differences were found in any of the cohorts.

CCR9+ Th cells, and mostly effector CCR9+ Th cells, from healthy controls and pSS patients produce high levels of CCL5 and CCR9+ Th cells respond more potently to IL-7 in the context of monocytes

CCL5 (RANTES) is elevated in CCR9+ Th cells at the transcriptomic level compared to the other subsets and met the selection criteria described above (Figure 5A). Because of its increased expression in pSS patients and potential key role in regulation of glandular inflammation in Sjögren-like disease in mice(49), CCL5 was studied in more detail using multiple technological platforms. The differential *CCL5* mRNA expression between HC and pSS patients could not be confirmed by qPCR (Figure 5B). Nonetheless, differential CCL5 expression between CCR9+ Th cells and the other subsets was replicated (p≤0.01). Since the number of CCR9+ Th cells and its ligand CCL25 are increased in the blood and salivary glands of pSS patients(10,26), we analyzed whether CCR9+ Th cells in the context of antigen presenting cells produce CCL5. To test this, FACS sorted CCR9+, CXCR5+ and CCR9-CXCR5- Th subsets were co-cultured with monocytes and CCL5 was measured in supernatants upon PMA/ionomycin restimulation. Significantly elevated levels of CCL5 were measured in the CCR9+ Th cell cultures as compared to CXCR5+ and CCR9-CXCR5- Th cells (p≤0.05, Figure 5C). Since antigen triggering and IL-7 stimulation, as previously demonstrated(31,50), may be key drivers of CCR9+ Th activation and thereby CCL5 secretion, cells were cultured with superantigen Staphylococcal Enterotoxin B (SEB) or IL-7. Interestingly, IL-7 strongly upregulated CCL5 production by co-cultured CCR9+ Th cells. Their CCL5 production is significantly higher than that of co-cultured CXCR5+ or CCR9-CXCR5- Th cells (both p<0.05). SEB even more strongly stimulated CCL5 production by CCR9+ Th cells, significantly higher than CXCR5+ Th cells but not significantly higher than CCR9-CXCR5- Th cells (p=0.18) (Figure 5D).

To ensure that CCL5 production is not merely monocyte dependent, using flow cytometry we evaluated CCL5 expression levels directly in CCR9+ Th cells vs. CCR9- Th cells in blood from HC and pSS patients (gating strategy, Supplementary figure 3). In support of the RNA sequencing data CCR9+ Th cells expressed higher CCL5 levels than CCR9- Th cells, both

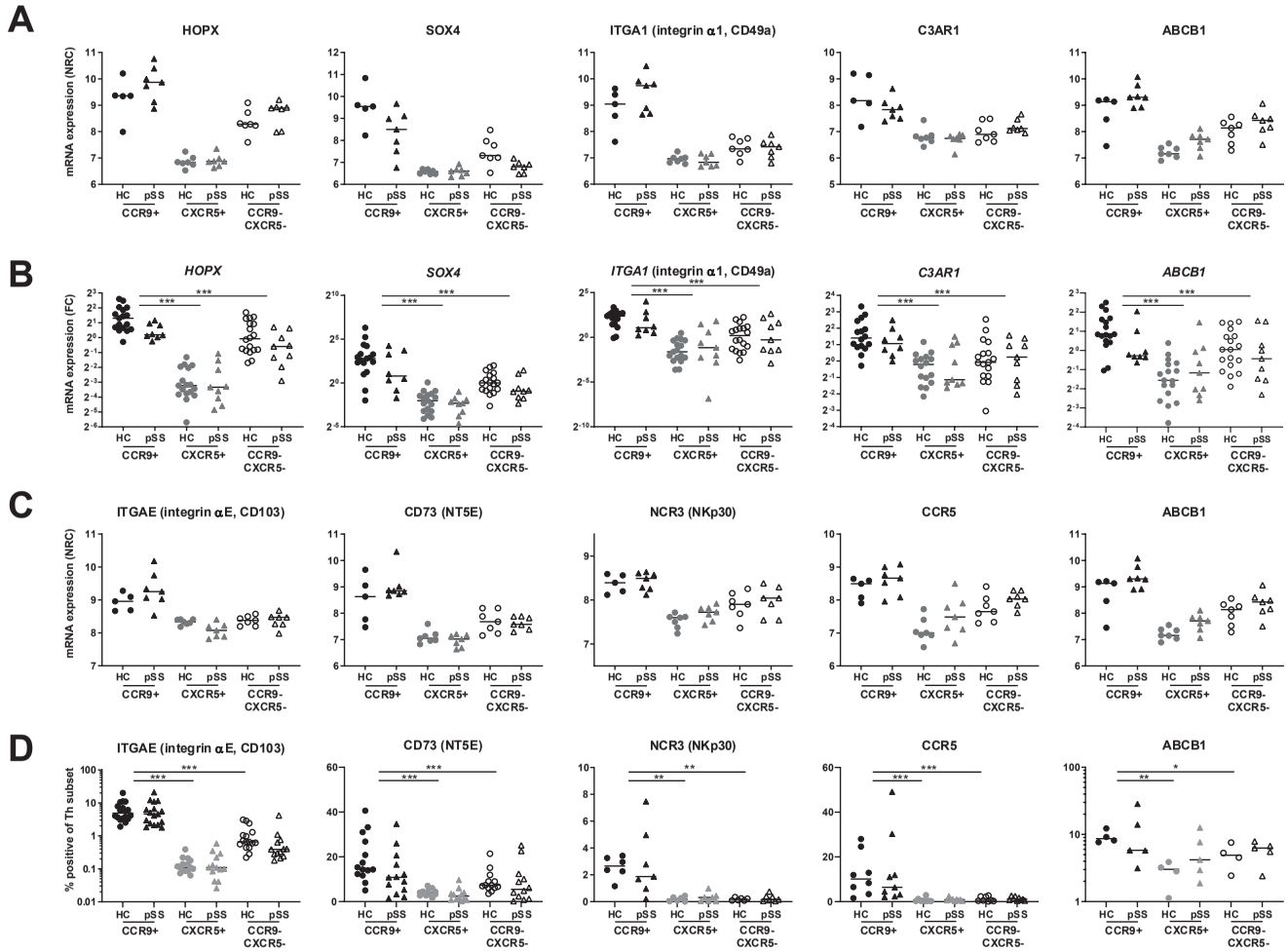

Figure 4 | Validation of selected target genes from the blue module in replication cohorts confirms differences between Th subsets, but not between HC and pSS patients. According to the criteria shown in Figure 3 and based on pathway enrichment analysis (Figure 2) and literature, genes were selected from the blue module and were replicated by qPCR and flow cytometry. RNA sequencing data is shown in (A) and (C), qPCR and flow cytometry data are shown in (B) and (D), respectively. HOPX, SOX4, ITGA1, C3AR1 and ABCB1 were evaluated by qPCR in an additional replication cohort (B). Genes encoding for surface proteins for which antibodies were available were replicated by flow cytometry: ITGAE (CD103), CD73 (NT5E), NCR3 (NKp30), CCR5 and ABCB1 (D). NRC: normalized read counts; FC: fold change, DN HC mean is set at 1. *, **, *** indicates statistical significance of $p<0.05$, 0.01, 0.001, respectively.

in HC and pSS patients (p=0.006 and p=0.003, respectively) (Figure 5E). In line with the RNA expression data CCL5 protein expression in CCR9+ Th cells in pSS patients was not significantly different from HC. However, we confirmed our earlier findings (26), showing that the frequency of CCR9+ cells in the Th cell subset is significantly higher in pSS patients compared to HC (1.92%±0.97 in HC vs 3.81%±1.25 in pSS patients, Figure 5F).

Since CCR9+ Th cells are enriched for effector memory and effector Th cells(26) we tested whether higher CCL5 expression was related to differentiation of Th cells. For this purpose CD45RO/CD27-defined naive, central memory, effector memory and effector Th cell subsets were analyzed.
We here show that CCL5 expression is significantly higher in more differentiated cells (compared to naive Th cells, Figure 5G). Also in these separate subsets the difference between CCR9+ and CCR9- Th cells is statistically significantly different (all p<0.05, Figure 5H). To determine if more differentiated Th cells release CCL5 more robustly upon stimulation, we compared CCL5 expression in the Th cell subsets after 4 hours PMA/ionomycin stimulation as compared to unstimulated Th cells. Histograms of a representative donor are shown in Figure 5I. Release of CCL5 is measured as reduced intracellular CCL5 expression as previously shown.(43) Effector Th cells release more CCL5 after stimulation compared to naive, central memory and effector memory cells (all p<0.05, Figure 5J). All CCR9+ subsets secrete more CCL5 after stimulation compared to CCR9- cells (p<0.05, except for effector memory cells p=0.063, Figure 5K).

Since it has been demonstrated that CCL5 secretion by CD8 T cells is rapidly induced (43) we also assessed whether this holds true for CCR9+ Th cells. For this purpose we performed a separate experiment testing the kinetics of CCL5 release measuring CCL5 at t=0, t=10 minutes and t=4 hours. Interestingly, we found that reduction of CCL5 expression after 10 minutes of stimulation was similar as compared to 4 hour stimulation (reduction at 10 minutes to 61%±20 versus 60%±24 reduction at 4 hours, p=0.583, n=12 HC). Hence, this indicated a rapid release of CCL5 also by CCR9+ Th cells. Finally, we also assessed whether increased CCL5 expression was a specific feature of IFN-γ/TNF-α-secreting effector Th cells and found that CCR9+ IFN-γ+TNF-α+ Th cells express higher CCL5 levels (MFI 589±231 vs 425±190 compared to CCR9+ IFN-γ-TNF-α- cells, p=0.018).

Discussion

In this study, for the first time transcriptomic analysis of CCR9+ Th cells was performed in both healthy controls and pSS patients, and compared to CXCR5+ Th cells. RNA sequencing analysis revealed multiple networks of differentially expressed genes between the Th subsets that were shared between healthy controls and pSS patients. Identified pathways involved in effector T cell function were upregulated in CCR9+ Th cells including genes associated with adhesion, chemotaxis, proliferation, TCR activation, drug response and complement activation. This was exemplified by high production of CCL5 by CCR9+ Th cells as compared to CCR9- Th cells, which was identified in all Th subsets but in particular in effector cells and induced upon antigen challenge and IL-7 stimulation.

Our strategy for selection of target genes to validate, used strict and robust criteria and was focused on upregulated genes in the CCR9+ Th cell subset in one of the CCR9-specific (blue) modules showing significant differences between the Th subsets. Using this strategy we identified a number of differentially expressed genes that were validated in additional cohorts on RNA and/or protein level. These include transcription (co)factors *HOPX* and *SOX4*, chemokine receptor *CCR5*, chemokine *CCL5*, adhesion molecules *ITGAE* (CD103, integrin αE) and *ITGA1* (CD49a, integrin α1), cytotoxicity receptor *NCR3* (NKp30), multidrug resistance gene *ABCB1* (MDR-1, P-glycoprotein), complement receptor *C3AR1* and inhibitory molecule *NT5E* (CD73).
Nonetheless, we realize that genes from the other modules potentially also could play key roles in the function of CCR9+ Th cells. For example, *LZTFL1* that has coherence within the black module encodes for a protein that is upregulated by all-trans retinoic acid and upon TCR signaling. Its overexpression has been shown to enhance NFAT mediated signaling, potentially contributing to the production of cytokines by Th cells.(51) Similarly, genes from the yellow module include the genes encoding for CCR9 and integrin α4, which have been indicated to represent key molecules for CCR9+ Th cells. Hence, future studies should replicate these genes and reveal their roles.
The same holds true for genes that are unique to CXCR5+ Th cells, in particular those that are significantly different between HC and pSS patients. In addition, the interaction between genes in identified networks should be studied.

Here we focused on the highly connected and robustly expressed genes in the blue module and their potential role in the pathogenicity of CCR9+ Th cells and their potential relevance to pSS immunopathology is discussed.
Interestingly, we identified transcription cofactor *HOPX* (homeobox only protein) to be strongly expressed by CCR9+ Th cells. *HOPX* previously was shown to be associated with Th1 activity. In humans, *HOPX* is highly upregulated in effector/memory Th1 cells and in mice *HOPX* is crucial for survival of Th1 cells. *HOPX*-deficient mice do not develop colitis or arthritis in models inducing these inflammatory conditions.(52) In addition, we demonstrated increased levels of *SOX4* in CCR9+ Th cells as compared to the other Th cells. *SOX4* inhibits Th2 responses and overexpression of *SOX4* during Th1 differentiation induces more IFN-γ-producing cells.(53) Together, this suggests that *HOPX* and *SOX4* may drive the high IFN-γ

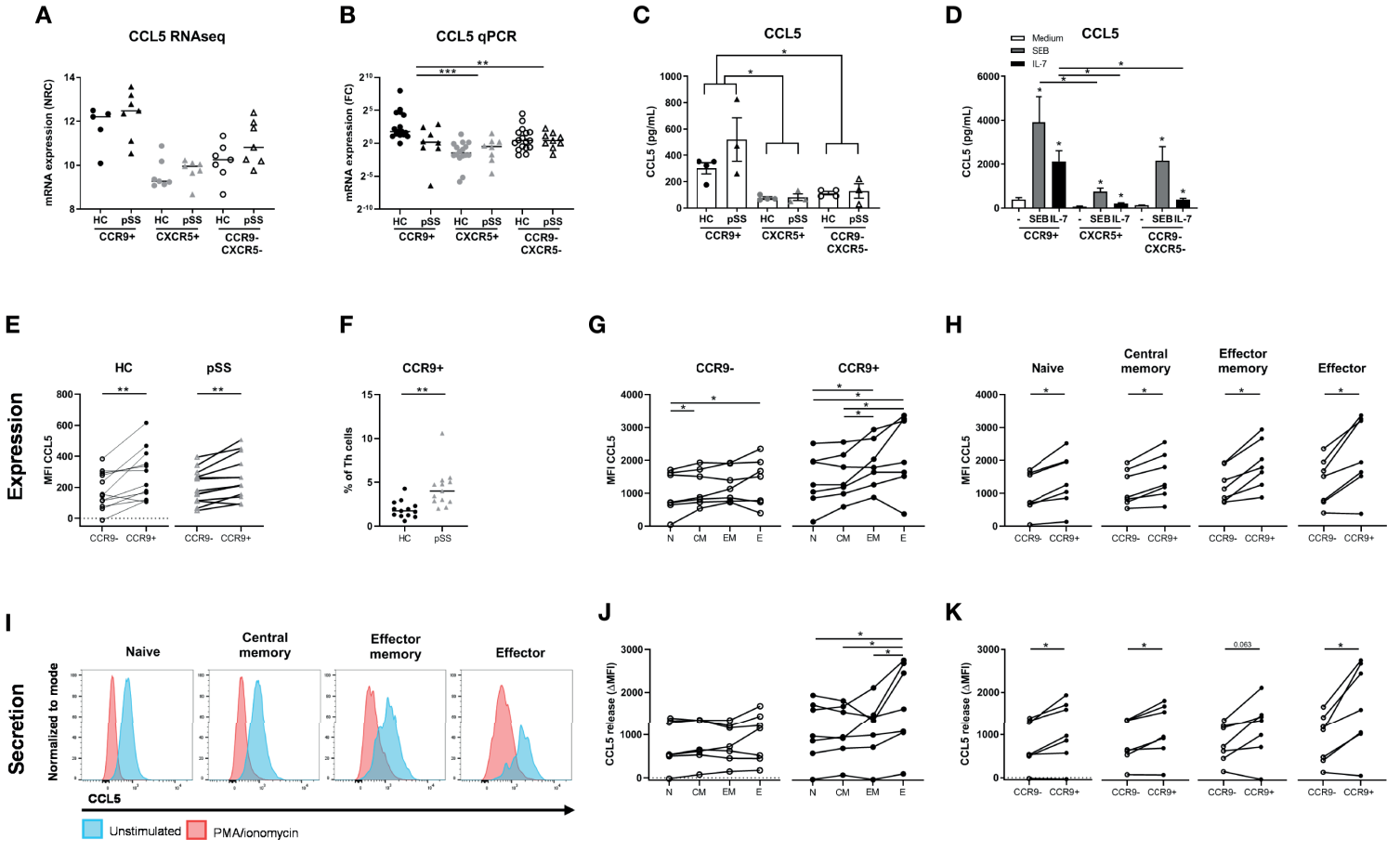

Figure 5 | Increased production and release of CCL5 by CCR9+ Th cells in pSS patients and controls is higher in effector cells and is enhanced by IL-7 and antigenic triggering. mRNA expression as assessed by RNA sequencing (A). In a replication cohort qPCR shows validation of significantly increased CCL5 mRNA in the CCR9+ Th cell subset as compared to CXCR5+ and CCR9-CXCR5- Th cell subsets (B). CCR9+, CXCR5+ and CCR9-CXCR5- Th cells from HC (n=4) and pSS patients (n=3) were cultured with monocytes for 72 hours followed by PMA/ionomycin restimulation (C). To determine the effect of antigen triggering (SEB=Staphylococcal Enterotoxin B) and IL-7 stimulation on CCL5 production by the Th cell subsets we pooled HC and pSS (D). CCL5 protein expression in Th cells from peripheral blood from healthy controls (n=13) and pSS patients (n=13) was analyzed ex vivo by flow cytometry in a 2nd replication cohort (E). CCR9 expression on Th cells was also assessed in this replication cohort by flow cytometry (F). In HC CCL5 expression by effector memory and effector Th cells is compared to naive Th cells, both for CCR9- and CCR9+ Th cells (G); and in all CD45RO/CD27 compartments CCL5 expression is compared between CCR9- and CCR9+ Th cells (H). Histograms from a representative donor show CCL5 expression upon 4h of stimulation with PMA/ionomycin in the different Th cell compartments. Blue and red shows CCL5 expression in unstimulated and PMA/ionomycin stimulated cells, respectively (I). CCL5 release expressed as the difference between unstimulated – the stimulated cultures (delta) between all four CD45RO/CD27 compartments for both CCR9- and CCR9+ Th cells (J) and within each compartment comparing CCR9- and CCR9+ Th cells (K). FC: fold change. Th cell phenotypic validation: naive (CD27+CD45RO-), central memory (CD27+CD45RO+), effector memory (CD27-CD45RO+), effector (CD27-CD45RO-) cells. MFI: mean fluorescence intensity. ΔMFI: deltaMFI (unstimulated minus PMA/ionomycin stimulated). *, **, *** indicates statistical significance of p<0.05, 0.01, 0.001, respectively.

production by CCR9+ Th cells, which we and others have previously shown.(13,26) However, future research is needed to confirm the role of *HOPX* and *SOX4* in regulating Th1-associated activity in CCR9+ Th cells.

In addition to *HOPX* and *SOX4*, *CCL5* and its receptor *CCR5* are upregulated in CCR9+ Th cells and have been associated with a Th1 phenotype.(54,55) Also, CCR5 has been implicated in dry eye disease. In a mouse model of experimental dry eye disease, desiccating stress potently stimulated the expression of Th1-attracting chemokines and their receptors on the ocular surface of C57BL/6 mice.(56) In human studies, CCR5 expression has been shown to increase in the conjunctival epithelium of patients with dry eye syndrome.(47,57)
Interestingly, CCL5 and its receptor CCR5 were increased in inflamed glands in Sjögren-like disease and blockade of CCL5 can significantly reduce disease.(49) The ligands for CCR5, CCL3 and CCL5 are elevated in pSS salivary glands, potentially facilitating recruitment of CCR5-expressing cells including CCR9+ Th cells.(48) In this paper we demonstrate higher expression and secretion of CCL5 in CCR9+ effector cells. Also when looking at IFN-γ/TNF-α –secreting cells we find CCR9+ Th cells express highest CCL5. Besides this we have demonstrated rapid release of CCL5 by CCR9+ Th cells and a strongly increased secretion of CCL5 by co-cultured CCR9+ Th cells as compared to CXCR5+ and DN Th cells. Interestingly, IL-7, which is a key early mediator of salivary gland inflammation(31–34) and is a crucial factor in lymphoid structure organization(38,58), significantly increased CCL5 production by CCR9+ Th cells to a higher level than CXCR5+ and CCR9-CXCR5- Th cells. As IL-7 has previously been shown to increase responsiveness of autoreactive T cells (59,60) this could implicate that IL-7-driven self-reactive T cell responses associated with CCL5 production plays a significant role in early inflammatory responses in pSS, attracting multiple leukocyte subsets to affected sites. Similarly, T cell receptor crosslinking by exogenous antigen as we demonstrate in this paper can strongly boost CCL5 production by CCR9+ Th cells.

Genes encoding for adhesion molecules *ITGAE* (CD103) and *ITGA1* (CD49a) were elevated on RNA level in CCR9+ Th cells as compared to the other Th cells. On protein level this corresponded with around 10% of CCR9+ Th cells expressing CD103 which is elevated as compared to the other subsets. CD103 is known as a marker for intraepithelial lymphocytes dimerizing with β7 to form αEβ7, and its ligand is E-cadherin, both of these molecules have been found to be elevated in pSS salivary glands.(61) Also, laminin, a ligand for CD49a, has been found to be upregulated in pSS salivary glands.(62) However, whereas we did find a subset of CCR9+ Th cells with increased expression of CD103, we could not find any evidence for increased surface expression of CD49a on the CCR9+ Th cells (data not shown). These data indicate that part of the CCR9+ Th cells are prone to adhere in the salivary gland of pSS patients, potentially mediated via ITGAE.

In the present study we demonstrate that *NCR3* gene expression is elevated in CCR9+ Th cells. The ligand for NCR3, B7H6, is present in pSS salivary glands potentially triggering CCR9+ Th cells present in the glands, and in addition a SNP in this gene was associated with pSS potentially contributing to the pathogenesis.(46) Identification of natural cytotoxicity receptor NCR3 on Th cells is unexpected, and indeed only a small percentage of CCR9+ Th cells expresses this molecule and levels are much higher on CD56+ NK cells (data not shown). Further investigation is needed to study whether this represents a functionally relevant expression by CCR9+ Th cells, potentially inducing IFN-γ production like in NK cells.

Elevated *NT5E* (CD73) gene expression was found in CCR9+ Th cells and on protein level CD73 (ecto-5'-nucleotidase) was expressed on 10-40% of CCR9+ Th cells. CD73 is an enzyme that dephosphorylates AMP into anti-inflammatory adenosine contributing to an anti-inflammatory milieu.(63) CD73 is expressed by regulatory T cells but can be upregulated on all Th cells upon activation.(64) As a homeostatic process, regulatory molecules are upregulated upon activation of T cells, and this may be the case for CD73 on the proinflammatory CCR9+ Th cells.

Another interesting molecule upregulated in a small subset of CCR9+ Th cells is *ABCB1*, a multidrug resistance gene. It encodes for P-glycoprotein or MDR-1 (multidrug resistance 1) which causes efflux of intracellular drugs and is associated with unresponsiveness to treatment in various diseases including systemic lupus erythematosus.(65) Some agents can induce expression of P-glycoprotein and some can inhibit its function, including cyclosporin-A. This suggests that increased expression of *ABCB1* on CCR9+ Th cells might contribute to resistance to inhibition by some drugs and might benefit from simultaneous inhibition of ABCB1 function (e.g. by cyclosporine-A).(66) Further research is needed to study the relevance of expression of *ABCB1* on CCR9+ Th cells and in pSS.

In some pSS patients, hypocomplementemia of in particular C3 and C4 is found, which is associated with lymphoma development.(67) It is generally hypothesized that low C3 and C4 levels are a result of consumption mediated by immune complexes. This complement activation/consumption is likely associated with formation of complement fragments such as C3a and C4a, the latter being elevated in pSS salivary glands.(68) Although not demonstrated on protein level or by functional experiments, *C3AR1* was found to be elevated in CCR9+ Th cells. Upregulated C3aR may contribute to CCR9+ Th cell activation since C3aR signaling has been shown to contribute to maintenance of effector functions via mTOR.(69)

Although robust differences between Th subsets were detected and replicated, a drawback of our study is that the cohort in which RNA sequencing was performed was rather small. Especially since differences in mRNA expression between HC and pSS patients were small, the number of donors may not have been sufficient to detect true differences. The most robust differences between HC and pSS were present in all Th subsets; gene expression differences between HC and pSS that were specific for the CCR9+ Th cell subset were smaller. Indeed, the small CCR9+ Th cell subset-specific differences between HC and pSS patients were unfortunately not validated. False discovery rate correction may have partly increased the chance of replicating positive results from RNA sequencing. In this respect, DEGs from the modules showing the largest differences between HC and pSS patients i.e. cyan, darkgreen including IFN induced genes, which indeed have been shown to be upregulated in part of pSS patients(70), and purple, in which the differences between HC and pSS were present in all Th subsets, may have given a higher chance of being replicated in additional cohorts.

In addition, in this study circulating CCR9+ Th cells were studied, but whether these are recirculating from the gut or the salivary glands and to what extent these reflect tissue CCR9+ Th cells is unclear. As a future perspective, bulk or single cell sorting of CCR9+ Th cells from salivary glands of pSS patients to perform RNA sequencing and TCR sequencing may reveal the local activation profile and possible autoreactivity of this proinflammatory Th subset. Also, further analyses may elucidate the specific and shared molecular features as compared to PD-1^{hi}CXCR5- Tph cells.(29,30)

Also, variation in gene expression data may partly be due to patient heterogeneity. Indeed differences were observed between patients used for discovery (RNA sequencing) and replication (qPCR, flow cytometry and in vitro). A larger proportion of patients of the RNA sequencing cohort was treated with immunosuppressants and there were differences in clinical parameters (LFS, presence of autoantibodies, ESSDAI scores, serum IgG levels). However, none of these clinical parameters showed statistically significant correlations with the expression of the target genes. Finally, the already strongly differentiated nature of (re) circulating CCR9+ effector Th cells in any individual may also have hampered the detection of a difference between HC and pSS patients. Previously we have documented strongly increased percentages of IFN-γ, IL-17, IL-21 and IL-10-producing cells in circulating CCR9+ vs CXCR5+ Th cells(26), which was similar in HC and pSS patients. The increased numbers of CCR9+ Th cells in the circulation and the salivary glands suggest that given the effector functions of CCR9+ Th cells these may significantly contribute to immunopathology. In the same line, our in vitro data suggest that activation of CCR9+ Th cells upon antigen challenge and increased IL-7 significantly could drive local inflammation. Future research should elucidate the generalized differentiated nature of circulating CCR9+ effector Th cells in additional inflammatory diseases.

Altogether the present study reveals that CCR9+ Th cells show many differentially expressed genes as compared to CXCR5+ Th cells and CCR9-CXCR5- Th cells, identifying novel effector molecules that reveal additional properties of these pathogenic cells. This is exemplified by CCL5, which may be a key mediator in early migration of inflammatory cells. Targeting predicted key molecules based on the results from this study might reveal novel therapeutic approaches to halt the pathogenic processes induced by CCR9+ Th cells.

Data availability statement

The RNA sequencing dataset generated for this study can be found in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE173635.

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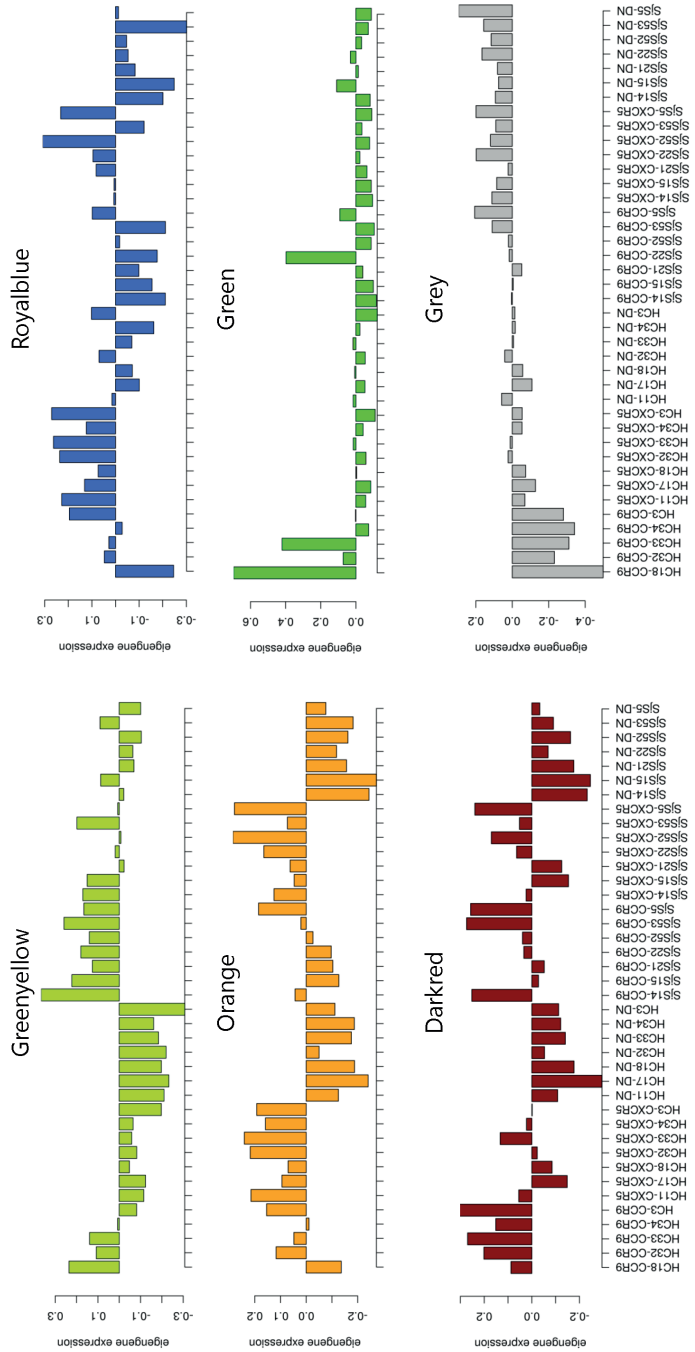
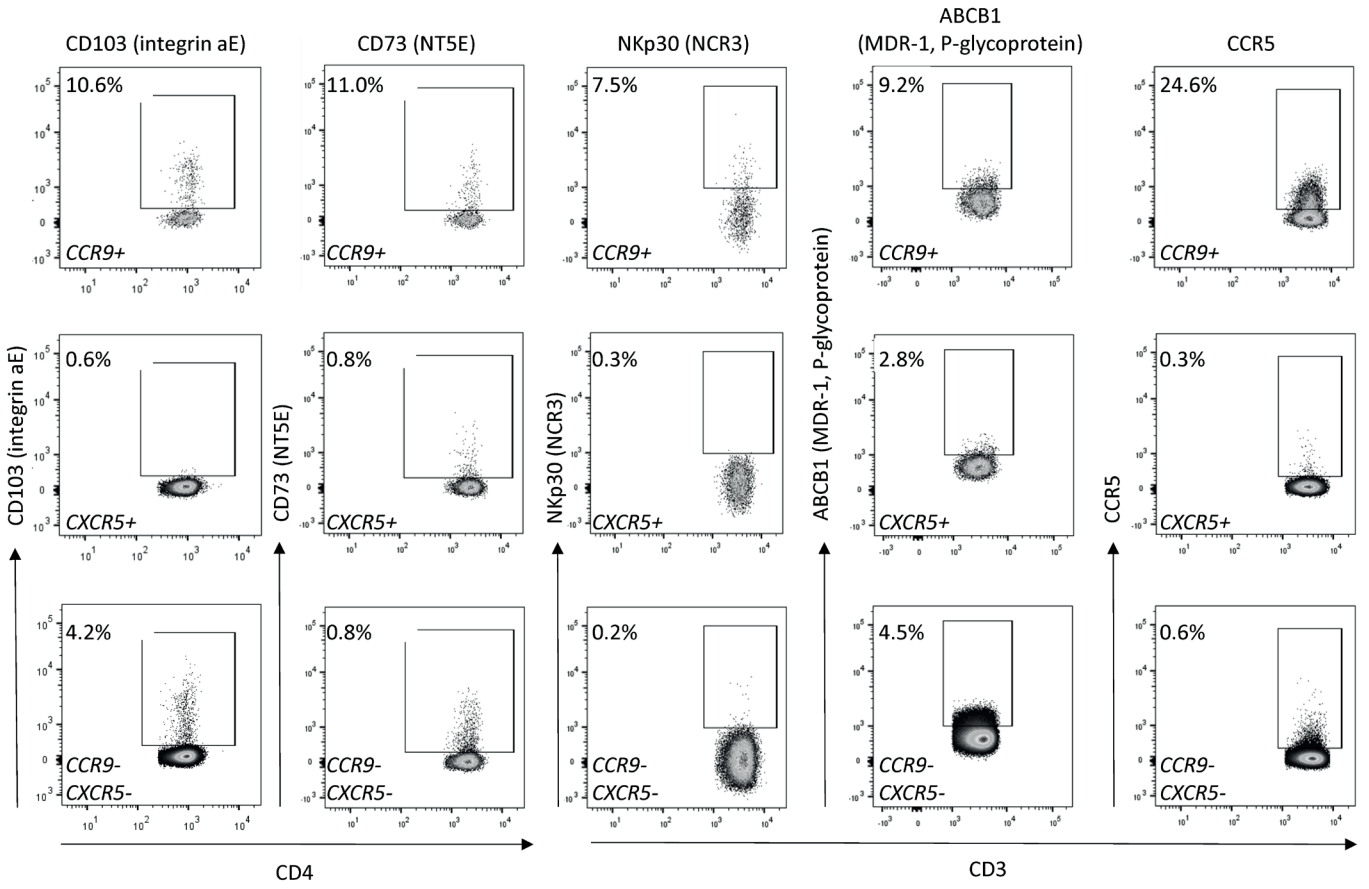
Supplementary material

Marker	Fluorochrome	Company	Clone
CD3	AF700	Sony Biotechnology	UCHT1
CD4	PerCP	Biolegend	RPA-T4
	eF780	eBioscience	RPA-T4
	BV785	Biolegend	RPA-T4
CXCR5	BV421	BD Biosciences	RF8B2
	PerCP-Cy5.5	Biolegend	J252D
CCR9	PE	R&D/Bio-Techne	248621
	APC	Biolegend	L053E8
NKp30	PE	BD Biosciences	P30-15
CD8	PerCP	BD Biosciences	SK1
	FITC	BD Biosciences	RPA-T8
CD56	FITC	Biolegend	HCD56
CD73	PE	BD Biosciences	AD2
CD45RO	BV711	Biolegend	UCHL1
	PE-Cy7	BD Biosciences	UCHL1
CD27	eF780	eBioscience	O323
	BV510	BD Biosciences	L128
CD103	FITC	Dako	Ber-ACT8
CD49a	APC	Sony Biotechnology	TS2/7
ABCB1	FITC	eBioscience	UIC2
CCR5	PE	eBioscience	eBioT21/8
CCL5	PE	Biolegend	VL1
IFN-gamma	PerCP-Cy5.5	eBioscience	4S.B3
TNF-alpha	BV421	BD Biosciences	Mab11

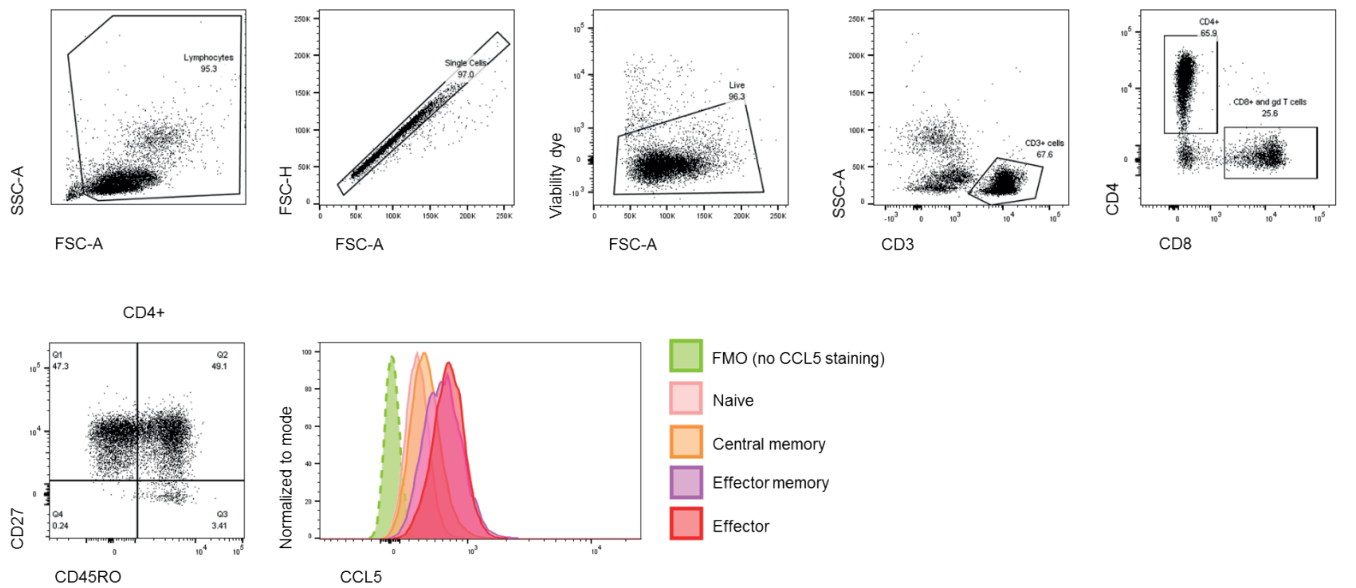
Supplementary Table 1 | Antibodies used for flow cytometry.



Supplementary figure 1 | Excluded modules. Using WGCNA analysis 15 modules were constructed. Based on less robust patterns of distinct eigengene expression between Th cell subsets or between HC and pSS 6 modules were excluded from further analysis. The grey module contains genes which were not assigned to any module.



Supplementary figure 2 | Validation of target genes by flow cytometry. Representative flow cytometry stainings of CCR9+ Th cells (top row), CXCR5+ Th cells (middle row) and DN Th cells (last row) are shown for integrin αE (ITGAE), NKp30 (NCR3), CD73 (NT5E), ABCB1 (MDR-1, P-glycoprotein) and CCR5.



Supplementary figure 3 | Gating strategy for intracellular flow cytometry staining of CCL5. Representative flow cytometry staining of healthy control. Top row, panel left to right: forward versus side scatter (FSC vs SSC); selection of single cells, selection of live cells using fixable viability dye; selection of CD3+ cells, followed by selection of CD4+ cells. Bottom row: CD45RO/CD27 staining of CD4+ cells and CCL5 expression in given subsets.

Chapter 3

CCL5 Release by CCR9+ CD8 T Cells: A Potential Contributor to Immunopathology of Primary Sjögren's Syndrome

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Abstract

Introduction. Increased CCL5 expression and CD8 T cells have been shown to be pivotal regulators of immunopathology in primary Sjögren's syndrome (pSS) and pSS-like disease. Increased CCL5 expression by CCR9+ CD4 T cells has previously been implicated as a contributor to immunopathology in pSS. The role of CD8 T cells and in particular CCR9+ CD8 T cells and their potential to secrete CCL5 has not previously been studied in pSS. In this study we investigated both CCR9 and CCL5 expression by CD8 T cells in pSS patients compared to healthy controls (HC).

Methods. CCR9 expression on CD8 T cells from peripheral blood was compared between patients with pSS and HC by flow cytometry. Intracellular CCL5 expression by naive, memory and effector CCR9- and CCR9+ CD8 T cells was assessed. In addition, the capacity and pace of CCL5 release upon T cell activation was determined for all subsets and compared with CD4 T cells.

Results. The frequency of circulating CCR9+ CD8 T cells in pSS patients is increased compared to HC. Antigen-experienced CD8 T cells, especially CCR9+ effector CD8 T cells, express the highest CCL5 levels, and release the highest levels of CCL5 upon activation. Memory and effector CD8 T cells of pSS patients express significantly less CCL5 and subsequently release less CCL5 upon stimulation compared to HC. CCR9+ CD8 T cells rapidly release CCL5 and significantly more than CCR9+ CD4 T cells.

Conclusion. CCR9+ CD8 T cells express more CCL5 than CCR9- CD8 T cells. CCL5 is rapidly released upon activation, resulting in reduced intracellular expression. Reduced CCL5 expression by an elevated number of antigen-experienced CCR9-expressing CD8 T cells in pSS patients points towards increased release in vivo. This suggests that CCL5 release by CCR9+ CD8 T cells contributes to immunopathology in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disorder, associated with inflammation of the exocrine glands and in some cases other organs, resulting in complaints of dryness of eyes and mouth and extraglandular manifestations.(1) Biopsies of salivary gland tissue of pSS patients show lymphocytic aggregates, mainly consisting of CD4 T cells, CD8 T cells and B cells.(1–4) However, also elevated numbers of cell types such as NK cells and NK T cells have been demonstrated in pSS salivary glands.(5–7) B cell hyperactivity is a hallmark feature of the disease, which is reflected by the production of autoantibodies, elevated serum IgG and the increased risk of developing lymphoma.(1,8)

T follicular helper (Tfh) cells are important contributors to B cell hyperactivity. Both Tfh cell numbers and typical Tfh-associated cytokines (e.g. IL-21, IL-4 and CXCL13) are increased in the salivary glands and in peripheral blood of pSS patients.(9–13) Furthermore, Tfh cell numbers are correlated to autoantibody production and disease severity, suggesting a significant role for these cells in the immunopathology of pSS.(9–13)

Recent work from our group and others has implicated a role for "Tfh-like" cells in pSS immunopathology, which do not express CXCR5, but are characterized by C-C chemokine receptor 9 (CCR9) expression. CCR9+ CD4 T cells induce CD8 T cell-dependent immunopathology in mucosa-associated tissue, including salivary glands in Sjögren-like disease in mice.(14) In pSS patients CCR9+ CD4 T cell numbers are increased in the salivary glands and blood compared to controls and express more IL-21, IL-4 and IFN-γ than CXCR5+ CD4 T cells.(14,15) Also, the ligand for CCR9, C-C chemokine ligand 25 (CCL25), is elevated in salivary gland tissue of pSS patients compared to controls.(15) In healthy human salivary gland tissue *CCL25* mRNA is not detectable.(16)

In our previous work studying CCR9+ CD4 T cells in pSS we identified genes that are specifically upregulated in CCR9+ CD4 T cells and were involved in effector T cell pathways compared to CXCR5+ and CCR9-CXCR5- CD4 T cells.(17) One of the identified genes is *CCL5*. CCL5 (regulated upon activation normal T cell expressed and secreted (RANTES)) is a chemokine which mediates trafficking and homing of e.g. T cells, monocytes and NK cells. It is associated with Th1 activity, just like its receptor CCR5.(17–19) The main producers of type I IFNs, pDCs, have an elevated expression of CCR5 in pSS patients compared to healthy controls (HC).(20) Both CCL5 and CCR5 are increased in salivary glands of pSS patients.(21,22) This increase in both is also seen in inflamed glands in Sjögren-like disease in a murine model, where blocking of CCL5 reduces disease.(23)

Interestingly, in previous studies CD8 T cells were found to be rapid and more potent producers of CCL5, originating from unique vesicles and released upon activation.(24,25) CD8 T cells through production of CCL5 have been shown to regulate inflammation in experimental models of arthritis. In particular CD103+CD69+ CD8 T resident memory (Trm) cells were found in elevated numbers in previously inflamed joints in rheumatoid arthritis (RA), and shown to initiate flares by recruiting circulating lymphocytes by CCL5 secretion. Inhibition of CCL5 release by these cells inhibited arthritis flares in a murine model of RA.(26)

In recent years numerous studies have shown that CD8 T cells are not merely cytotoxic killers, but can orchestrate inflammation. In this respect CXCR5+ CD8 T cells were found to display Tfh features.(27) In line with these observations in a Sjögren-like disease mouse model knockdown of CD8α showed diffuse infiltration instead of focal infiltration with no acinar atrophy, duct damage, fibrosis nor leukocyte infiltration, and similar results were found after therapeutic depletion of CD8 from salivary glands, resulting e.g. in improvement of saliva production.(4) Furthermore, whereas CD4 T cells predominate in lymphocytic infiltrates in the salivary glands, one subset of CD8 T cells, the CD103+CD69+ CD8 T resident memory (Trm) cells outnumber CD103+CD69+ CD4 Trm cells and show elevated IFN-γ production in Sjögren-like disease in mice.(4) In pSS, circulating activated CD8 T cells correlate with a multi-omics-based pSS disease signature and HLA-DR-expressing CD8 T cells correlate to disease activity, as captured by the EULAR Sjögren's syndrome disease activity index (ESSDAI).(28,29) Altogether these data suggest an important role of CD8 in orchestrating pSS immunopathology.

Previously, we and others have demonstrated increased CCL25 in mucosa-associated tissues, including salivary glands of pSS patients compared to non-Sjögren sicca (nSS) patients in conjunction with increased CCR9-expression (on CD4-positive and CD4-negative T cells).(14,15) Using CyTOF it was established that CD8 T cells are significantly increased in pSS patients with a lymphocytic focus score of 1 (LFS≥1) as compared to nSS patients without foci (LFS=0).(29) To the best of our knowledge previously no associations of CD8 T cells or CCR9+ CD8 T cells with B cell hyperactivity associated markers, including Tfh cells, in pSS and nSS patients have been reported. Given the pivotal role of CD8 T and CCR9+ T cells in Sjögren-like disease, the important proinflammatory role of CCL5 and CD8 T cells secreting CCL5, and the fact that CD8 T cells can display features of Tfh cells, in this study we investigated the role of CD8 T cells, and in particular CCR9+ CD8 T cells, in CCL5 secretion in pSS. To this end we studied the association of CD8 T cells with Tfh-associated lymphocytic foci. In addition, we studied the frequency of CCR9+ CD8 T cells in pSS patients versus HC, CCL5 expression and secretion of CD8 T cell subsets in pSS versus HC and CCL5 release by CD8 T cells as compared to CD4 T cells.

Methods

Patients and healthy controls

As previously described (30), for assessment of inflammatory cells, including CD8 T cells by epigenetic cell counting, salivary gland tissue was collected from n=13 non-Sjögren sicca (nSS) patients and n=29 pSS patients. For flow cytometry n=7 pSS patients and n=13 age and sex matched healthy controls (HC) were included. All patients were diagnosed by a rheumatologist. The pSS patients all met the 2016 ACR-EULAR criteria.(31) The nSS patients were defined as patients with sicca complaints, who do not fulfill the classification criteria, do not have a connective tissue disease and are anti-SSA negative, with LFS=0. All patients and healthy controls were included in the University Medical Center Utrecht (UMC Utrecht). The UMC Utrecht Medical Research Ethics Committee (METC) approved the study (reference 14/589 for usage of surplus salivary gland tissue and reference 13/697 for work with peripheral blood). All participants gave written informed consent. Demographic and clinical data are presented in Table 1.

	Epigenetic cell counting		Flow cytometry	
	nSS (n=13)	pSS (n=29)	HC (n=13)	pSS (n=7)
Female, n (%)	10 (77)	26 (90)	13 (100)	7 (100)
Age, years	47 ± 14	53 ± 13	52 ± 11	55 ± 9
Anti-Ro/SSA positive, n (%)	0	20 (69)	-	5 (71)
Anti-La/SSB positive, n (%)	0	9 (31)	-	3 (43)
ANA positive, n (%)	4 (31)	20 (69)	-	5 (71)
Lymphocytic focus score (foci/4mm²)	0	2.3 ± 1.4	-	2.7 ± 1.5
IgA positive plasma cells (%)	75 ± 14	49 ± 16	-	51 ± 20
Schirmer (mm/5min)	4.8 ± 7.1	6.2 ± 6.7	-	0.3 ± 0.4
Serum IgG (g/L)	11.0 ± 2.9	16.3 ± 8.1	-	12.8 ± 2.8
ESSDAI score (0-123)	-	3.9 ± 3.2	-	4.1 ± 3.0
ESSPRI score (0-10)	-	4.7 ± 2.2	-	6.3 ± 1.1
Immunosuppressants, n	3	8	-	2
Hydroxychloroquine, n	3	6		1

Table 1 | Participants' characteristics. Mean ±SD are shown unless stated otherwise. nSS: non-Sjögren sicca; pSS: primary Sjögren's syndrome; HC: healthy controls; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index

Epigenetic cell counting

Epigenetic cell counting was performed by Epiontis GmbH (Berlin, Germany), full methodology was as previously reported.(30,32) In brief, epigenetic cell counting quantifies cell frequencies based on cell-specific methylome markers of cellular DNA. Using bisulfite converted DNA as substrate, qPCR-assays were performed for the selected cell type-specific demethylated loci and for a locus known to be demethylated in all cell types (GAPDH). For the present analyses, data of epigenetic-based cell counts are presented as the percentage of cell-specific demethylation divided by GAPDH locus demethylation within salivary gland tissue DNA samples and multiplying that ratio by 100.(32) Thus, CD3 T cells, B cells, CD4 and CD8 T cells, as well as regulatory T cells, Tfh and Th17 cells were assessed.

Flow cytometry

Fresh peripheral blood mononuclear cells (PBMCs) from Lithium-heparinized blood were isolated using Ficoll density gradient centrifugation. Fresh PBMCs were first stained with fixable viability dye eF780 (eBioscience) for 10 minutes at 4°C and after washing the cells were incubated with fluorochrome-conjugated antibodies against CD3, CD4, CD8, CCR9, CCL5, CD27, CD45RO, IFN-γ and TNF-α for 25 minutes at 4°C (details of used antibodies are listed in Supplementary table 1. Gating strategies are shown in Supplementary Figure 1). Intracellular staining of CCL5, IFN-γ and TNF-α was performed according to the Fixation-Permeabilization protocol from the manufacturer (eBioscience) after 4 hours incubation with phorbol myristate acetate (PMA), ionomycin and Brefeldin A. All samples were measured on a BD LSRFortessa using DIVA software version 8.0.1. Analysis of the obtained FCS files was performed using FlowJo™ Software (BD Life Sciences). It was previously shown that CCL5 release is not blocked by Golgi complex blockade, such as Brefeldin A and subsequently reduced expression is a consequence of increased release. (24,25) A medium control was taken along to calculate CCL5 release. The release was defined by the difference between CCL5 expression in the medium condition minus CCL5 expression after stimulation (deltaCCL5).

Statistical analysis

Mann-Whitney U test (for comparison between pSS and HC) and Wilcoxon-non parametrical paired test were used. For correlations Pearson's correlation and Spearman's rho were used where appropriate. Data were analyzed using FlowJo™ Software, Graphpad Prism 8, IBM SPSS Statistics 26. Statistical significance was considered for differences at $p \leq 0.05$.

Results

CD8 T cells are abundantly present in salivary glands in pSS and correlate with lymphocytic foci and Tfh cell numbers

We performed new analyses on epigenetic cell counting data of tissue DNA from labial salivary gland tissue from nSS and pSS patients. So far we have not been able to assess CCR9-specific CD8 T cell counts in tissue due to technical limitations. However, we were able to assess percentages of CD8 T cells, which were significantly higher in pSS patients compared to nSS patients (Figure 1A). Furthermore the percentage of CD8 T cells in the salivary gland tissue significantly correlated with the lymphocytic focus score (LFS), a hallmark clinical parameter in pSS (Figure 1B). Also, CD8 T cell numbers significantly correlated with Tfh cell numbers (Figure 1C). Interestingly, significant correlations were also found between CD8 T cell and both B cell numbers (Spearman's rho=0.44 p<0.01) and serum IgG (Spearman's rho=0.45, p<0.01) (Supplementary Figure 2). Finally, increased CD8 T cell numbers correlated with reduced percentages of IgA found in the salivary gland tissue which is a consequence of increased IgG and IgM-producing plasma cells (Spearman's rho=-0.41, p<0.01, Supplementary Figure 2).

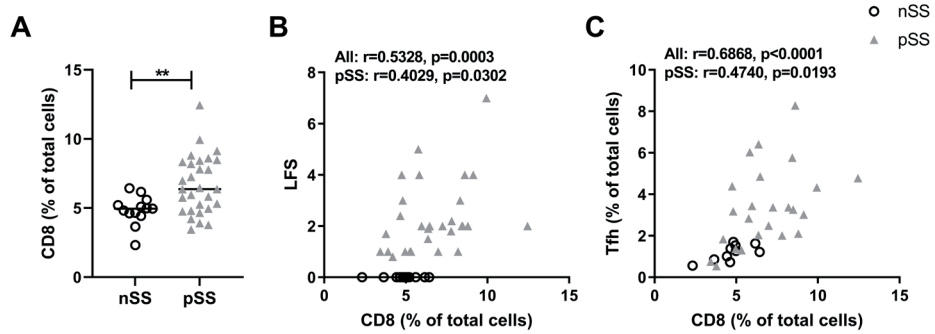

Figure 1 | CD8 T cells are abundantly present in salivary glands in pSS and correlate with lymphocytic foci and T follicular helper cell numbers. Epigenetic cell counting from labial salivary gland tissue DNA was used to determine (A) the percentage of CD8 T cells in nSS versus pSS patients. (B) Spearman's correlation between CD8 T cell percentages and lymphocytic focus scores (LFS). (C) Spearman's correlation between CD8 T cell percentages and Tfh cell percentages. nSS: non-Sjögren sicca; pSS: primary Sjögren's syndrome; LFS: lymphocytic focus score; Tfh: T follicular helper

CD8 effector T cells, in particular CCR9+ CD8 effector T cells, express high levels of CCL5 that is reduced in pSS patients as compared to healthy controls

After confirming the local abundance of CD8 T cells in salivary gland tissue of pSS patients we next determined the expression of CCL5 in circulating CD8 and CD4 T cells. We found higher expression of CCL5 in CD8 T cells compared to CD4 T cells (Figure 2A). In addition, increased percentages of CCR9+ CD8 T cells were observed in pSS patients compared to HC (Figure 2B). pSS patients with anti-SSA-antibodies in particular had higher percentages of CCR9+ CD8 T cells compared to HC (median 4.7 [IQR 3.4 – 5.9] for pSS patients and median 1.6 [IQR 1.3 – 3.8] for HC, p=0.03), which was not observed for SSA- patients (median 3.6 [IQR 3.5-3.7]). In this relative small cohort no significant correlations of CCR9+ CD8 T cells with ESSDAI, sIgG, and LFS were found.

CCR9+ CD8 T cells expressed higher levels of CCL5 compared to CCR9- CD8 T cells. When analyzing total CD8 T cells no significant difference was seen in CCL5 expression in pSS versus HC (Figure 2C). However, when analyzing CD27/CD45RO-defined cell compartments memory and effector CD8 T cells from pSS patients were shown to have significantly reduced CCL5 expression as compared to HC (Figure 2D shows the results for CCR9+ CD8 T cells, and Supplementary Figure 3 the results for CCR9- CD8 T cells and CD8 total).
Both in HC and pSS patients the CCL5 expression is increased in antigen-experienced cells. CCR9+ effector CD8 T cells express the highest level of CCL5 (compared to naive, central memory and effector memory cells, Figure 2E). Similar results are found when defining effector profile based on IFN-γ/TNF-α co-expression. A significantly higher expression of CCL5 is found in IFN-γ+TNF-α+ (double positive) CCR9+ CD8 T cells as compared to IFN-γ-TNF-α- (double negative) CCR9+ CD8 T cells. Just as for CD27/CD45RO-defined effector cells, IFN-γ+TNF-α+ (double positive) CCR9+ CD8 T cells from pSS patients express significantly less CCL5 compared to HC (Figure 2F).

Effector (memory) cells have the highest capacity to release CCL5 in HC and this release is reduced in pSS patients

In order to evaluate CCL5 release we compared the medium condition to 4 hours stimulation with PMA/ionomycin. Upon this stimulation CD8 T cells express less CCL5 compared to unstimulated cells (Figure 3A). In HC a significant difference was seen in CCL5 release between CCR9- and CCR9+ CD8 T cells in all subsets except for the effector memory subset. CCR9+ effector CD8 T cells released most CCL5 compared to the other subsets. Associated with lower CCL5 expression levels in pSS memory and effector CD8 T cell subsets (as compared to HC - Figure 2D), these differences in CCL5 release were not seen for the pSS patients. In contrast, naive CD8 T cells that did not significantly differ in CCL5 expression from HC ex vivo, showed significantly different CCL5 release upon stimulation in both HC and pSS patients (Figure 3B).

No significant differences in CCL5 release from naive and central memory CD8 T cell subsets were found between pSS patients and HC. From effector memory and effector CD8 T cells from pSS patients less CCL5 release was detected than from HC (Figure 3C).

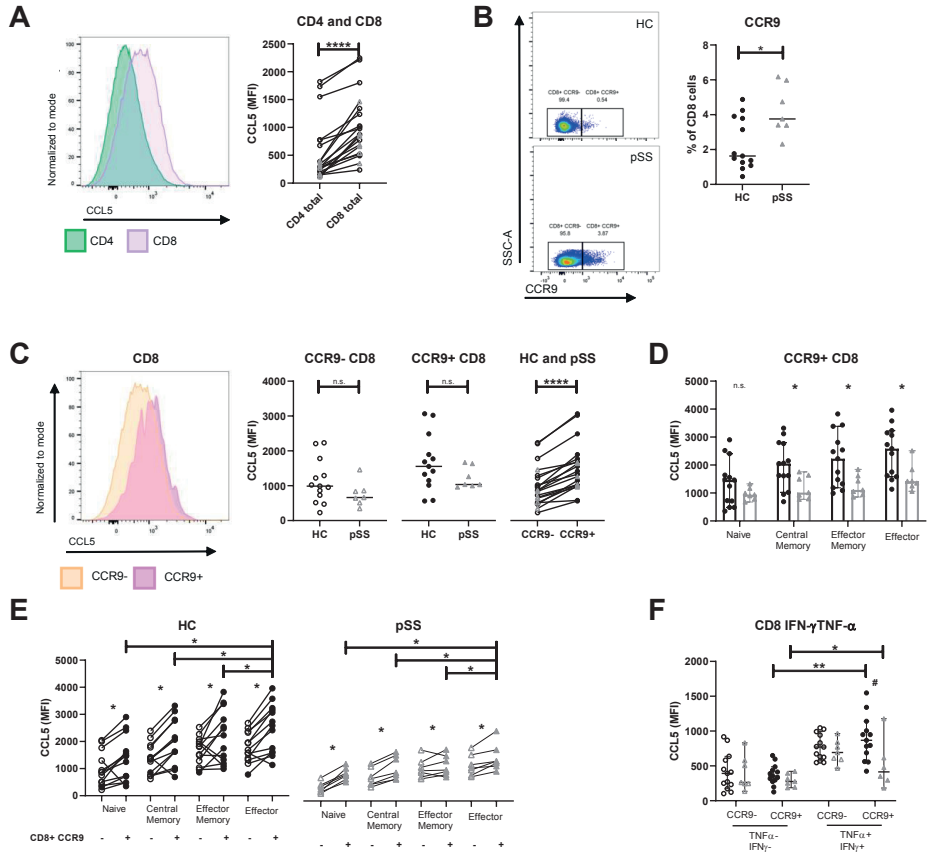

Figure 2 | CCL5 expression is highest in CCR9+ CD8 T cells, of which pSS patients have significantly higher cell numbers. Memory and effector CCR9+ cells from pSS patients express reduced CCL5 levels compared to HC. Flow cytometric analyses comparing for HC (n=13) and pSS patients (n=7). (A) CCL5 expression on CD4 versus CD8 T cells. (B) Percentage of CCR9-expressing CD8 T cells from PBMCs of HC and pSS (representative plots of CCR9 expression from a HC and a pSS patient). (C) CCL5 expression compared for CCR9- and CCR9+ CD8 T cells in HC and pSS patients and between CCR9- and CCR9+ CD8 T cells. (D) CCL5 expression in CD27/CD45RO-defined cell subsets within the CCR9+ CD8 T cells. MFI depicted as median with 95% confidence interval. CCL5 expression for HC and pSS comparing CCR9- and CCR9+ CD27/CD45RO-defined CD8 T cell subsets (E) and in IFN-γ/TNF-α double negative and double positive CCR9- and CCR9+ T cells (F). Representative histograms show CCL5 expression normalized to mode. Naive: CD27+CD45RO-; Central Memory: CD27+CD45RO+; Effector Memory: CD27-CD45RO+; Effector: CD27-CD45RO-. MFI: mean fluorescence intensity. pSS: primary Sjögren's syndrome; HC: healthy controls; PBMCs: peripheral blood mononuclear cells. n.s.: not significant. *, **, ***, **** indicates statistical significance of p<0.05, 0.01, 0.001, 0.0001, respectively. # indicates significant difference with p<0.05 between pSS and HC in figure 2F.

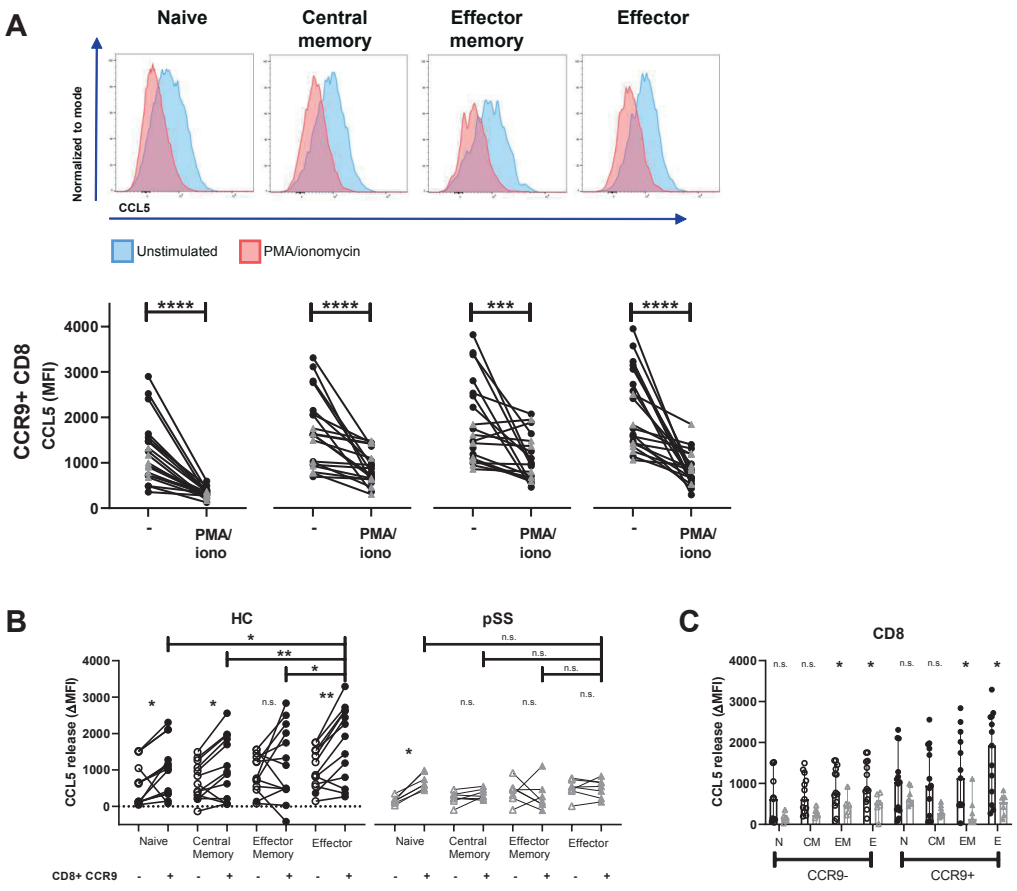

Figure 3 | Effector (memory) cells of pSS patients release less CCL5 after stimulation. CCR9+ CD8 T cells from HC release more CCL5 than CCR9- CD8 T cells, which is highest in effector cells. CD27/CD45RO-defined CD8 T cell subsets from n=13 HC and n=7 pSS were analyzed using flowcytometry. (A) Representative histograms show CCL5 expression (normalized to mode) of unstimulated (blue) versus PMA/iono stimulated (red) cells with below quantification for CCR9+ CD8 T cells. (B) Difference in CCL5 expression (ΔMFI CCL5) between unstimulated and stimulated CD8 T cells. Graph shows differences in CCR9- and CCR9+ CD8 T cell subsets, both for HC and for pSS patients. (C) Bar plot showing differences between HC and pSS in ΔMFI CCL5 in CCR9- and CCR9+ CD8 T cell subsets. Naive (N): CD27+CD45RO-; Central Memory (CM): CD27+CD45RO+; Effector Memory (EM): CD27-CD45RO+; Effector (E): CD27-CD45RO-. MFI: mean fluorescence intensity. ΔMFI: deltaMFI (CCL5 difference of 4h unstimulated-4h stimulated); pSS: primary Sjögren's syndrome; HC: healthy controls; PMA/iono: phorbol myristate acetate/ionomycin. *, **, ***, **** indicates statistical significance of p<0.05, 0.01, 0.001, 0.0001, respectively.

CCR9- and CCR9+ CD8 T cells release CCL5 equally effective and rapidly, but CCR9+ CD8 T cells release significantly more CCL5 within 10 minutes than CCR9+ CD4 T cells

To assess the pace of release we compared ex vivo CCL5 expression with 10 minutes and 4 hours of PMA/ionomycin stimulation. This showed neither relative differences between CCR9- and CCR9+ CD8 T cells, nor differences between pSS patients and HC (Figure 4AB), but when looking at mean fluorescence intensities (MFI) CCR9+ CD8 T cells displayed the highest release (median MFI decrease 419 and 995 for CCR9+ CD8 T cells versus 258 and 278 for CCR9- CD8 T cells at 10 minutes and 4 hours respectively).

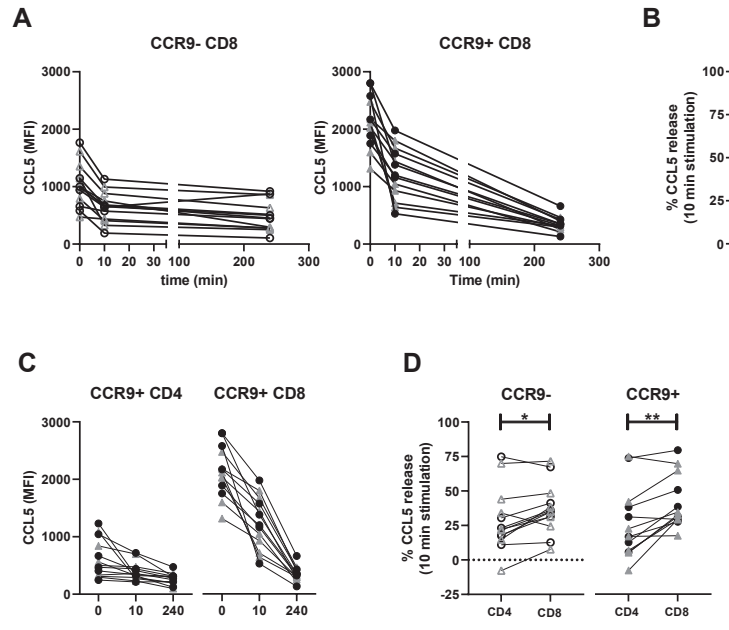

Figure 4 | Rapid relative but not absolute CCL5 release is comparable between HC and pSS. CCR9+ CD8 T cells release more CCL5 than CCR9+ CD4 T cells. For n=6 HC and n=7 pSS the MFI CCL5 was determined at t=0, t=10 minutes and t=4 hours. (A) Graphs showing MFI CCL5 over time for both CCR9- and CCR9+ CD8 T cells. (B) Comparison of CCL5 release after 10 minutes stimulation with phorbol myristate acetate (PMA) and ionomycin between HC and pSS patients in CCR9- and CCR9+ CD8 T cells. (C) Graphs depicting timepoints t=0, t=10m and t=4h (unscaled) for CCR9+ CD4 T cells and CCR9+ CD8 T cells combining HC and pSS patients. (D) difference between CD4 and CD8 T cells in CCL5 release after 10 minutes stimulation for CCR9- and CCR9+ cells. MFI: mean fluorescence intensity. pSS: primary Sjögren's syndrome; HC: healthy controls; n.s.: not significant. *, **, ***, **** indicates statistical significance of p<0.05, 0.01, 0.001, 0.0001, respectively.

When comparing with CCR9+ CD4 T cells also no differences in CCL5 release were found between pSS and HC (Figure 4C). Yet, there was a difference in release within 10 minutes, with CCR9+ CD8 T cells releasing significantly more CCL5 than CCR9+ CD4 T cells (Figure 4D, median 33% [IQR 29-51] and 17% [IQR 7-38], respectively). This corresponds to a decrease in median MFI of 419 in CCR9+ CD8 T cells versus a decrease of 68 in CCR9+ CD4 T cells.

Discussion

This study demonstrates that CD8 T cells are elevated in the salivary gland tissue of pSS patients compared to nSS patients and that their numbers are correlated to the lymphocytic aggregates and the number of Tfh cells and B cells. Activation induces release of CCL5, which is reflected by reduced intracellular CCL5 levels. CD8 T cells secrete higher CCL5 levels than CD4 T cells, with the highest levels found in and secreted by effector CD8 T cells, in particular those cells expressing CCR9. The frequency of CCR9+ CD8 T cells is increased in pSS patients. CCL5 expression and subsequently release of CCL5 upon in vitro activation was higher in circulating CD8 T cells from healthy controls than pSS patients.

Using epigenetic cell counting we found elevated numbers of CD8 T cells in salivary gland tissue of pSS patients compared to nSS patients. The CD8 T cell presence in lymphocytic infiltrates of pSS patients has been implicated in salivary gland dysfunction, possibly by inducing apoptosis of acinar epithelial cells.(33) Many of the CD8 T cells in the salivary glands of pSS patients are considered Trm cells, expressing both CD103 and CD69.(4,33) Whereas CD8 Trm cells could serve a “traditional” CD8 direct cytotoxic role, it has also been suggested that they rather regulate local inflammation by producing cytokines, but are not terminally differentiated and can e.g. gain properties fitting to memory T cells from the circulation.(34,35) Another indication towards a more orchestrating CD8 function is found in “Tfh-like” CD8 T cells as have been described in Hodgkin lymphoma. CXCR5+ICOS+ CD8 T cells were found secreting cytokines such as IL-4, IL-21 and CXCL13, whereas little to no IFN-γ, perforin or Granzyme B was secreted.(27) In our data we find significant correlations between CD8 T cell numbers and Tfh and B cell numbers in the salivary glands, the lymphocytic focus score and percentage of IgA+ cells in the lymphocytic aggregate and serum IgG levels. Although we cannot discriminate whether CD8 T cells, e.g. by secretion of CXCL13, contribute to formation of the foci, the strong cytokine producing potential in this study strongly suggests that they could play a key role.
Earlier work in our group has shown that CCR9+CXCR5- “Tfh-like” CD4 T cells form a distinct group of cells, which show a potent proinflammatory phenotype and have an increased frequency in peripheral blood and salivary gland tissue of pSS patients.(15) Chemokine CCL5 was identified as a potential target, and was highest expressed by effector CCR9+ CD4 T cells.(17) CD8 T cells, however, are described as a more potent producer of CCL5(36,37): a finding we confirmed in this study for both HC and pSS patients. We found that CCR9+ CD8 T cells and particularly those with an effector (memory) profile have the highest CCL5 levels. As prolonged T cell stimulation reduces CCR9 expression, we have experienced difficulties in finding CCR9 expression and mapping CCL5, because of its rapid release is challenging. Hence, the expression and release of CCL5 by (CCR9-expressing) CD8 T cells in the inflamed glands remains to be established. However, in juvenile idiopathic arthritis (JIA) isolated synovial CD8 T cells were found to rapidly release high levels of CCL5 upon TCR stimulation, without new protein synthesis, and synovial CD8 T cells showed high levels of CCL5 protein and increased CCL5 mRNA as compared to CD8 T cells from peripheral blood.(38) In analogy to rheumatoid arthritis (RA) synovial fluid T cells, which were shown to be almost completely antigen-experienced memory/effector cells(39), this suggests that in the salivary glands of pSS patients the results we have found in blood will be even more pronounced. In support of this we demonstrated that effector CD8 T cells were rapid and high CCL5 producers.

Although CCR9+ effector CD8 T cells expressed the highest CCL5 levels, also in all other CD27/CD45RO-defined CD8 T cell subsets a significantly higher CCL5 expression was found in CCR9+ cells as compared to CCR9- CD8 T cells, which we previously also demonstrated for CD4 T cells.(17) Since CCR9 is a chemokine receptor that initiates migration of T cells towards CCL25 under both physiological and inflammatory conditions and CCL5 is a chemokine that recruits several types of leukocytes to inflamed sites, the high expression of CCL5 in CCR9-expressing cells indicates that facilitating recruitment of other inflammatory cells is a main feature of these cells in immunopathology.

Between pSS patients and controls several significant differences in CCL5 expression and release were found. Memory and effector CCR9+ CD8 T cells from pSS patients have a significantly lower CCL5 expression than from HC. Also, the subsequent release of CCL5 by these cells is lower in pSS patients than in controls. However, since after 10 minutes of stimulation the relative release of CCL5 is similar between pSS patients and controls, this indicates that CCR9-expressing cells in pSS patients did not lose the ability to quickly release CCL5. Together, these results could indicate that in the proinflammatory environment of pSS some CCL5 is already released by CD8 T cells, yet these cells retain their ability to release CCL5 and are not exhausted. Together these findings match the increased levels of CCL5 in pSS patients. One study has found elevated serum CCL5 in pSS patients with an elevated ESR.(40) In addition, several studies have shown increased expression of CCR5 by pDCs, cDC2s, and CCR9+ CD4 T cells.(17,20) Both CCL5 and CCR5 were shown to be increased in salivary glands of pSS patients and thus increased release of CCL5 by CD8 T cells could play a key role in regulation of chemotaxis and subsequent inflammation.(21,22)

This is supported by the fact that blockade of CCL5 in inflamed glands halts Sjögren-like disease and that blockade of CCL5 by CD8 T cells halts experimental arthritis.(23,26)

What exactly triggers the release of CCL5 is unknown, but several autoantigens have been suggested to initiate T cell activation in pSS and could trigger CCL5 release.(41) In addition, several viruses such as EBV and HTLV-1 have been demonstrated in pSS and could trigger inflammation.(42–44) Additionally, induction of cytokines such as IL-7 and IL-15, which are known to be upregulated in the glands of pSS patients, could induce cytokine-activated T cells and trigger release of CCL5. Such cytokines could arise upon innate triggering or following stimulation by type I and type II IFNs.(45,46) Our data show that increased expression of CCL5 could arise from increased frequencies of effector T cells co-expressing IFN-γ and TNF-α, and specifically those co-expressing CCR9. The rapid release of CCL5 by CD8 T cells in all cases could play an important role in the initiation of the immunopathology in pSS.

Besides differences in CCL5 expression also the difference in CCR9+ CD8 T cell numbers stands out. Just as for CCR9+ CD4 T cells, increased percentages of CCR9+ CD8 T cells were observed in pSS patients compared to healthy controls. To the best of our knowledge this is the first paper demonstrating that CCR9-expressing CD8 T cell numbers are elevated in pSS patients. Considering the increased expression of CCL25 in the inflamed salivary glands this suggests that similarly to CCR9+ CD4 T cells the increased CCR9+ CD8 T cells could home to the glands. In addition, we found a very strong correlation between Tfh cells and CXCL13.(30) Interestingly, we recently also demonstrated co-expression of CCR9 and CXCR5 on a small percentage of circulating CD8 T cells (manuscript in preparation). This suggests that at least a proportion of CCR9+ CD8 T cells can migrate to the lymphocytic infiltrates. These cells through release of CCL5 could further stimulate inflammation.

Even though our study is performed on samples from a small number of participants, our results clearly indicate differences between pSS patients and controls, not only in frequency of CCR9-expressing CD8 T cells, but also in these cells' expression and release of CCL5. Given the well-described strong recruiting abilities of CCL5 and the potent anti-inflammatory effect of targeting CCL5, in particular CD8-produced in several experimental models, this might present a new factor that plays a key role in pSS immunopathology.

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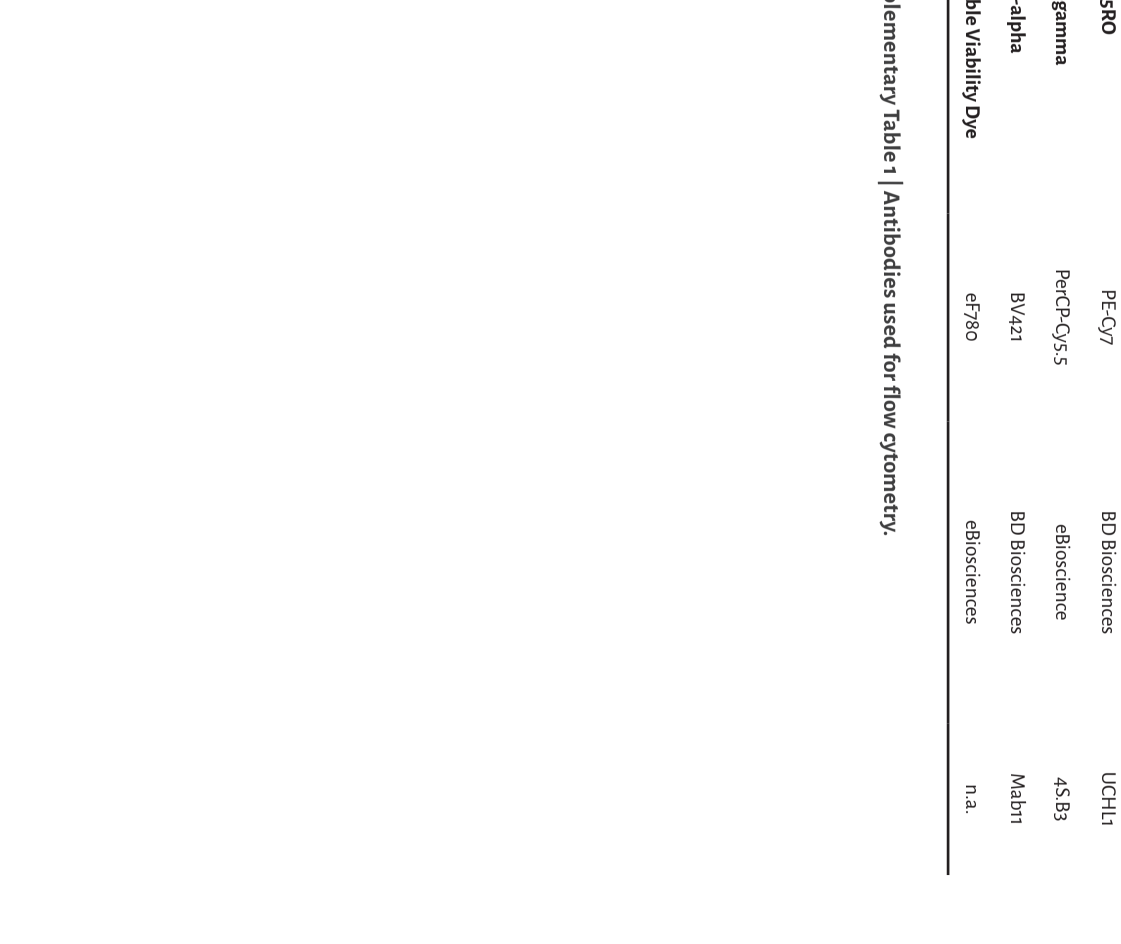
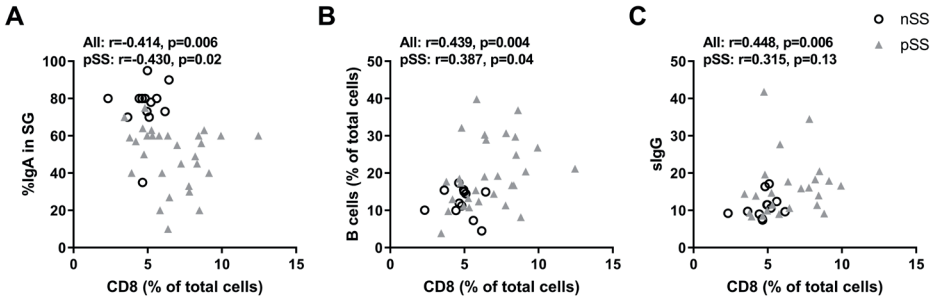
Supplementary Material

Marker	Fluorochrome	Company	Clone
CD3	AF700	Sony Biotechnology	UCHT1
CD4	BV785	Biolegend	RPA-T4
CD8	FITC	BD Biosciences	RPA-T8
CCR9	APC	Biolegend	L053E8
CCL5	PE	Biolegend	VL1
CD27	BV510	BD Biosciences	L128
CD45RO	PE-Cy7	BD Biosciences	UCHL1
IFN-gamma	PerCP-Cy5.5	eBioscience	4S.B3
TNF-alpha	BV421	BD Biosciences	Mab11
Fixable Viability Dye	eF780	eBiosciences	n.a.

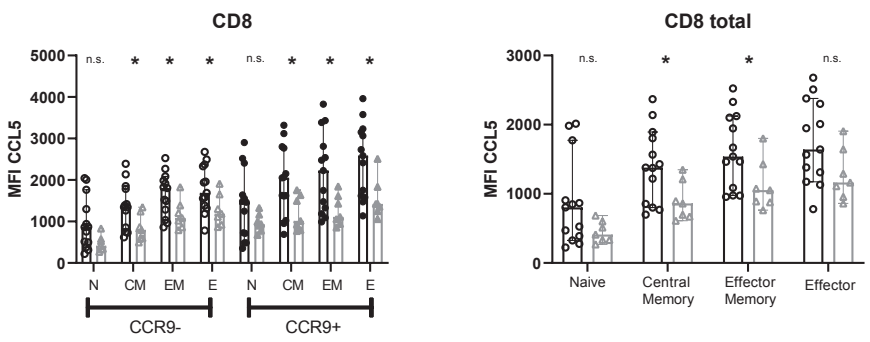
Supplementary Table 1 | Antibodies used for flow cytometry.



Supplementary Figure 1 | Gating strategy. Upper two rows show representative flow cytometry (intracellular) staining gating strategies of unstimulated cells; bottom two rows of PMA/ionomycin-stimulated cells. Both conditions shown are after 4 hours of culture. Top (first) row, panels left to right: forward versus side scatter (FSC vs SSC); selection of single cells, selection of live cells using fixable viability dye; selection of CD3+ cells, followed by selection of CD8+ single positive cells. Top second row: CD27/CD45RO staining of CD8+ cells, CCR9-expression on CD8+ T cells and intracellular IFN-γ/TNF-α expression in unstimulated CCR9- and CCR9+ CD8+ T cells. Bottom first row, from left to right: PMA/ionomycin-stimulated cells with FMO for both IFN-γ and TNF-α in CCR9- and CCR9+ CD8 T cells, with below the respective full stained samples. Bottom, colored plots, from left to right: dotplot overlay and below histograms of CCL5 expression in given subsets comparing full stain with FMO stain.



Supplementary Figure 2 | CD8 T cell number correlates to percentage IgA and B cell number in salivary gland tissue, and to serum IgG. Epigenetic cell counting from labial salivary gland tissue DNA was used to determine (A) Spearman's correlation between CD8 T cell percentages and percentage of IgA positive cells in salivary gland tissue. (B) Spearman's correlation between CD8 T cell percentages and B cell percentages. (C) Spearman's correlation between CD8 T cell percentages and level of IgG in serum (in g/L). nSS: non-Sjögren sicca; pSS: primary Sjögren's syndrome; SG: salivary gland; sIgG: serum IgG.



Supplementary Figure 3 | MFI CCL5 pSS vs HC in subsets. Flow cytometric analyses comparing for HC (n=13) and pSS patients (n=7). CCL5 expression in pSS patients and HC in CD27/CD45RO-defined cell subsets (A) within both CCR9- and CCR9+ CD8 T cells and (B) within CD8 total. MFI depicted as median with 95% confidence interval. Naive: CD27+CD45RO-; Central Memory: CD27+CD45RO+; Effector Memory: CD27-CD45RO+; Effector: CD27-CD45RO-.MFI: mean fluorescence intensity. pSS: primary Sjögren's syndrome; HC: healthy controls. n.s.: not significant. *, **, ***, **** indicates statistical significance of p<0.05, 0.01, 0.001, 0.0001, respectively.

Chapter 4

CCR9/CXCR5 co-expressing CD4 T cells are increased in primary Sjögren's syndrome and are enriched in PD-1/ICOS-expressing effector T cells

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Abstract

Objectives. Primary Sjögren's syndrome (pSS) is an autoimmune disease characterised by B cell hyperactivity. CXCR5+ follicular helper T cells (Tfh), CXCR5-PD-1^{hi} peripheral helper T cells (Tph) and CCR9+ Tfh-like cells have been implicated in driving B cell hyperactivity in pSS, however, their potential overlap has not been evaluated. Our aim was to study the overlap between the two CXCR5- cell subsets and to study their PD-1/ICOS expression as compared to "true" CXCR5/PD-1/ICOS-expressing Tfh cells.

Methods. CXCR5- Tph and CCR9+ Tfh-like cell populations from peripheral blood mononuclear cells of pSS patients and healthy controls (HC) were compared by flow cytometry. PD-1/ICOS expression from these cell subsets was compared to each other and to CXCR5+ Tfh cells, taking into account their differentiation status.

Results. CXCR5- Tph cells and CCR9+ Tfh-like cells, both in pSS patients and HC showed limited overlap. PD-1/ICOS expression was higher in memory cells expressing CXCR5 or CCR9. However, the highest expression was found in CXCR5/CCR9 co-expressing T cells, which are enriched in the circulation of pSS patients.

Conclusion. CXCR5- Tph and CCR9+ Tfh-like cells are two distinct cell populations that both are enriched in pSS patients and can drive B cell hyperactivity in pSS. The known upregulated expression of CCL25 and CXCL13, ligands of CCR9 and CXCR5, at pSS inflammatory sites suggests concerted action to facilitate migration of CXCR5+CCR9+ T cells, which are characterised by the highest frequencies of PD-1/ICOS positive cells. Hence, these co-expressing effector T cells may significantly contribute to the ongoing immune responses in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterised by lymphocytic infiltration of exocrine glands, and B cell hyperactivity as a hallmark of the disease. Increased B cell activity is often reflected by glandular B cell infiltration and increases in local IgM and IgG-producing plasma cells, autoantibody production, systemic hypergammaglobulinemia and in 5-10% of the patients B cell lymphoma development.(1–4)

Several CD4 T cell subsets have been described that can contribute to B cell hyperactivity. Follicular helper T cells (Tfh) are C-X-C chemokine receptor 5-expressing (CXCR5+) CD4 T cells that were shown to be key drivers of B cell hyperactivity in many inflammatory conditions, including pSS.(5–8) In more recent years, also several CD4 T cell subsets that lack CXCR5 expression (CXCR5-), such as peripheral T helper cells (Tph) and C-C motif chemokine receptor 9-expressing (CCR9+) follicular helper-like T cells (CXCR5-CCR9+ T cells or CCR9+ Tfh-like cells), were described with potent B cell activating features.(9,10) These cells were found to have Tfh-like activities in several inflammatory diseases including autoimmune diseases such as rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE).(9,11) The present study aimed to study the frequencies and phenotypic features of these three B cell activating cell types to reveal potential commonalities and dissimilarities.

Tfh cells are canonically defined as memory CD4 T cells, with expression of transcription factor Bcl6, surface expression of CXCR5, ICOS, and PD-1, and secretion of IL-21.(12,13) Even though cells defined in this way best resemble activated Tfh cells found in lymphoid structures, in some studies – including those studying Sjögren's syndrome – (memory) CXCR5+ CD4 T cells without extensive further phenotyping are pragmatically also considered Tfh cells.(7,14) Elevated frequencies of circulating PD-1/ICOS/CXCR5-expressing Tfh cells have been reported in pSS patients and were associated with increased B cell activity and autoimmunity in these patients.(5,15–19) In salivary glands of pSS patients CXCR5, CXCR5-expressing CD4 T cells, and the chemokine specifically mediating migration of CXCR5-expressing cells to the glands, CXCL13, are overexpressed.(7,20–27) Increased expression of CXCL13 is associated with increased lymphoid aggregates (foci) and increased organisation into ectopic lymphoid structures (ELS), and is subsequently associated with increased B cell hyperactivity and lymphoma development.(26–29) PD-1/ICOS/CXCR5-expressing Tfh cells, epigenetically quantified, were found to robustly correlate with lymphocytic focus scores, CXCL13 expression, and B cell hyperactivity.(24)

B cell activating Tph cells are defined as CXCR5-PD-1^{hi} memory CD4 T cells, and were found to be increased in the blood of pSS patients.(16,30,31) The cells do not express Bcl6, are PD-1^{hi}, can co-express ICOS and secrete high levels of CXCL13 and IL-21.(9,11,32) In pSS

patients Tph cells in peripheral blood are more abundant than in healthy controls (HC), and a correlation was found between CXCR5-PD-1^{hi} cells (not gated on memory) and plasmablasts, and rheumatoid factor positivity.(31)

CXCR5-CCR9+ Tfh-like cells are potent B cell activating cells, which produce high amounts of e.g. IL-21 and IFN-γ.(10,33) pSS patients have a higher frequency of CCR9+ Tfh-like cells in their circulation, and these cells frequently demonstrate an effector phenotype, e.g. with elevated PD-1/ICOS expression, high CD127 (IL-7Rα) expression, and IFN-γ and CCL5 production.(10,34) In addition, in salivary glands of pSS patients numbers of CCR9+ CD4 T cells are elevated, and CCL25, the cytokine mediating chemotaxis of CCR9+ cells, is overexpressed.(10) Increased CCL25 levels in secretomes of salivary gland tissue were associated with autoimmunity (SSA positivity), and increased B cell hyperactivity (serum IgG levels), and levels of IL-21 and soluble IL-7R.(25)

It is unknown to what extent overlap between Tfh, Tph, and CCR9+ Tfh-like cells exist. Also, the expression of typical Tfh/Tph/CCR9+ Th cell-associated markers PD-1 and ICOS on these cell subsets, including CXCR5/CCR9 co-expressing cells, has not been simultaneously studied on all these cell subsets. In this study we firstly evaluated the overlap of CCR9+ Tfh-like cells and Tph cells. Then we compared the expression of hallmark activation markers PD-1 and ICOS on the four different CXCR5/CCR9-defined cell subsets (Tfh, Tph, CCR9+ Tfh-like cells, and CXCR5/CCR9 co-expressing cells) in pSS patients and HC.

Methods

Participant inclusion

Peripheral blood mononuclear cells (PBMCs) were collected from n=12 pSS patients and n=11 age and sex matched healthy controls (HC). All pSS patients were diagnosed by a rheumatologist and met the 2016 ACR-EULAR criteria.(35) All participants were included in the University Medical Center Utrecht (UMC Utrecht). The Medical Research Ethics Committee (METC) of the UMC Utrecht approved the study (reference number 13/697). All participants gave written informed consent. An overview of the demographic and clinical data is shown in Table 1.

Flow cytometry

Fresh PBMCs from Lithium-heparinized blood were isolated using Ficoll density gradient centrifugation. After collection PBMCs were frozen and stored in liquid nitrogen until further use. PBMCs were thawed according to protocol and then stained with fixable viability dye eF780 (eBioscience) for 10 minutes at 4°C. Then the cells were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD8, CCR9, CXCR5, PD-1, ICOS, CD27, and CD45RO for 25 minutes at 4°C (details of used antibodies in Supplementary Table S1). Fluorescence minus one (FMO) controls were taken along to check marker expression. All samples were acquired on a BD LSRFortessa (BD Biosciences) using BD FACSDiva software v.8.0.1. (BD Biosciences). FlowJo™ v10.8 Software (BD Life Sciences) was used for data analysis.

Gating of PD-1^{hi} cells

For flow cytometric analysis of Tph cells memory CD4 T cells were gated on PD-1^{hi}. The PD-1^{hi} gate was determined based on the histogram of PD-1 staining in memory versus naïve T cells combined with gating based on the PD-1/ICOS plot in memory CD4 T cells (Supplementary Figure S1). This results in PD-1^{hi} gating in accordance with previously described gating by Rao et al. with approximately 1% of memory PD-1^{hi} CD4 T cells in HC.(9,36)

	HC (n=11)	pSS (n=12)
Female, n (%)	11 (100)	12 (100)
Age, years	55 (49-60)	59 (54-64)
Anti-Ro/SSA positive, n (%)		8 (67)
Anti-La/SSB positive, n (%)		4 (33)
ANA positive, n (%)		7 (58)
Lymphocytic focus score (foci/4mm²)		1.8 (1.1-2.4)
IgA positive plasma cells (%)		65 (40-81)
Schirmer (mm/5min)		0 (0-1)
Serum IgG (g/L)		13.6 (11.4-15.2)
ESSDAI score (0-123)		4 (2-5)
ESSPRI score (0-10)		6.3 (5.3-7.3)
Immunosuppressants use, n		3
Hydroxychloroquine, n		2

Table 1 | Participants' characteristics. Medians with interquartile range (Q1-Q3) are shown, unless specified otherwise. HC: healthy controls; pSS: primary Sjögren's syndrome; anti-Ro/SSA: anti-Ro/Sjögren's syndrome related antigen A antibody; anti-La/SSB: anti-La/Sjögren's syndrome related antigen B antibody; ANA: antinuclear antibody; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity score; ESSPRI: EULAR Sjögren's syndrome patient reported index.

Statistical analysis

Mann-Whitney U test (for unpaired analyses) and Wilcoxon non-parametrical paired test were used (for paired analyses). For correlations Spearman's rho was used. Data were analysed and visualised using IBM SPSS Statistics 26 and Graphpad Prism 8. Statistical significance was considered for differences at $p \leq 0.05$.

Results

In this study we defined Tph cells as CXCR5-PD-1^{hi} memory (CD45RO+), CCR9+ Tfh-like as CXCR5-CCR9+, and CCR9+ Tfh as CXCR5+CCR9+ memory CD4 T cells, as previously described (refs). CXCR5+CCR9+ co-expressing cells were not specified on memory phenotype. Tfh cells were defined as CXCR5+ memory, and "true" Tfh cells as CXCR5+PD-1+ICOS+ memory cells.

CXCR5-PD-1^{hi} Tph cells and CXCR5-CCR9+ Tfh-like cells are more abundant in pSS patients, but these cell types show little overlap in blood

From 12 female pSS patients and 11 age-matched female HC Tph cells and CCR9+Tfh-like cells were studied. In pSS patients the number of Tph cells was significantly increased compared to HC (median 0.53% versus 0.23% of CD4 T cells, p=0.049) (Figure 1A). Also the number of CCR9+ Tfh-like cells was significantly elevated in pSS patients as compared to HC, i.e. approximately 2.7% in pSS patients versus 1.7% in HC (medians, p=0.019) (Figure 1B). CCR9 expression was equally distributed between pSS patients and HC in Tph cells. Only a modest number of 2.1% (1.2-3.2%) of Tph cells express CCR9 (median with interquartile range) (Figure 1C). Enrichment of CCR9 in PD-1^{hi} and PD-1^{int} as compared to PD-1- was observed (Supplementary Figure S2). In summary, from CD4 T cells in peripheral blood of both pSS patients and HC 0.011% (0.007-0.019%) expressed CD45RO+CXCR5-CCR9+PD-1^{hi} (median with interquartile range, Figure 1D). Of this specialised cell subset a trend was seen for higher abundance in pSS patients (median values 0.009% and 0.013% for HC and pSS, respectively, p=0.12).

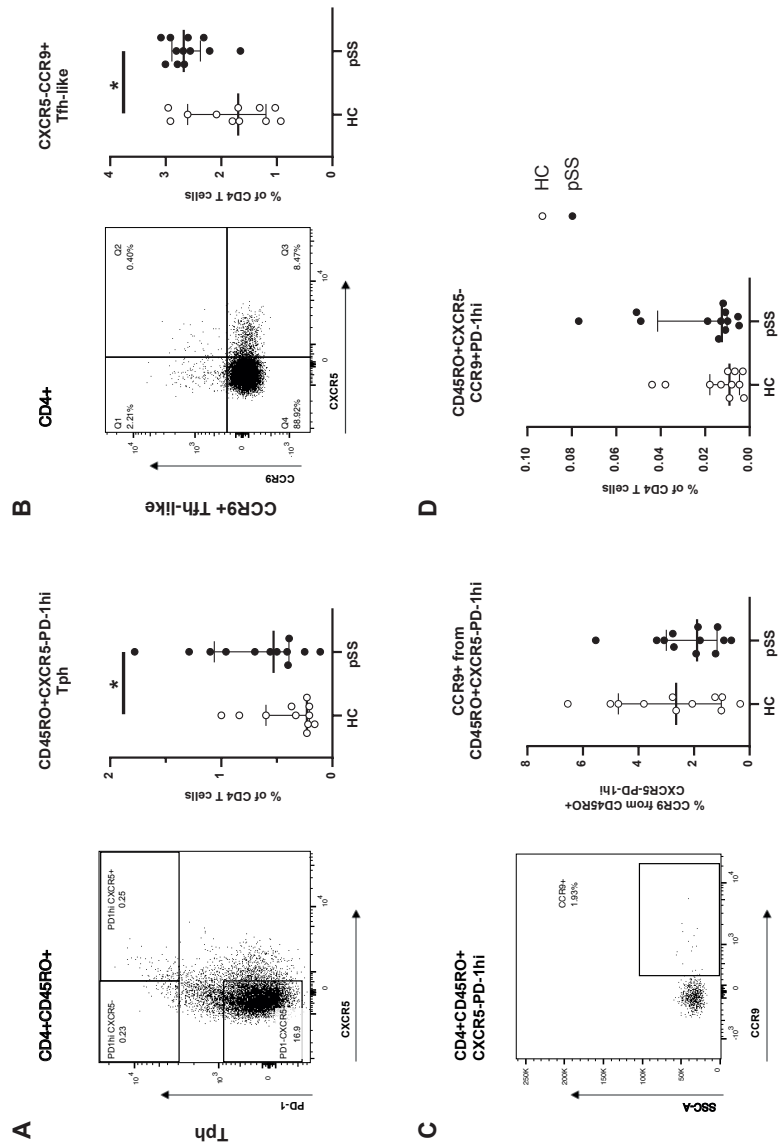

Figure 1 | Tph and CCR9+ Tfh-like cells in pSS patients and HC show minimal overlap. Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs). (A) Representative dot plot and quantification of circulating Tph cells (CXCR5-PD-1^{hi} memory CD4 T cells) in pSS patients and HC as percentages of CD4 T cells. (B) The frequency of CCR9+ Tfh-like cells in CD4 T cells in pSS patients and HC is shown. (C) The percentage of CCR9-expressing cells within Tph cells are depicted in a representative plot and quantified for all donors. (D) CXCR5-CCR9+PD-1^{hi} memory cells expressed as the frequency of total CD4 T cell population in pSS patients and HC. Plots show medians plus interquartile ranges. Memory is defined as CD45RO+. HC: healthy control; pSS: primary Sjögren's syndrome. Tph: T peripheral helper cell (memory CXCR5-PD-1^{hi} CD4 T cell); CCR9+ Tfh-like: C-C motif chemokine receptor 9-expressing T follicular helper-like cell (CXCR5-CCR9+ CD4 T cell) * p-value <0.05

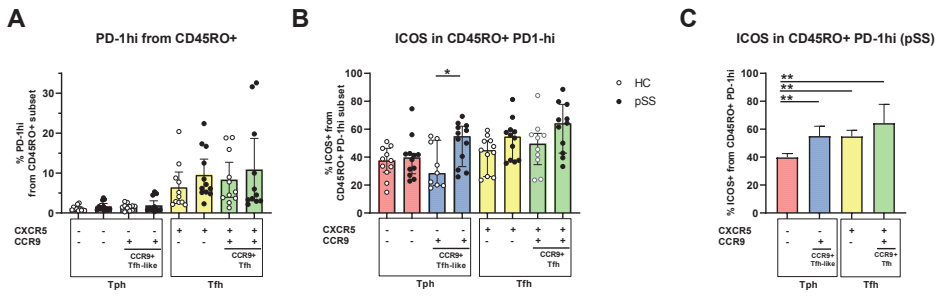

Figure 2 | CCR9 and CXCR5/CCR9 co-expressing memory PD-1^{hi} cells display increased ICOS expression. (A) The frequency of PD-1^{hi}-expressing cells in the memory (CD45RO+) compartment of CXCR5/CCR9-defined cell subsets for pSS patients and HC. (B) ICOS expression is shown of PD-1^{hi} memory subsets for pSS patients and HC. (C) Comparison of ICOS expression between the memory PD-1^{hi} CXCR5/CCR9-defined subsets in pSS. Plots show medians plus interquartile ranges. HC: healthy control; pSS: primary Sjögren's syndrome. Tph: T peripheral helper cell (memory CXCR5-PD-1^{hi} CD4 T cell); CCR9+ Tfh-like: C-C motif chemokine receptor 9-expressing T follicular helper-like cell (CXCR5-CCR9+ CD4 T cell); ICOS: inducible T cell co-stimulator; PD-1: programmed death-1; *, ** indicates p-value <0.05, 0.01, respectively.

PD-1^{hi} expression is highest in CXCR5+ memory cells, CCR9 and CXCR5/CCR9 co-expressing memory PD-1^{hi} cells show increased ICOS expression

After having determined that Tph cells and CCR9+ Tfh-like cells have limited overlap, and that Tph cells are mainly CXCR5-CCR9-PD-1^{hi} memory cells, we compared the number of PD-1^{hi} cells among all memory subsets and the expression of ICOS within PD-1^{hi} memory cell subsets.

The percentage of PD-1^{hi} memory cells was not different in pSS patients compared to HC. CXCR5+ (Tfh) cells are characterised by the highest frequency of PD-1^{hi} cells, either with or without CCR9 co-expression (Figure 2A, PD-1^{hi} expression did differ between CXCR5+ T cell subsets compared to CXCR5-CCR9- and CXCR5-CCR9+ T cells, both p<0.0001.) No difference in PD-1^{hi} expression was seen between CXCR5+CCR9- and CXCR5+CCR9+ cells (p=0.57).

Within the PD-1^{hi} populations, cells expressing either CXCR5 and/or CCR9 of pSS patients showed a trend of higher ICOS expression compared to HC, but this was only significant in CCR9+ Tfh-like cells (28.6% and 55%, medians for HC and pSS respectively, p=0.02, Figure 2B). Taking into consideration that CCR9+ Tfh-like cells have a low percentage of PD-1^{hi} cells, this implicates that the ratio between PD-1^{hi} and ICOS is quite different in these cells as compared to CXCR5+ cells (Figure 2B, Supplementary Figure S3A).

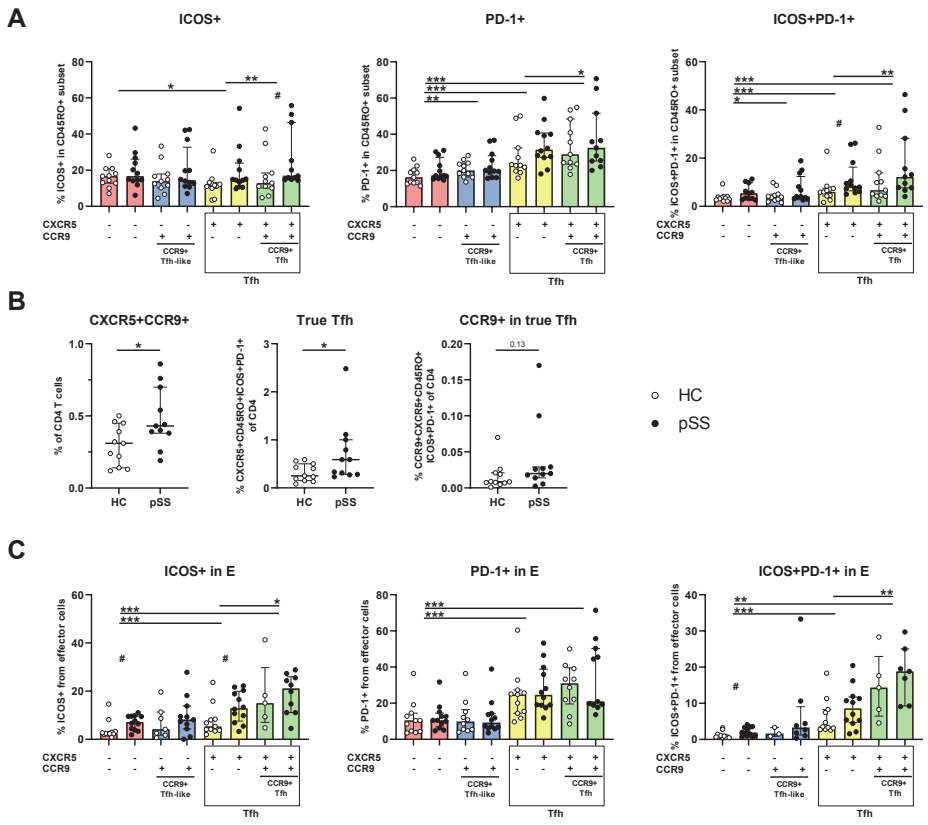

Figure 3 | CXCR5/CCR9 co-expressing memory and effector cells are enriched in PD-1+ICOS+ cells. (A) Expression of ICOS, PD-1 and PD-1+ICOS+ in memory (CD45RO+) CXCR5/CCR9-defined cell subsets in pSS patients and HC, comparing the CXCR5-CCR9- ("double negative" - DN) subset to the three other subsets and the CXCR5+CCR9+ ("double positive" - DP) subset to the CXCR5+CCR9- subset. (B) Abundance of CXCR5+CCR9+ T cells, true Tfh cells, and CCR9-expressing true Tfh cells from CD4 total in pSS patients and HC. (C) Expression of ICOS and PD-1 by CD27-CD45RO- effector cell subsets in pSS patients and HC, comparing the DN subsets with the other subsets and the DP subset with the CXCR5 single positive subset. Plots show medians plus interquartile ranges. HC: healthy control; pSS: primary Sjögren's syndrome. CCR9+ Tfh-like: C-C motif chemokine receptor 9-expressing T follicular helper-like cell (CXCR5-CCR9+ CD4 T cell); ICOS: inducible T cell co-stimulator; PD-1: programmed death-1; *, **, *** indicates p-value <0.05, 0.01, 0.001, respectively. # p-value <0.05 in comparison pSS vs HC (Mann Whitney test)

Given the pronounced upregulation of ICOS on CCR9+ Tfh-like cells we next compared ICOS expression between sion within the PD-1^{hi} subset was seen for CXCR5/CCR9 co-expressing cells (CCR9+ Tfh) in pSS patients (p=0.08, and p=0.06 compared to CXCR5+CCR9- and CXCR5-CCR9+ cells, respectively) (Figure 2C). Significantly increased ICOS expression in HC was only seen for CXCR5-expressing Tfh cells compared to CXCR5-CCR9- cells (Supplementary Figure S3B).

CXCR5/CCR9 co-expressing memory and effector cells are enriched for ICOS+PD-1+ cells, and are enriched in pSS

So far, in the above-mentioned analyses we adhered to the Tph definition of PD-1^{hi} memory cells. However, we next studied the expression of ICOS and PD-1 in CXCR5/CCR9-defined cells, since PD-1 expression has more frequently been used as a marker in cell phenotyping than PD-1^{hi} expression. For this we focused on memory cells to allow comparison of the CCR9 and CXCR5-defined subsets. In fact, memory and effector cell populations in our study showed an overall significantly increased PD-1 and ICOS expression as compared to naive cells (Supplementary Figure S4). While there was a trend for increased ICOS and PD-1 expression in pSS patients compared to HC for CXCR5-expressing cells, this was only significant for PD-1/ICOS co-expression in CXCR5+CCR9- cells, and ICOS expression of CXCR5+CCR9+ co-expressing cells (Figure 3A). When pooling patients and controls, in memory cells of the four different CXCR5/CCR9-defined subsets CXCR5-CCR9- ("double negative"- DN) cells showed the lowest expression of PD-1 and ICOS+PD-1+ (as compared to the three other subsets). ICOS expression was only significantly different in CXCR5+CCR9- cells as compared to DN cells (Figure 3A). Furthermore we wanted to evaluate if a difference was seen between the two Tfh cell subsets (i.e. CCR9- and CCR9+ memory CXCR5+ cells). PD-1, ICOS, and PD-1/ICOS co-expression was significantly higher on CCR9+ Tfh cells than on CCR9- Tfh cells (in all three comparisons $p<0.05$, Figure 3A).

As we established that higher expression of PD-1/ICOS was found on CXCR5/CCR9 co-expressing cells, we next evaluated if CXCR5+CCR9+ cell numbers were different in pSS patients compared to HC. Indeed, CXCR5+CCR9+ CD4 T cells were more abundant in pSS patients than in HC (Figure 3B). In addition, the number of true Tfh cells (CXCR5+ICOS+PD-1+ memory CD4) was also increased in pSS patients compared to HC ($p=0.03$, Figure 3B). Within these true Tfh cells 3.6% (2.9-6.8%) CCR9+ cells were observed (median with interquartile range). Calculated as percentages of CD4 T cells a trend was seen for higher CCR9-expressing true Tfh cells in pSS patients as compared to HC ($p=0.13$, Figure 3B).

In earlier work from our group CXCR5/CCR9-defined cells were not predefined on memory phenotype.(10,34) It was found that approximately up to 10% of CCR9+ Tfh-like cells has an effector phenotype (CD27-CD45RO-).(10) Given the potential important role of such cells in immunopathology, we also studied PD-1/ICOS expression within this effector subset. Similar to CD45RO+ memory cells, from the four CXCR5/CCR9-defined cell types CXCR5-CCR9- effector cells show lowest expression, and CXCR5+CCR9+ cells show highest expression of PD-1/ICOS (Figure 3C).

Increased numbers of CCR9+ Tfh-like cells and true Tfh cells are associated with autoimmunity

Finally, we evaluated if the four main cell subsets (Tph cells, true Tfh cells, CCR9+ Tfh-like cells, and CXCR5+CCR9+ T cells) might be associated with B cell hyperactivity or are associated to clinical parameters (ESSDAI, LFS, percentage IgA in minor salivary gland biopsy). In this small study population we did not find significant correlations of any of the subsets with ESSDAI or local inflammatory parameters. The percentage of both true Tfh cells and of CCR9+ Tfh-like cells from CD4 T cells was significantly higher in pSS patients with anti-SSA-antibodies as compared to HC (Figure 4).

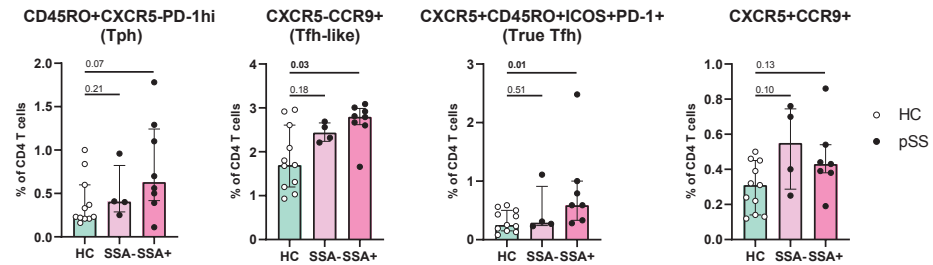

Figure 4 | Association of Tph, Tfh-like, true Tfh, and CCR9+ Tfh cell subsets with anti-SSA antibodies. Using PBMCs for flow cytometric analysis the abundance of each cell subset from CD4 T cells is depicted. HC in green, pSS patients in pink. Anti-SSA negative patients (SSA-) in light pink, and anti-SSA positive patients (SSA+) in dark pink. Plots show medians (interquartile range). HC: healthy control; pSS: primary Sjögren's syndrome; anti-SSA: anti-Sjögren's syndrome related antigen A antibody/anti-Ro. p-values shown are results of Wilcoxon non-parametrical paired test, with in bold significant differences.

Discussion

In this study we evaluated the frequency and phenotype of CCR9+ Tfh-like cells and Tph cells in peripheral blood to study the overlap between these populations in pSS. Typical markers associated with Tfh activity, PD-1 and ICOS, were assessed on these two CXCR5- cell subsets and on CXCR5+ Tfh cells. Our results show that the overlap between both memory CXCR5- populations is limited. From four CXCR5/CCR9-defined populations PD-1^{hi} expression was most pronounced on CXCR5+ cells, whereas ICOS expression is markedly higher in both CXCR5 and CCR9 single positive and double positive cells. Furthermore, we show that CXCR5+CCR9+ CD4 T cells are more abundant in pSS patients as compared to HC, and these cells are enriched in PD-1/ICOS expression in both the memory and effector cell subsets. Finally, we demonstrate that cell numbers of CCR9+ Tfh-like cells and true CXCR5+ memory PD-1+ICOS+ cells are increased in pSS patients with anti-SSA-antibodies when compared with HC.

The minimal overlap between Tph cells and CCR9+ Tfh-like cells was not anticipated, since earlier work from our group shows that CCR9+ Tfh-like cells express upregulated PD-1 levels, especially in pSS patients.(10) In addition, both cell subsets share B cell stimulating functional properties and are characterized by enrichment of IL-21 secreting cells, and elevated expression of ICOS, CCR5, and CD127.(9,10,34,36) This suggests that while functional properties may overlap, the chemokine receptor profile that CCR9+ cells acquire identify them as a separate population, indicating that previous reports studying Tph or CCR9+ cells have likely studied separate populations. The expression of CCL25 by inflamed epithelial cells could direct CCR9+ cells to the inflamed site.(37) CXCL13 expressed in e.g. germinal centers (GCs) or ectopic lymphoid structures (ELS) directs migration of CXCR5+ cells towards these specialized locations in the glands.(26,27)

It is unclear how chemotaxis of Tph cells is regulated but on RNA level increased expression of *CCR2*, *CCR3*, *CCR5*, *CXCR3*, *CX3CR1* and *CXCR6* in Tph cells as compared to CXCR5+ and PD-1- cells has been reported.(9) Ligands for several of these chemokine receptors, e.g. CCL5, CXCL10, and CX3CL1 (fractalkine) are elevated in pSS patients' salivary glands.(38–40) Hence it is very likely that these overexpressed chemokines can facilitate migration of Tph cells to the site of inflammation and contribute to immunopathology given their activated status.

Despite the fact that the CD4 T cell subsets described in the present study might be directed to specific areas in the inflamed tissues in pSS, they share functional properties such as ICOS and IL-21 expression. Hence, inhibiting these cell types might be achieved by targeting common surface-expressed proteins such as ICOS. In fact, all cell types studied here can be targeted by blocking ICOS/ICOSL interactions. Cells with ICOS expression can interact with cells expressing ICOSL (e.g. activated B cells). By blocking this pathway the stimulatory signal could be blocked, resulting e.g. in less release of IL-21, and blockade of activation and further differentiation of Tph, Tfh and CCR9+ Tfh-like cells, thereby preventing B cell hyperactivity.(41–43)

Our results in pSS fit to the RNA sequencing results from circulating memory CD4 T cells in RA patients (sorted based on PD-1^{hi} versus PD-1-, and CXCR5 expression), which indicated that the genes for ICOS and PD-1 (*ICOS* and *PDCD1* genes, respectively) were upregulated most strongly in CXCR5+PD-1^{hi} cells.(9) Our results show that in PD-1^{hi} cells from pSS patients PD-1/ICOS protein expression is the highest in CXCR5+CCR9+ Tfh cells. Of note, for *CCR9* gene expression a similar pattern was found with RNA sequencing, being most upregulated in CXCR5+PD-1^{hi} cells.(9) This is in line with earlier findings from our group showing that *ICOS* and *PDCD1* gene expression are highest in CXCR5+CCR9- CD4 T cells and lowest in CXCR5-CCR9- T cells.(34) Unfortunately, CXCR5+CCR9+ T cells were not studied here.

In the present study we confirmed findings of earlier studies showing that the abundance of Tph cells, CCR9+ Tfh-like cells, and true Tfh cells are elevated in pSS patients as compared to HC. In addition, we show that the number of CD4 T cells co-expressing CXCR5/CCR9, is elevated in pSS patients. These CXCR5+CCR9+ T cells are enriched for PD-1/ICOS expression, particularly effector cells. Both CXCL13 and CCL25 are overexpressed in pSS and associated with B cell hyperactivity, presence of autoantibodies, and inflammatory mediators such as IL-21 and IFN-γ.(10,26,27) Given the tight connection between PD-1/ICOS co-expression and effector functions such as IL-21 secretion, this seems to indicate that even though these CXCR5/CCR9 chemokine receptor co-expressing cells form a small subset, these cells have very potent B cell activating potential. Their chemokine receptor co-expression indicates these cells are primed to traffic to inflamed glands, and to interact with B cells in GCs. Future research should further determine functional properties of CXCR5+CCR9+ cells, preferably both in matched samples from circulation and salivary gland tissue.

Despite implications for further research, our study does have some limitations. As a study that started out as an evaluation of overlap between Tph cells and CCR9+ Tfh-like cells we included a small study population, indicating that definite conclusions about associations with clinical features are difficult to draw. In addition, we only performed phenotypic evaluation of peripheral blood samples and did not evaluate tissue samples. Nonetheless, we and others in previous studies have indicated enrichment of CCR9, CXCR5 and PD-1-expressing Th cells at inflammatory sites, including the inflamed glands of pSS patients.(9,10,24,33) Given the fact that the circulating cell populations we studied largely included memory/effector T cells that are known to home to inflammatory sites our data might give important clues for their role in pSS immunopathology.

In conclusion, our study demonstrates little overlap between Tph cells and CCR9+ Tfh-like cells, and that CXCR5+CCR9+ memory and especially effector CD4 T cells are enriched in PD-1/ICOS expression. Besides Tfh cells and the two more recently described CXCR5- cell subsets, i.e. Tph cells and CCR9+ Tfh-like cells, these CXCR5/CCR9 co-expressing cells may play a significant role in B cell hyperactivity in pSS.

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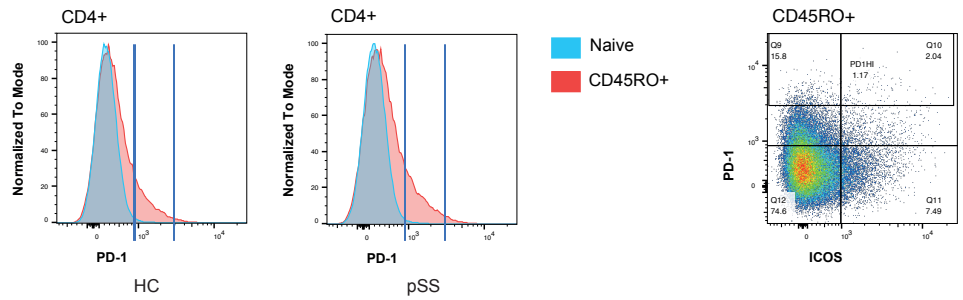
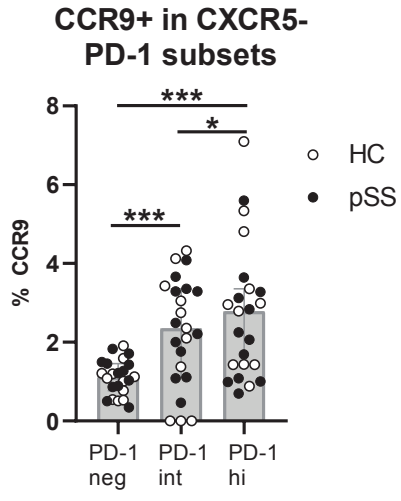
Supplementary Material

Marker	Fluorochrome	Company	Clone
CD3	AF700	Sony Biotechnology	UCHT1
CD4	BV785	Biolegend	RPA-T4
CD8	PE	Biolegend	RPA-T8
CCR9	APC	Biolegend	L053E8
CXCR5	PerCP-Cy5.5	Biolegend	J252D4
CD27	BV510	BD Biosciences	L128
CD45RO	PE-Cy7	BD Biosciences	UCHL1
ICOS	FITC	Biolegend	C398.4A
PD-1	BV711	BD Biosciences	EH12.1
Fixable Viability Dye	eF780	eBioscience	n.a.

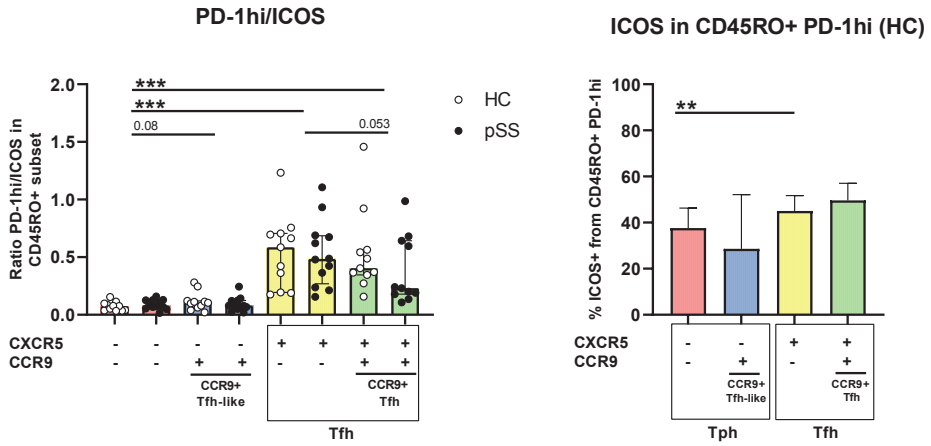
Supplementary Table S1 | Antibodies used for flow cytometry.



Supplementary figure S1 | PD-hi staining. Representative histograms of PD-1 staining in naive and memory (CD45RO+) CD4 T cells from one HC and one pSS patient. Vertical lines in histograms indicate gating of PD-1 negative, PD-1^{intermediate}, and PD-1^{hi} populations, as can also be seen in representative ICOS/PD-1 dotplot from memory CD4 T cells. HC: healthy control; pSS: primary Sjögren's syndrome; ICOS: inducible T cell co-stimulator; PD-1: programmed death-1.

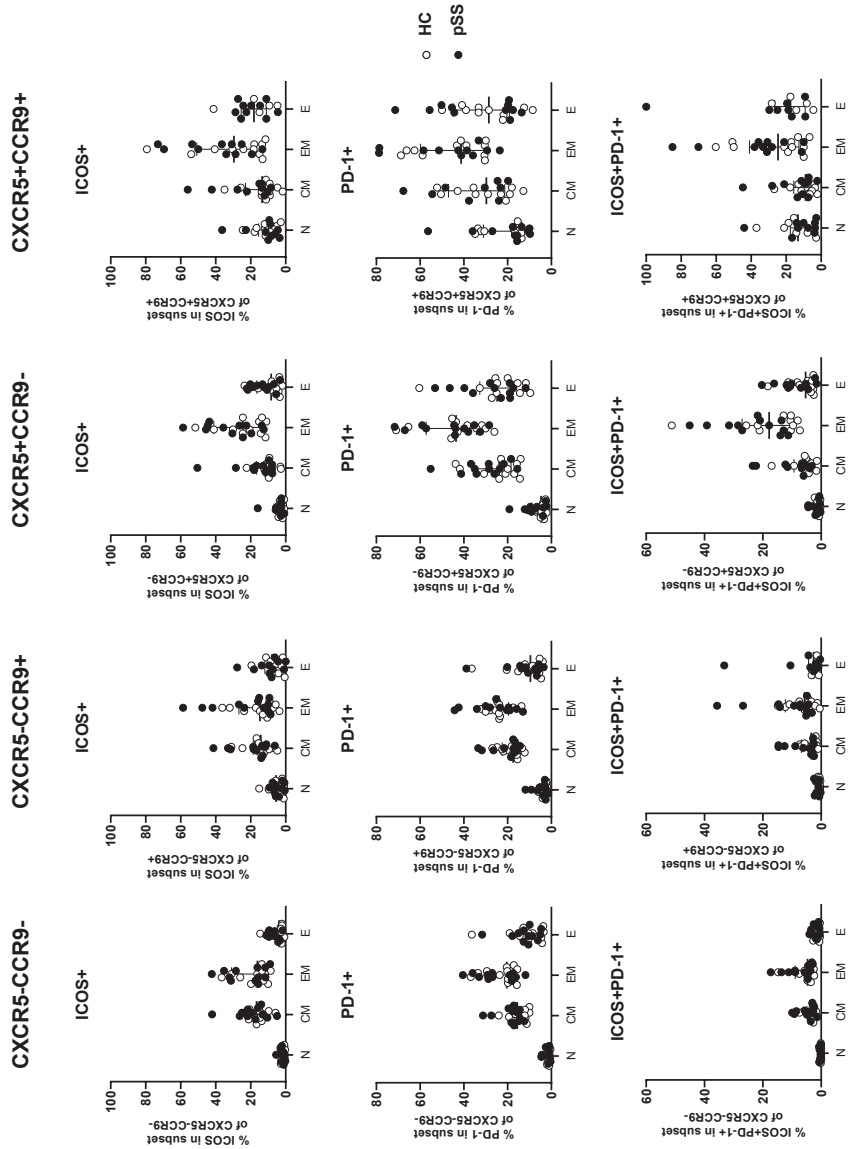


Supplementary Figure S2 | The percentage of CCR9-expressing cells is associated with PD-1 expression. CCR9 expression is evaluated on total (including naive and memory) CXCR5- CD4 T cells with negative, intermediate, and high PD-1 expression. HC: healthy control; pSS: primary Sjögren's syndrome. CCR9: C-C motif chemokine receptor 9; PD-1: programmed death-1; neg: negative; int: intermediate; hi: high. *, *** indicates p-value <0.05, 0.001, respectively.



Supplementary Figure S3 | CXCR5-expressing cells have a higher PD-1^{hi}/ICOS ratio. Increased ICOS expression in HC was only seen for CXCR5-expressing Tfh cells compared to CXCR5-CCR9- cells. (A) Ratios of PD-1^{hi}/ICOS in memory population of all four CXCR5/CCR9-defined cell subsets in pSS patients and HC. (B) Comparison of ICOS expression between the memory PD-1^{hi} CXCR5-CCR9- subset compared to the three other subsets in HC. Plots show medians plus interquartile ranges. HC: healthy control; pSS: primary Sjögren's syndrome. Tph: T peripheral helper cell (memory CXCR5-PD-1^{hi} CD4 T cell); CCR9+ Tfh-like: C-C motif chemokine receptor 9-expressing T follicular helper-like cell (CXCR5-CCR9+ CD4 T cell); ICOS: inducible T cell co-stimulator; PD-1: programmed death-1. **, *** indicates p-value <0.01, 0.001, respectively.

Supplementary Figure S4 | ICOS+, PD-1+, and ICOS+PD-1+ are significantly lower in naive cells in all CXCR5/CCR9-defined cell subsets. Expression of ICOS, PD-1, and ICOS+PD-1+ in naive (N), central memory (CM), effector memory (EM), and effector (E) cells in all four CXCR5/CCR9-defined cell subsets. All comparisons between N cells and CM/EM/E cells are significant within the same CXCR5/CCR9-defined cell subset (p<0.01), except for ICOS expression between N and E in CXCR5-CCR9+ and CXCR5+CCR9+ cells (p=0.19 and p=0.43, respectively), and ICOS+PD-1+ expression between N and E cells in CXCR5+CCR9+ subset (p=0.21). Plots show medians plus interquartile ranges. Naive (N): CD27+CD45RO-; Central memory (CM): CD27+CD45RO+; Effector memory (EM): CD27-CD45RO+; Effector (E): CD27-CD45RO-.



Chapter 5

Do IL-7R+ innate cells orchestrate autoimmune pathology?

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Immunopathology in autoimmune diseases is promoted by complex interactions between many types of immune cells, and adequate disease inhibition relies on targeting molecules that orchestrate these interactions. One such molecule, IL-7 , has well-known effects on lymphoid cells, but could this cytokine also affect innate immune cells to induce immunopathology?

Refers to Kim, S.J. et al. Macrophages are the primary effector cells in IL-7-induced arthritis. *Cell. Mol. Immunol*. https://doi.org/10.1038/s41423-019-0235-z (2019)

IL-7 is a cytokine that is well-known for its lymphopoietic function. In humans, IL-7 receptor (IL-7R) deficiency affects T cell lymphopoiesis and is associated with reduced T cell-dependent B cell activity.(1) Over the past few decades, a role for IL-7 in immune reactions against infections and tumours has emerged, as has a contribution of IL-7 to the immunopathogenesis of numerous autoimmune rheumatic diseases.(1) The list of IL-7R+ cells that can contribute to disease in response to IL-7 is growing. Whereas previous studies have shown IL-7 to be involved in T cell-dependent immune responses in several disease models(1), a new study by Kim et al.(2) suggests a role for IL-7 in innate immune responses.

In their study, Kim et al.(2) demonstrate that IL-7 contributes to arthritis in a T cell-independent manner by using RAG-deficient mice, which lack T cells and B cells. In this IL-7-mediated model of arthritis, IL-7R+ synovial macrophages were strongly associated with joint inflammation, and IL-7 enhanced inflammation and osteoclastogenesis in the absence of T cells and B cells. In vitro, lipopolysaccharide (LPS)-primed macrophages expressed IL-7R, rendering them responsive to IL-7, which increased their production of TNF, IL-6, CC-chemokine ligand 2 (CCL2) and CCL5. These results(2) support previous data showing that IL-7R expression on monocytes and macrophages strongly correlates with disease activity scores in patients with rheumatoid arthritis (RA), and that IL-7 mediates myeloid cell chemotaxis.(3) The increase in IL-7R+ monocytes and macrophages that occurs in several inflammatory diseases (such as RA and primary Sjögren syndrome (pSS)) might therefore contribute to IL-7-mediated immunopathology.

An unsolved question from the study by Kim et al.(2) is whether other innate cells, such as plasmacytoid dendritic cells (pDCs) and innate lymphoid cells (ILCs), also contribute to IL-7-mediated inflammatory arthritis. These cell types are present in RAG-deficient mice and contribute to inflammatory responses in several rheumatic diseases. In vitro, virus-induced activation primes pDCs to secrete type I interferon (IFN) in response to IL-7.(4) Type I (and type II) IFNs in their turn can induce IL-7 production by epithelial cells, and intranasal administration of IL-7 in mice causes recruitment of pDCs to sites such as the lungs or lacrimal glands.(1) These results indicate that IL-7-mediated chemotaxis might occur for pDCs, as has been shown for monocytes, although this direct role for IL-7 in pDC chemotaxis still needs to be confirmed.

ILCs are another innate cell type involved in IL-7-mediated inflammation.(5) In addition to the three non-cytolytic subtypes (ILC1s, ILC2s and ILC3s), this group of cells also includes cytolytic natural killer (NK) cells. All ILCs express IL-7R, are increased in number in rheumatic diseases and potentially have a role in the immunopathology of these diseases. Numerous studies indicate potent proinflammatory functions for ILCs.(5) Notably, ILC3s are important in IL-7-mediated lymphoid structure formation and are thought to have a vital role in the formation of ectopic lymphoid structures, which characteristically form in several rheumatic diseases, including RA, psoriatic arthritis, pSS and systemic lupus erythematosus.(5) Thus, although the data from Kim et al.(2) are strongly suggestive of an important contribution from macrophages in arthritis pathogenesis in RAG-deficient mice, these findings(4,5) suggest that innate cells such as pDCs and ILCs might also contribute to inflammatory responses independently of T cells and B cells. However, the relative roles of these cell types in the arthritis model used by Kim et al.(2) and in systemic autoimmune diseases remains to be demonstrated.

The comparative contributions of direct (T cell-independent) and indirect (T cell-dependent) effects of IL-7 on innate cells have not previously been studied in many disease models. In their paper, Kim et al.(2) clearly demonstrate how IL-7 orchestrates interactions between IL-7R-expressing innate and lymphoid cells. In this study(2), full-blown inflammation (characterized by strongly increased secretion of proinflammatory cytokines and chemokines, including CCL2 and CCL5, and an increased number of proinflammatory IL-7R+ macrophages and osteoclasts) required the presence of T cells. Joint erosion was also only induced in the presence of T cells and was dependent on IL-7, suggesting some crosstalk between innate and lymphoid cells. Underscoring this cellular crosstalk, in wild-type mice, IL-7-induced IFN-γ production (which mostly indicates T helper 1 (Th1) cell activity) in the arthritic joint was strongly dependent on the priming of macrophages with LPS2, which enabled them to stimulate T cell activity. In line with these data, monocytes and activated type 2 classical (myeloid) CD1c+ dendritic cells from patients with RA were previously shown to robustly activate both naive and effector T cells upon stimulation with IL-7.(1,6) Vice versa, IL-7 induces a potent T cell-dependent activation of myeloid cells that is associated with enhanced osteoclastogenesis.(1,2) Similarly, IL-7 triggers T cell-dependent B cell activation, inducing synergistic B cell and T cell proliferation upon co-stimulation of B cells with TLR7.(1)

In addition to IL-7R+ innate cells and ILCs, discrete populations of tissue-resident IL-7R+ T cells with innate-like properties can contribute to immunopathology. For example, mucosa-

associated tissues harbour IL-7R+ mucosal-associated invariant T (MAIT) cells and IL-7R+ CC-chemokine receptor 9 (CCR9)+ T cells.(7,8) The numbers of these cells are increased in target tissues in several autoimmune diseases (such as in the salivary glands of patients with pSS) and are imprinted to home to mucosa and mucosa-associated sites by specific chemokine receptors and adhesion molecules, including CCR9 and integrin α4β7.(7) MAIT cells, unconventional innate-like T cells with an invariant T cell receptor, are important in defending the body against bacteria and can be activated by bacterial antigens to produce Th1 cell-associated and Th17 cell-associated cytokines and to become cytotoxic.(7,8) CCR9+ T follicular helper (Tfh)-like cells that highly express IL-7R produced large amounts of effector molecules such as IL-21, IFN-γ and IL-17 and induced B cell activation comparably to Tfh cells in vitro.(7) A colitogenic population of CCR9+ T helper cells has also been identified that had innate-like properties.(9) In addition, a substantial proportion of effector CCR9+ T helper cells express CD161, a lectin-like receptor that is also expressed by ILCs, suggesting potential innate-like functions for CCR9+ T helper cells.(10) The unique functions of these IL-7R+ cells are under investigation to reveal their specific roles in the immunopathology of autoimmune disease.

Overall, the paper by Kim et al.(2) indicates that innate IL-7R+ effector cells can contribute to the immunopathology of systemic autoimmunity. Overexpression of IL-7 can promote complex interactions between IL-7R+ innate cells (macrophages, monocytes and pDCs), ILCs (including NK cells) and T cells with innate-like features to activate IL-7R– cells (such as B cells and monocytes), thereby inducing immunopathology in autoimmune rheumatic diseases (Fig. 1). Blockade of the IL-7/IL-7R pathway successfully inhibits the spread of autoimmunity, B cell hyperactivity and specific immunopathology in different organs in many experimental models of disease.(1) Such targeting could be of particular interest when potent anti-inflammatory drugs have failed, such as in patients with RA who do not respond to abatacept treatment (which targets T cell co-stimulation). The addition of IL-7 completely overruled T cell inhibition by abatacept in vitro(6), suggesting that targeting IL-7 signalling might be an alternative strategy to inhibit T cells in patients with IL-7-mediated disease. Drugs targeting IL-7 or IL-7R are expected to enter the clinical arena soon.

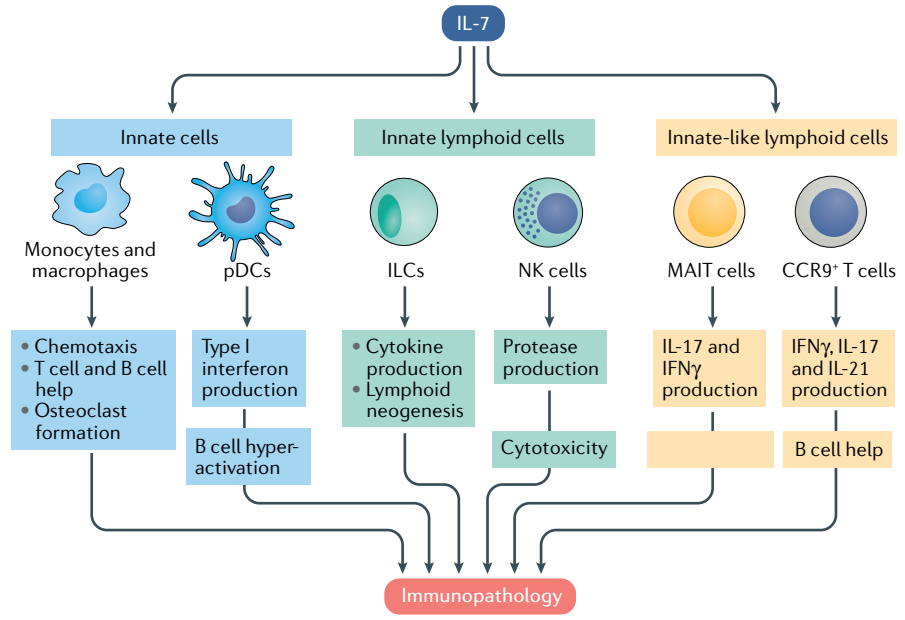

Figure 1 | IL-7R+ innate cells in rheumatic autoimmune diseases promote immunopathology. IL-7 is produced by stimulated tissue cells such as fibroblasts, epithelial cells and endothelial cells, as well as by dendritic cells (not shown), and binds to IL-7 receptor (IL-7R)-expressing innate cells (monocytes, macrophages and plasmacytoid dendritic cells (pDCs)) and innate lymphoid cells (ILCs; including natural killer (NK) cells). In addition, IL-7 regulates the activity of lymphoid cells that have innate functions (such as mucosal-associated invariant T (MAIT) cells and CC-chemokine receptor 9 (CCR9)+ T cells). Through direct and indirect activation of cells with innate functions, IL-7 orchestrates immunopathology by promoting cell recruitment, cytokine and protease secretion, B cell hyperactivity, lymphoid neogenesis, cytotoxicity and osteoclast formation.

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Chapter 6

In patients with primary Sjögren's syndrome innate-like MAIT cells display upregulated IL-7R, IFN-γ, and IL-21 expression and have increased proportions of CCR9 and CXCR5-expressing cells

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Abstract

Introduction. Mucosal-associated invariant T (MAIT) cells might play a role in B cell hyperactivity and local inflammation in primary Sjögren's syndrome (pSS), just like previously studied mucosa-associated CCR9+ and CXCR5+ T helper cells. Here, we investigated expression of CCR9, CXCR5, IL-18R and IL-7R on MAIT cells in pSS, and assessed the capacity of DMARDs to inhibit the activity of MAIT cells.

Methods. Circulating CD161+ and IL-18Rα+ TCRVα7.2+ MAIT cells from pSS patients and healthy controls (HC) were assessed using flow cytometry, and expression of CCR9, CXCR5, and IL-7R on MAIT cells was studied. Production of IFN-γ and IL-21 by MAIT cells was measured upon IL-7 stimulation in the presence of leflunomide (LEF) and hydroxychloroquine (HCQ).

Results. The numbers of CD161+ and IL-18Rα+ MAIT cells were decreased in pSS patients compared to HC. Relative increased percentages of CD4 MAIT cells in pSS patients caused significantly higher CD4/CD8 ratios in MAIT cells. The numbers of CCR9 and CXCR5-expressing MAIT cells were significantly higher in pSS patients. IL-7R expression was higher in CD8 MAIT cells as compared to all CD8 T cells, and correlated to several clinical parameters. The elevated production of IL-21 by MAIT cells was significantly inhibited by LEF/HCQ treatment.

Conclusion. Circulating MAIT cell numbers are decreased in pSS patients. Given their enriched CCR9/CXCR5 expression this may facilitate migration to inflamed salivary glands known to overexpress CCL25/CXCL13. Given the pivotal role of IL-7 and IL-21 in inflammation in pSS this indicates a potential role for MAIT cells in driving pSS immunopathology.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease clinically characterized by dryness of eyes and mouth, fatigue and myalgia.(1) Hallmark features of pSS are B cell hyperactivity and lymphocytic infiltration of exocrine glands.(2,3) B cell hyperactivity is reflected by e.g. elevated serum IgG, the presence of autoantibodies in the circulation, elevated numbers of IgM/IgG plasma cells in the salivary glands, and an elevated risk of developing lymphoma.(4,5)

Several T cell subsets have been studied in pSS for their potential to drive B cell hyperactivity, and to organize ectopic lymphoid structures (ELS).(6–9)
T follicular helper (Tfh) cells are B cell activating T cells that express CXCR5, and that can reside in germinal centers (GCs) in lymph nodes.(10) The number of Tfh cells is elevated in salivary glands and in peripheral blood of pSS patients, just like the levels of Tfh-associated cytokines such as CXCL13 (which CXCR5+ cells migrate towards), IL-21, and IL-4.(6,7,11–14) Tfh cell numbers correlate to autoantibody levels, and disease severity.(14–18) Increased expression of ligand CXCL13 in the glands is associated with a higher number of lymphocytic foci and increased organization into ELS.(6,7,9)
In the context of pSS also two Tfh-like cells subsets have been studied recently, T peripheral helper (Tph) cells, and CCR9+ Tfh-like cells, both lacking CXCR5. Tph cells are CXCR5-PD-1^{hi} memory T cells, produce high levels of IL-21 and CXCL13, like Tfh cells, and are found in elevated numbers in blood of pSS patients, and in the salivary glands of pSS patients with GCs in their gland tissue.(18–20) CCR9+ Tfh-like cells are cells that are enriched in mucosa-associated tissues, and are potent B cell activating T cells that have elevated expression of ICOS, PD-1, CCL5 and IL-7R, and also produce high amounts of IFN-γ, and IL-21.(21–23) The number of CCR9+ Tfh-like cells is elevated in peripheral blood of pSS patients. In (mucosa-associated) salivary glands of pSS patients both CCR9+ T cell numbers, and levels of its ligand CCL25 are elevated.(21–24) In secretomes of salivary gland tissue CCL25 levels were also found to be associated with SSA positivity, B cell hyperactivity (serum IgG levels), and levels of IL-21 and soluble IL-7R.(25)

Other cells that are enriched in mucosal tissues and that are increasingly studied for their potential role in autoimmune disease are mucosal-associated invariant T (MAIT) cells. MAIT cells are unconventional innate-like effector T cells that become activated by binding to MHC class I-like molecule MR1.(26) MAIT cells express a semi-invariant T cell receptor (TCR) TCRVα7.2-Jα12/20/33 with a limited amount of options of Vβ chains.(27–30) MAIT cells are abundant in e.g. peripheral blood, liver, gastro-intestinal tract, and mesenteric lymph nodes, and play an important role in mucosal immunity against infections, e.g. by rapidly producing cytokines, such as IFN-γ, IL-21, TNF-α, IL-17, perforin, and granzyme B.(31–37)

MAIT cells are activated both in TCR-dependent and TCR-independent ways, including stimulation by IL-7, IL-12, and IL-18.(37–40) In humans MAIT cells predominantly express CD8, or express neither CD8 nor CD4 (±70-80% and ±15%, respectively), and just a few percent of MAIT cells express CD4.(28,35,41,42) MAIT cells can be identified in blood and tissues based on the expression of CD3 and TCRVα7.2 in combination with either CD161 and/or IL-18Rα.(32,35,43) Furthermore high levels of CCR6, CXCR6, IL-7Rα (CD127), ABCB1 and NKG2D were found.(35) Also CCR9-expressing MAIT cells have been identified, and were found to be enriched in mucosal sites such as the colon, with similar CCR9 expression between colonic CD8 MAIT cells and non-MAIT CD8 T cells.(44) Finally MAIT cells in a human in vitro model have been shown to stimulate B cell activity associated with production of IL-21, IL-10, and IL-6, and in a lupus-like mouse model MAIT-driven B cell activation and autoantibody production has been shown.(36,45–47)

In pSS patients the number of circulating CD161+TCRVα7.2+ MAIT cells was decreased compared to controls, whereas in salivary gland tissue the number of (CD161+)TCRVα7.2+ MAIT cells was elevated as compared to non-Sjögren sicca patients and mild sialoadenitis patients (without pSS).(48,49) pSS patients had significantly more circulating CD4, and naive CD8 CD161+TCRVα7.2+ MAIT cells, which was not the case for CD161-TCRVα7.2+ T cells.(48) MAIT cells from salivary glands of pSS patients produced increased levels of IL-17, an effect that was validated in vitro upon stimulation of MAIT cells with IL-7.(49)

Currently, the number of studies on MAIT cells in pSS is limited and the precise mechanisms by which MAIT cells could instigate pSS immunopathology are not known. In the present study in pSS patients and healthy controls we investigated CCR9 (in comparison with CXCR5) as a receptor that might facilitate migration to mucosa-associated tissues such as salivary glands. In addition, IL-18Rα+ MAIT cells, and IL-18 and IL-7 receptor expression on MAIT cells in pSS patients and HC were studied. Also the capacity of IL-7 to induce IL-21 and IFN-γ by MAIT cells was studied, as well as the potential of disease-modifying antirheumatic drugs (DMARDs) leflunomide and hydroxychloroquine to inhibit this production.

Methods

Patients and controls

Peripheral blood mononuclear cells (PBMCs) were collected from n=12 primary Sjögren's syndrome (pSS) patients and n=11 healthy controls (HC). Controls were age and sex matched to patients. All pSS patients were diagnosed by a rheumatologist or clinical immunologist and met the 2016 ACR-EULAR criteria.(50) All participants were included in the University Medical Center Utrecht (UMC Utrecht) and gave written informed consent. The Medical Research Ethics Committee (METC) of the UMC Utrecht approved the study (reference number 13/697). Demographic and clinical data are shown in Table 1. Fresh PBMCs were isolated from Lithium-heparinized blood using density gradient centrifugation on Ficoll-Paque™ Plus (GE Healthcare Life Sciences). Collected PBMCs were frozen and stored in liquid nitrogen until further use.

Cultures

From 5 individuals (n=3 HC and n=2 pSS patients) 0,5.10^6 thawed PBMCs were cultured overnight with optimal IL-7 concentrations as previously demonstrated as biologically relevant (10ng/ml, Peprotech)(51,52). Leflunomide (33μM, biologically active metabolite A77 1726, MedChemExpress, Monmouth Junctions, USA), hydroxychloroquine (10μM, Sigma-Aldrich), or the combination of both drugs were tested at clinically relevant concentrations inducing optimal inhibition of lymphocyte activation as previously demonstrated.(53,54) In our hands upon in vitro cultures, and following T cell activation, CCR9 was strongly reduced after 48 hours of culture. Hence, to appreciate cytokine secretion by CCR9+ T cells we cultured for 24 hours. For T cell cytokine analyses all samples were restimulated with phorbol myristate acetate (PMA) and ionomycin for 4 hours, in the presence of Brefeldin A. Medium control was taken along. The pSS patients whose PBMCs were used for this culture were not prescribed immunosuppressive drugs.

	HC (n=11)	pSS (n=12)
Female, n (%)	11 (100)	12 (100)
Age, years	55 (49-60)	59 (54-64)
Anti-Ro/SSA positive, n (%)		8 (67)
Anti-La/SSB positive, n (%)		4 (33)
ANA positive, n (%)		7 (58)
Lymphocytic focus score (foci/4mm²)		1.8 (1.1-2.4)
IgA positive plasma cells (%)		65 (40-81)
Schirmer (mm/5min)		0 (0-1)
Serum IgG (g/L)		13.6 (11.4-15.2)
ESSDAI score (0-123)		4 (2-5)
ESSPRI score (0-10)		6.3 (5.3-7.3)
Immunosuppressants use, n		3

Table 1 | Participants' characteristics. Medians with interquartile range (Q1-Q3) are shown, unless specified otherwise. Used immunosuppressants are hydroxychloroquine (n=2) and methotrexate (n=1). HC: healthy controls; pSS: primary Sjögren's syndrome; anti-Ro/SSA: anti-Ro/Sjögren's syndrome related antigen A antibody; anti-La/SSB: anti-La/Sjögren's syndrome related antigen B antibody; ANA: antinuclear antibody; ESSDAI: European League Against Rheumatism (EULAR) Sjögren's syndrome disease activity score; ESSPRI: EULAR Sjögren's syndrome patient reported index.

Flow cytometry

PBMCs from all donors were thawed and stained with fixable viability dye eF780 (eBioscience), after which the cells were stained with fluorochrome-conjugated antibodies against CD3, CD4, CD8, CD45RO, CD161, CCR9, CXCR5, TCRVα7.2, IL-7Rα (CD127), and IL-18Rα (CD218α). For intracellular staining after overnight stimulation cells were fixed/permeabilized using Fixation/Permeabilization Concentrate and Diluent (Cat #00-5123-43, #00-5223-56, eBioscience) according to manufacturer's protocol. In this panel IL-7Rα and IL-18Rα were replaced with IL-21 and IFN-γ. Details of used antibodies can be found in Supplementary Table 1. All samples were acquired on a BD LSRFortessa (BD Biosciences) using BD FACSDiva software v.8.0.1 (BD Biosciences). For data analysis FlowJo™ Software v10.8 (BD Life Sciences) was used. MAIT cells were defined using either CD161+ or IL-18Rα+, combined with TCRVα7.2+ CD3 cells. Gating of MAIT cells (CD161+ and IL-18Rα+ in combination with TCRVα7.2+) within CD4/CD8 populations is shown in Supplementary Figure 1.

Statistical analysis

Data were analyzed and visualized using IBM SPSS Statistics 26 and Graphpad Prism 8. Mann-Whitney U test (for unpaired analyses) and Wilcoxon non-parametrical paired test were used (for paired analyses). For correlations Spearman's rho was used. Statistical significance was considered for differences at $p<0.05$.

Results

The number of CD161+ and IL-18Rα+ MAIT cells is decreased in pSS patients, and MAIT cells have a significantly different CD4/CD8 ratio in pSS patients

To define MAIT cells from live CD3 cells TCRVα7.2+ expression was analyzed together with either CD161+ or IL-18Rα+ (representative plots from HC and pSS patient in Figure 1A). The numbers of CD161+, IL-18Rα+, and co-expressing (memory) MAIT cells were all significantly decreased in pSS patients compared to HC (Figure 1B). Whereas in the total CD3 population CD4 T cells were the largest subset, in MAIT cells CD8 cells represented the largest subset, followed by CD4-CD8- and CD4 MAIT cells (Figure 1C). Increased numbers of CD4 MAIT cells were found in pSS patients as compared to controls ($p=0.02$ for CD161+, and $p=0.049$ for IL-18Rα+ MAIT cells, Figure 1C). This resulted in significantly skewed CD4/CD8 ratios for CD3 cells and MAIT cells from 2.2 (1.7-4.6) (median with interquartile range using pooled HC and pSS data) for CD3 cells to below 0.50 (median) in MAIT cell subsets (Figure 1D). In addition, in pSS patients a significantly higher CD4/CD8 MAIT cell ratio compared to HC was observed, in line with the increased CD4 MAIT cell percentages in pSS patients (Figure 1D).

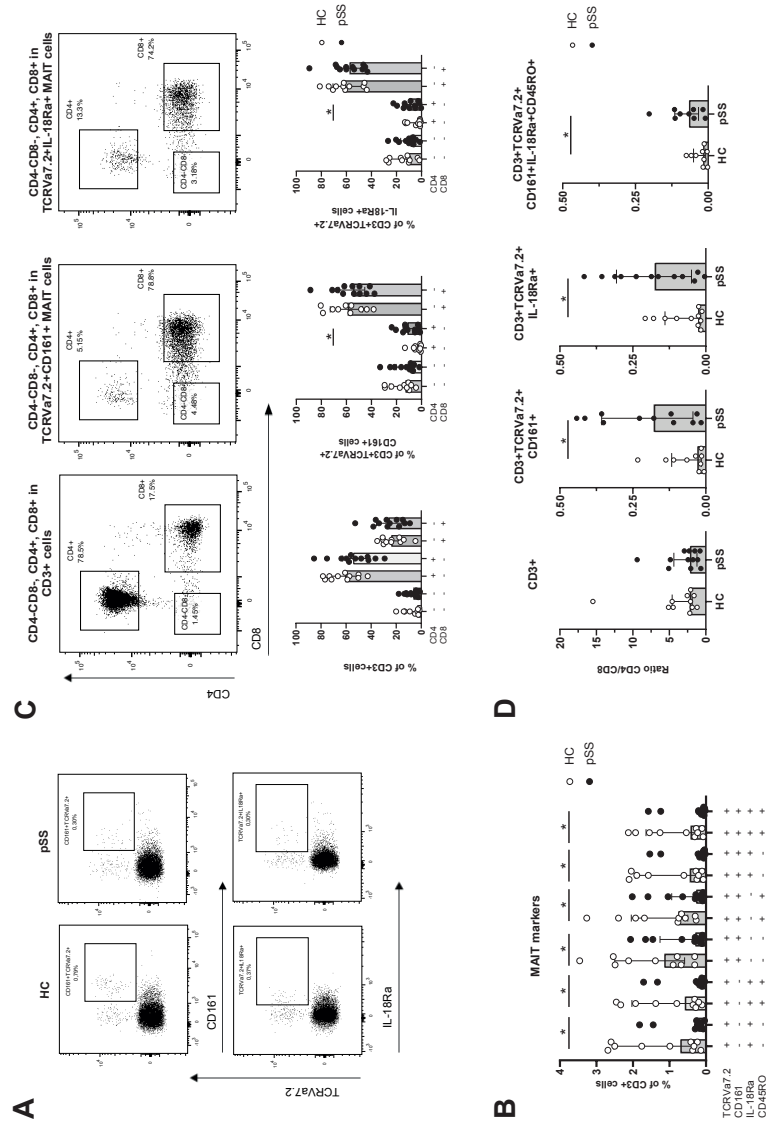

Figure 1 | Reduced frequencies and shift in CD4/CD8 ratio of antigen-experienced CD161+/IL-18Rα+ MAIT cells in pSS patients. (A) Representative flow cytometry plots from a HC and a pSS patient of MAIT cell staining. Gates are shown of TCRVα7.2+CD161+ and TCRVα7.2+IL-18Rα+ within CD3+ cells. (B) MAIT cell subsets defined by TCRVα7.2 expression in combination with CD161, IL-18Rα, and CD45RO, are shown as percentages of the CD3+ T cell population in pSS patients and controls. (C) Representative dot plots of CD4/CD8 staining of all CD3+ cells, CD161+ and IL-18Rα+ TCRVα7.2+ MAIT cells, with quantification below the representative dot plots. (D) CD4/CD8 ratio in CD3+ cells and MAIT cells defined by TCRVα7.2+CD161+, TCRVα7.2+IL-18Rα+, and combination of MAIT markers TCRVα7.2, CD161, IL-18Rα, and CD45RO in pSS patients and HC. Bar plots show all individuals and median (interquartile range). HC: healthy control; pSS: primary Sjögren's syndrome. * indicates statistical significance of $p<0.05$

CCR9-expressing MAIT cells are mainly observed in CD4 MAIT cells and are rare in CD4-CD8- and CD8 MAIT cell populations

Earlier work from our group and others has shown increased CCR9 expression on CD4 and CD8 T cells in pSS compared to HC.(21–24) In this study we confirmed these findings (representative dot plots in Figure 2A). In fact, in most CD3 populations (CD3 total, CD4 T cells, CD8 T cells, CD161+ MAIT cells and IL-18Rα+ MAIT cells) the percentage of CCR9-expressing cells was elevated in pSS patients (Figure 2B). Within both CD161+ and IL-18Rα+ MAIT cell subsets CD4 cells expressed most CCR9. In addition, significantly increased CCR9 expression in pSS patients was found for CD161+ and IL-18Rα+ CD8 MAIT cells (Figure 2C). Overall the proportions of CCR9-expressing cells follow the same skewing in CD4-CD8-, CD4, and CD8 MAIT cells as in the respective total populations. Interestingly, for CXCR5 expression similar results were found. CXCR5 expression was elevated in pSS compared to HC in CD161+ and IL-18Rα+ MAIT cell subsets, with the highest expression of CXCR5 in CD4 MAIT cells (Supplementary Figure 2 and 3).

IL-7R expression is increased on CD8 MAIT cells, and is associated with inflammatory parameters. IL-21 production by MAIT cells is increased, and in the context of IL-7 activation reduced by LEF and HCQ

CD8 MAIT cells showed a significantly increased IL-7R expression compared to the total CD8 population (Figure 3A). This increase was also seen in CD3, CD4-CD8-, and CD4 MAIT cells (Supplementary Figure 4). Of note, CCR9+ CD8 T cells showed a significant increase in IL-7R expression ($p<0.001$) compared to CCR9- CD8 T cells, which was not the case for IL-7R expression of CCR9+ CD8 MAIT cells compared to CCR9- CD8 MAIT cells, showing comparable high expression (Figure 3A). For CXCR5-expressing MAIT cells a similar pattern of IL-7R expression was seen as for CCR9+ cells (Supplementary Figure 5). In pSS patients, a decreased expression of IL-7R on CD161+ CD8 MAIT cells significantly correlated to increased lymphocytic focus scores (LFS), decreased percentage of IgA+ plasma cells in minor salivary gland tissue (IgA%), and increased serum IgG levels (Figure 3B). Also, IL-7R expression on CD161+ CD8 MAIT cells was significantly decreased in pSS patients with anti-SSA-autoantibodies (Figure 3B).

Since DMARDs leflunomide (LEF) and hydroxychloroquine (HCQ) were recently shown to be efficacious in vivo, and in vivo block inflammatory activity(54), we tested the capacity of these drugs to target MAIT activity. For this purpose we cultured ex vivo IL-7-stimulated cells with LEF and HCQ, and evaluated IFN-γ and IL-21 production (Supplementary Figure 6). CD161+ CD8 MAIT cells produced more IL-21 compared to (CCR9+) non-MAIT CD8 T cells (median 12.2% versus 3.8% ($p=0.009$) and versus 5.4 ($p=0.048$) for CD8 and CCR9+ CD8 T cells respectively, Figure 3C). Unfortunately, it was not feasible to directly evaluate CCR9-expressing CD161+ CD8 MAIT cells due to limited CCR9 cell counts within this population.

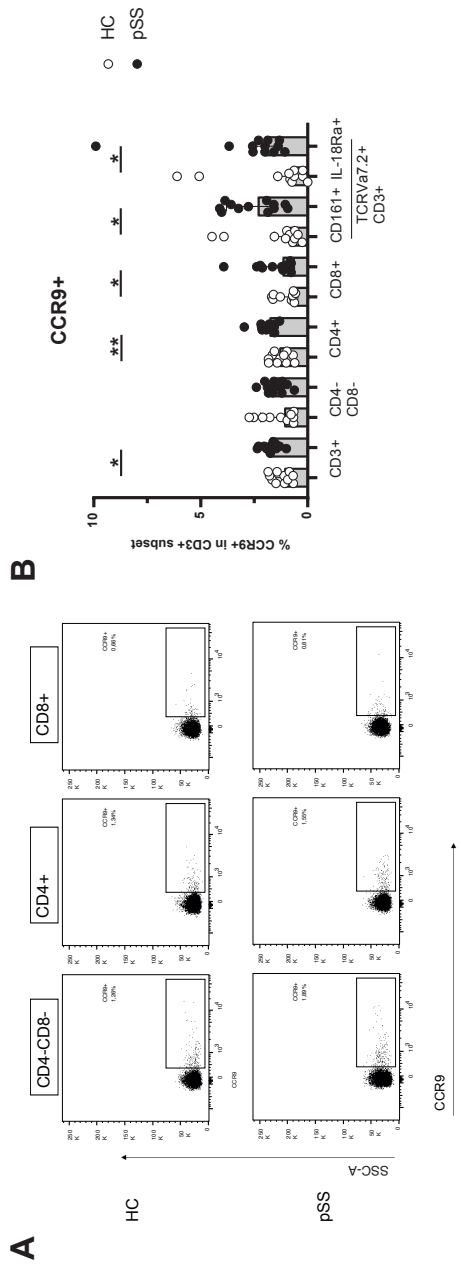

Figure 2 | Increased frequencies of CCR9-expressing cells among circulating MAIT cells in pSS patients. (A) Representative flow cytometry plots of CCR9 expression on CD4-CD8-, CD4+, and CD8+ T cells from one pSS patient and one HC. (B) CCR9 expression is compared between pSS patients and healthy controls in different CD3+ cell subsets, including CD161 and IL-18Rα-expressing MAIT cells. (C) In CD4-CD8-, CD4+, CD8+ cells from CD161+ and IL-18Rα+ MAIT cells CCR9 expression is quantified. Bar plots show median (interquartile range). HC: healthy control; pSS: primary Sjögren's syndrome. *, ** indicates statistical significance of $p<0.05$, $p<0.01$, respectively.

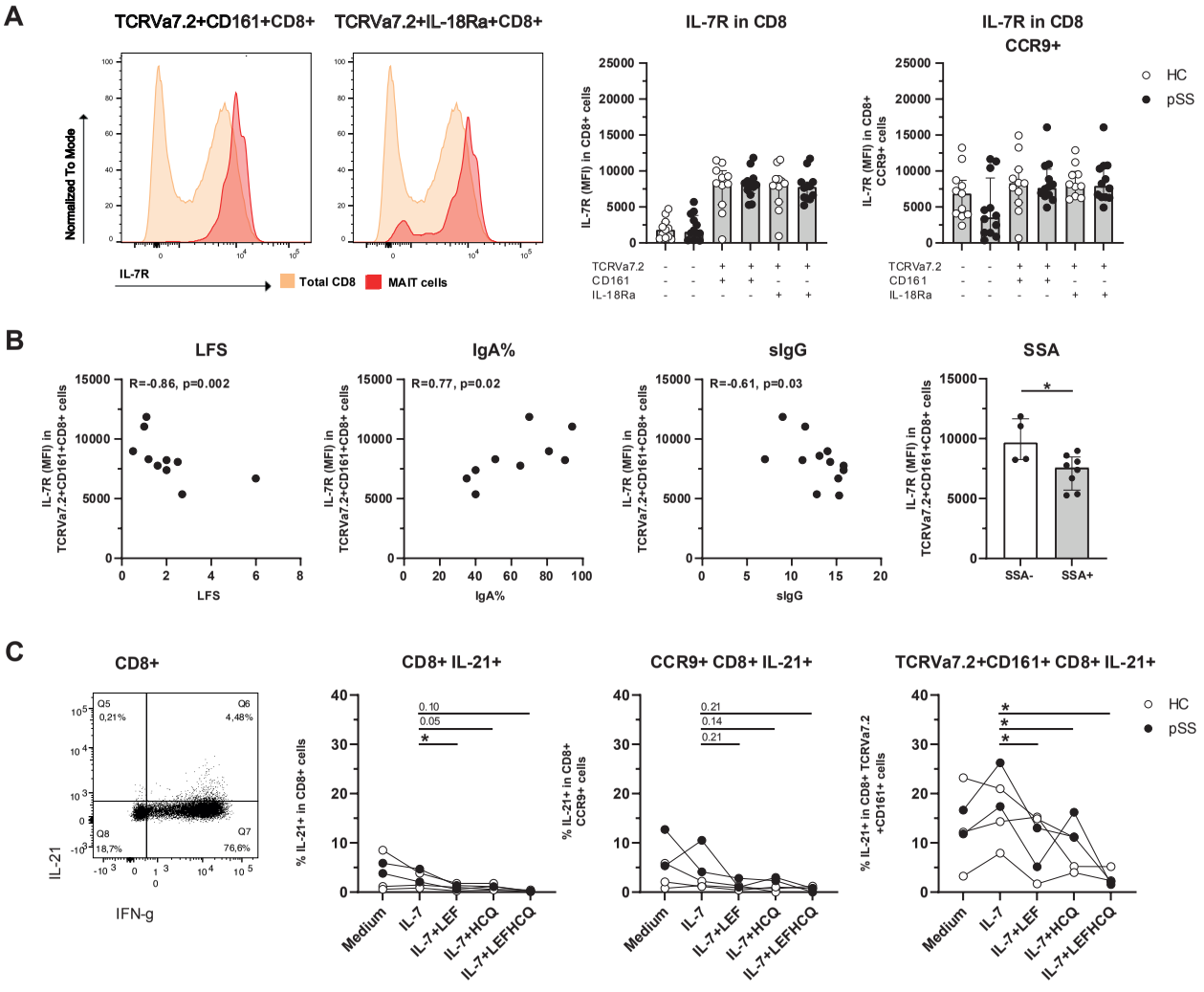

Figure 3 | Expression of IL-7R is increased on CD8 MAIT cells, and this is associated with inflammatory parameters. IL-21 production by MAIT cells is increased and in IL-7-stimulated conditions reduced by leflunomide and hydroxychloroquine. (A) Representative histograms of IL-7R expression on CD161+ and IL-18Rα+ TCRVα7.2+ CD8 MAIT cells compared to IL-7R expression in CD8 total population. Bar plots showing medians (interquartile range) of IL-7R on CD8 (MAIT) cells and CCR9+ CD8 (MAIT) cells in pSS patients and controls. (B) Correlation between IL-7R expression on TCRVα7.2+CD161+ CD8 MAIT cells and local (LFS and percentage of IgA-producing plasma cells), and systemic (sIgG) clinical parameters. Bar graph shows comparison of IL-7R expression on TCRVα7.2+CD161+ CD8 MAIT cells in pSS patients who are SSA-autoantibody positive or negative. (C) Representative dot plot of IFN-γ/IL-21 expression in CD8 cells. IL-21 expression of CD8 cells, CCR9+ CD8 cells, and TCRVα7.2+CD161+ CD8 MAIT cells was measured upon overnight stimulation with IL-7, or IL-7 in combination with either leflunomide, hydroxychloroquine or a combination of both drugs. HC: healthy control; pSS: primary Sjögren's syndrome; LFS: lymphocytic focus score; IgA%: percentage of IgA+ plasma cells in minor salivary gland biopsy (the percentage reduces when the percentage of IgM and IgG-producing plasma cells increases); sIgG: serum immunoglobulin G; SSA: anti-Sjögren's syndrome related antigen A antibody; LEF: leflunomide; HCQ: hydroxychloroquine. * indicates statistical significance of $p<0.05$.

However, the results of CD8 T cells, CCR9+ CD8 T cells and CD161+ CD8 MAIT cells could be evaluated and showed a similar reduction in IL-21 expression in the presence of LEF and/or HCQ, which was statistically significant for conditions in MAIT cells (Figure 3C). Like IL-21, IFN-γ was also significantly higher in CD161+ CD8 MAIT cells as compared to CD8, and CCR9+ CD8 T cells (mean 84.9% versus 55.0% (p=0.004), and 31.6% (p=0.003), respectively). However, in this short-term culture no significant effect on IFN-γ production was observed in response to LEF/HCQ treatment (data not shown).

Discussion

In this study we demonstrated decreased circulating CD161+ as well as IL-18Rα+ MAIT cell numbers in pSS patients. Additionally, it was established that also memory subsets of CD161+ and IL-18Rα+ MAIT cells were significantly lower in the circulation of pSS patients. In addition, we found that proportions of CCR9-expressing cells are elevated in MAIT cells of pSS patients, and follows the same skewing in CD4-CD8-, CD4, and CD8 MAIT cells as in the respective total populations. Furthermore, expression of IL-7R was found to be increased on CD8 MAIT cells as compared to the general CD8 T cell population. The expression of IL-7R on CD161+ MAIT cells correlated to the LFS, the percentage of IgA+ plasma cells in minor salivary gland tissue, and serum IgG levels, and was associated with the presence of anti-SSA-antibodies. IL-21 production by CD8 MAIT cells stimulated with IL-7 was significantly reduced by LEF and HCQ treatments.

In line with earlier reports we found reduced CD161+ MAIT cells, which are at least partly caused by increased migration to inflammatory sites, since MAIT cells were found increased in the salivary glands of pSS patients as compared to controls.(48,49) In addition, we for the first time demonstrate that IL-18Rα+ MAIT cells are decreased in pSS patients compared to controls. Also, CD161+ MAIT cells express high levels of the IL-18Rα as compared to non-MAIT cells in both pSS patients and HC (Supplementary figure 7), allowing them to strongly respond to IL-18 produced by innate cells. In pSS patients increased IL-18 production by macrophages, dendritic cells, and epithelial cells has been demonstrated and was associated with increased immune activation.(55–57) This indicates that increased IL-18Rα-expressing MAIT cells at the inflammatory sites in pSS could form another target for IL-18-mediated immune activation.

In accordance with earlier work, we found that in pSS patients the number of CD4 MAIT cells is elevated as compared to controls.(48) Our data corroborate previous studies demonstrating that as compared to CD3 T cells MAIT cells have a strongly skewed CD4/CD8 T cell ratio with skewing towards CD8 T cells. (28,35,41,42) Here we demonstrate that for both CD161 and IL-18Rα-expressing MAIT cells in pSS patients this is even further skewed. As compared to non-MAIT CD8 cells CD8 MAIT cells produce high levels of IFN-γ and IL-21. Currently, it is unclear to what extent CD8 T cells and CD8 MAIT cells contribute to immune activation in pSS patients. Nonetheless, it was observed that CD8 T cells were found to associate with lymphocyte aggregates and Tfh numbers in salivary glands of pSS patients.(24) Hence, we anticipate that increased CD8 MAIT cells may also significantly contribute to immune activation in the glandular lymphocytic foci given their effector profile. In addition, their innate-like properties, responding to specific environmental/infectious antigens suggests their capacity to respond in an early phase, perhaps (in certain cases) playing a crucial role in initiating immune responses.

Overexpressed CCL25 in salivary glands is associated with the presence of SSA-autoantibodies and increases in lymphocyte foci, B cell hyperactivity, and salivary gland secretome IL-21 and sIL-7R levels.(22) We here demonstrate that together with increased proportions of CCR9-expressing total CD4 and CD8 T cells, also proportions of CCR9-expressing CD161+ and IL-18Rα+ CD8 MAIT cells were significantly increased in pSS patients. Interestingly, the strongest increases in CCR9-expressing cells were found in CD4 CD161+ MAIT cells in pSS patients. The elevated levels of CCR9 expression in circulating (memory) MAIT cells of patients with pSS, a disease with inflammation in mucosa-associated tissues, fit to the observation that colonic MAIT cells with an activated memory phenotype express CCR9 and other chemokine receptors such as CCR6.(44)
In a similar pattern as CCR9-expressing MAIT cells, increased proportions of CXCR5-expressing MAIT cells were found. CXCR5 expression on CD161+ and IL-18Rα+ MAIT cells was significantly increased in pSS patients. CXCL13, the ligand for CXCR5, is overexpressed in salivary glands of pSS patients, and increased expression of CXCL13 is associated with increased B cell hyperactivity, and development of B cell lymphoma.(6,7,58) Together this suggests that overexpressed CCL25 and CXCL13, facilitating the migration of CCR9 and CXCR5-expressing MAIT cells, in addition to classical Tfh cells and CCR9 Tfh-like cells, could contribute to immunopathology in pSS patients.

Significantly increased IL-7R expression was observed on MAIT cells from both HC and pSS patients. Interestingly, reduction of IL-7R expression on CD8 MAIT cells was associated with local and systemic immune parameters, including lymphocytic focus scores and B cell hyperactivity. Since IL-7 causes IL-7R downregulation this observation could reflect increased IL-7 levels in pSS patients.(59,60) Both systemically and in the inflamed glands of pSS patients increased IL-7 was associated with inflammatory parameters, and disease activity parameters.(61–63) Alternatively, TCR-induced activation, which is another trigger of IL-7R downregulation, might be related to reduced IL-7R expression.(64) Thus IL-7R downregulation might reflect activation of MAIT cells either via IL-7 stimulation or TCR cross-linking.

Immunohistochemical assessment of CCR9 and CXCR5 expression, as well as CD161 and IL-18Rα expression, on MAIT cells in the salivary gland of pSS patients is currently lacking. This might however be challenging due to low cell frequencies, but also due to potential downregulation of chemokine receptor CCR9. In our hands CCR9 expression was strongly reduced upon T cell activation after 48 hours of in vitro culture. Thus, considering immune activation and binding to its ligand CCL25 (which is elevated in pSS salivary gland tissue), assessment of CCR9+ T cells in inflamed salivary glands may be a challenge. The same uncertainty holds true for MAIT cell markers. For circulating cells it has been established that most MAIT cells will be identified using CD3 TCRVα7.2+CD161+ expression.(28) Whether this is also the case for MAIT cells in salivary gland tissue of pSS patients still needs to be confirmed. Also, which subset of MAIT cells predominates locally (CD4, CD8 or CD4-CD8-) in pSS patients still is unknown. Enrichment of CD4-CD8- cells up to 40-50% of MAIT cells in mucosal tissues previously has been demonstrated and could match with the low frequencies seen in blood.(65,66) However, this still needs to be evaluated in pSS.

In this study we have demonstrated that MAIT cells in the circulation of pSS patients are antigen-experienced effector T cells that are characterized by high expression of IL-7R, IFN-γ, and IL-21. Our data suggest that at least part of the activity of MAIT cells can be targeted by DMARD treatment such as leflunomide and hydroxychloroquine combination therapy. The increased proportion of CCR9 and CXCR5-expressing MAIT cells, and overexpression of their ligands in inflamed tissues in these patients suggests that these cells with innate properties might contribute to the immunopathology in pSS.

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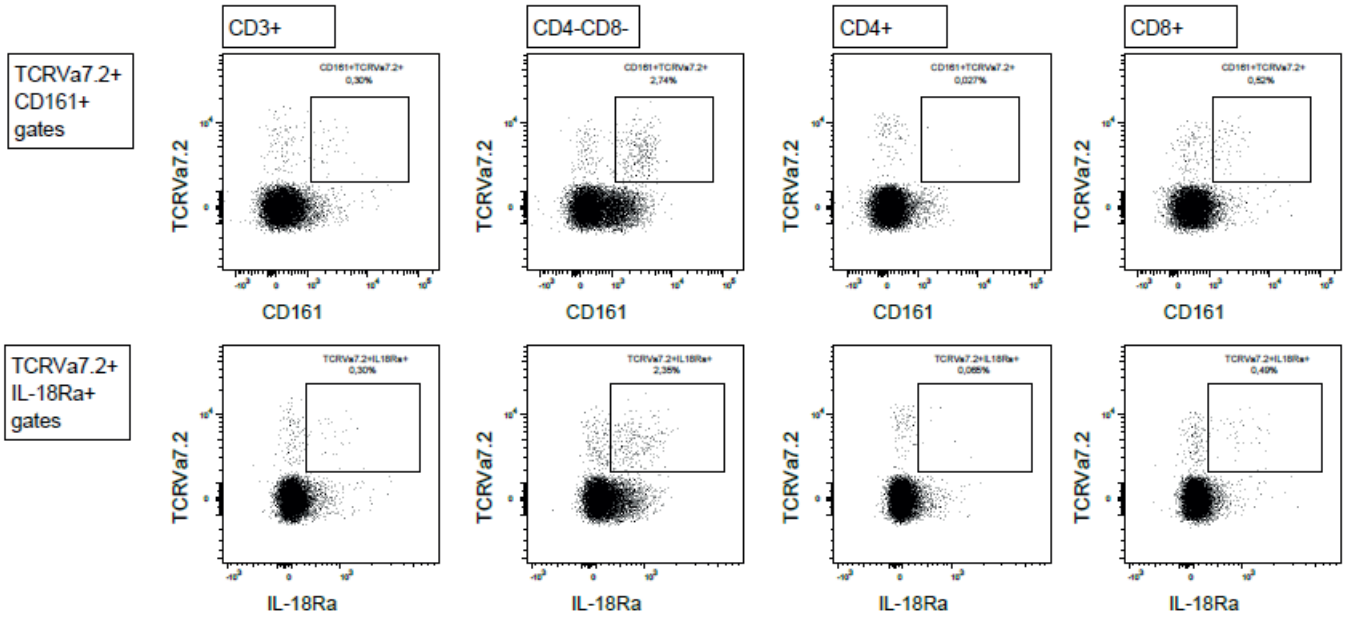
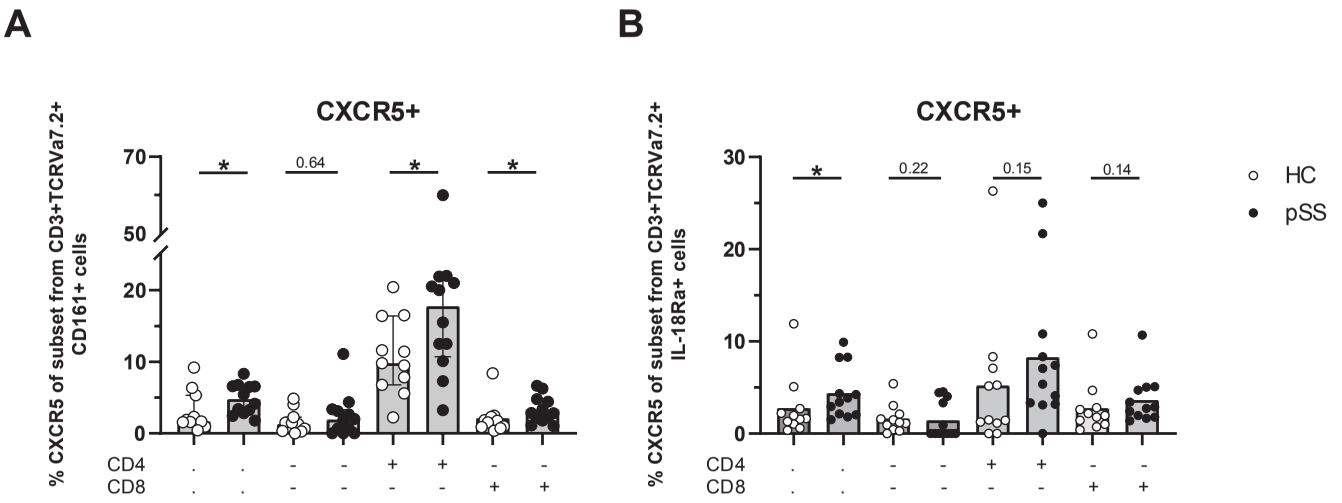
Supplementary Material

Marker	Fluorochrome	Company	Clone
CD3	AF700	Sony Biotechnology	UCHT1
CD4	BV785	Biolegend	RPA-T4
CD8	PE	Biolegend	RPA-T8
CCR9	APC	Biolegend	L053E8
CXCR5	PerCP-Cy5.5	Biolegend	J252D4
CD45RO	BV711	BD Horizon	UCHL1
CD127 (IL-7Rα)	PE	BD Phamingen	HIL-7R-M21
CD161	BV510	BD Horizon	DX12
TCRVα7.2	PE-Cy7	Biolegend	3C10
IL-18Rα (CD218α)	FITC	eBioscience	H44
IL-21	PE	BD Pharmingen	3A3-N2.1
IFN-γ	FITC	BD FastImmune	25723.11
Fixable Viability Dye	eF780	eBioscience	n.a.

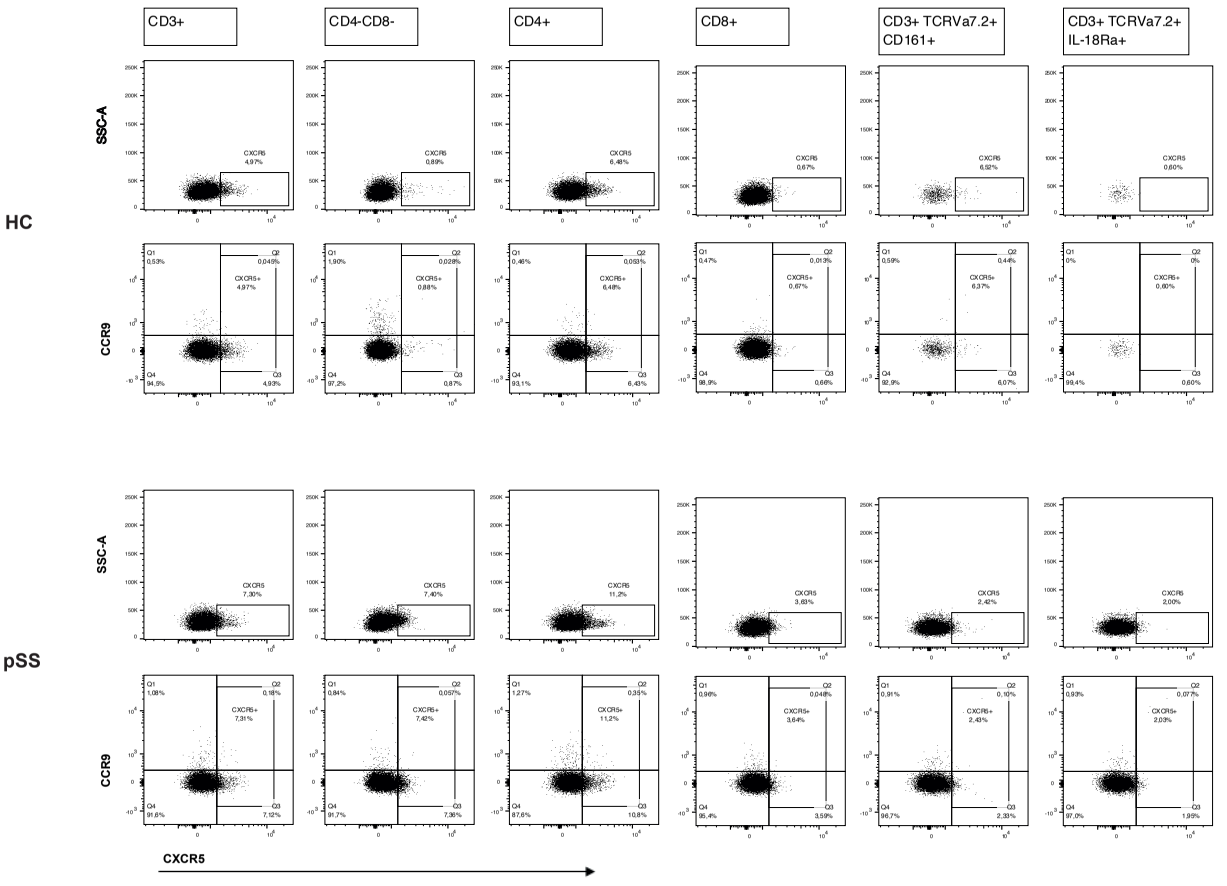
Supplementary Table 1 | Antibodies used for flow cytometry.



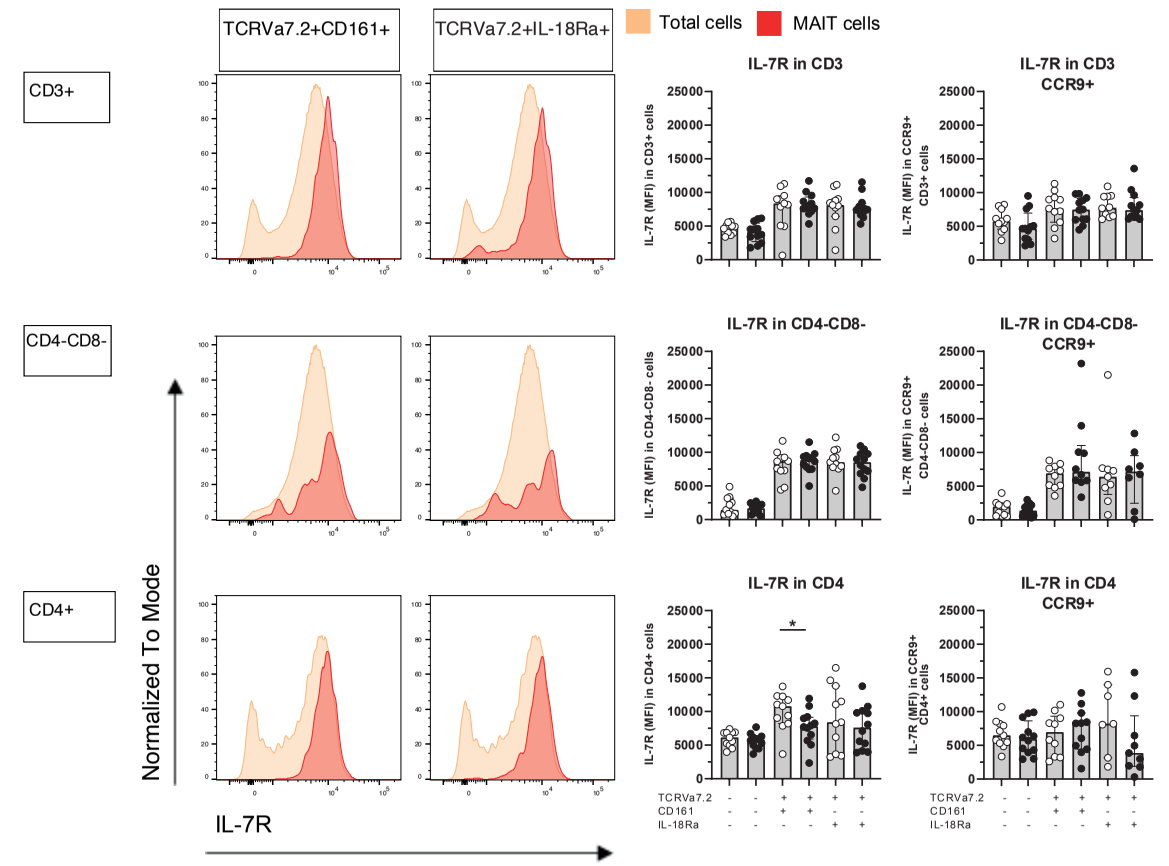
Supplementary Figure 1 | Gating strategy MAIT cells. Representative flow cytometry plots and gating of CD161+ and IL-18Rα+ TCRVα7.2+ MAIT cells within CD3, CD4-CD8-, CD4+, and CD8+ T cells from a representative pSS donor.



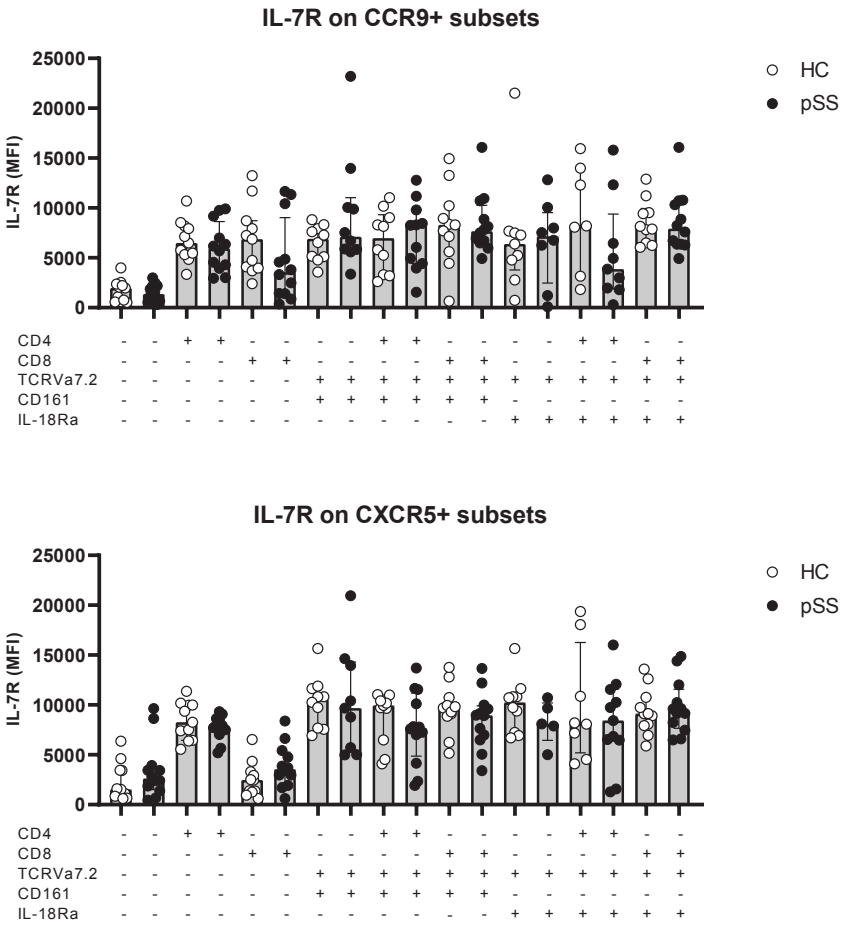
Supplementary figure 2 | Increased frequencies of CXCR5-expressing cells among circulating MAIT cells in pSS patients. (**A**) CXCR5 expression in TCRVα7.2+CD161+ MAIT cells, and CD4/CD8 TCRVα7.2+CD161+ MAIT subsets. (**B**) CXCR5 expression in TCRVα7.2+IL-18Rα+ MAIT cells, and CD4/CD8 TCRVα7.2+IL-18Rα+ MAIT subsets. HC: healthy control; pSS: primary Sjögren's syndrome. * indicates statistical significance of p<0.05.



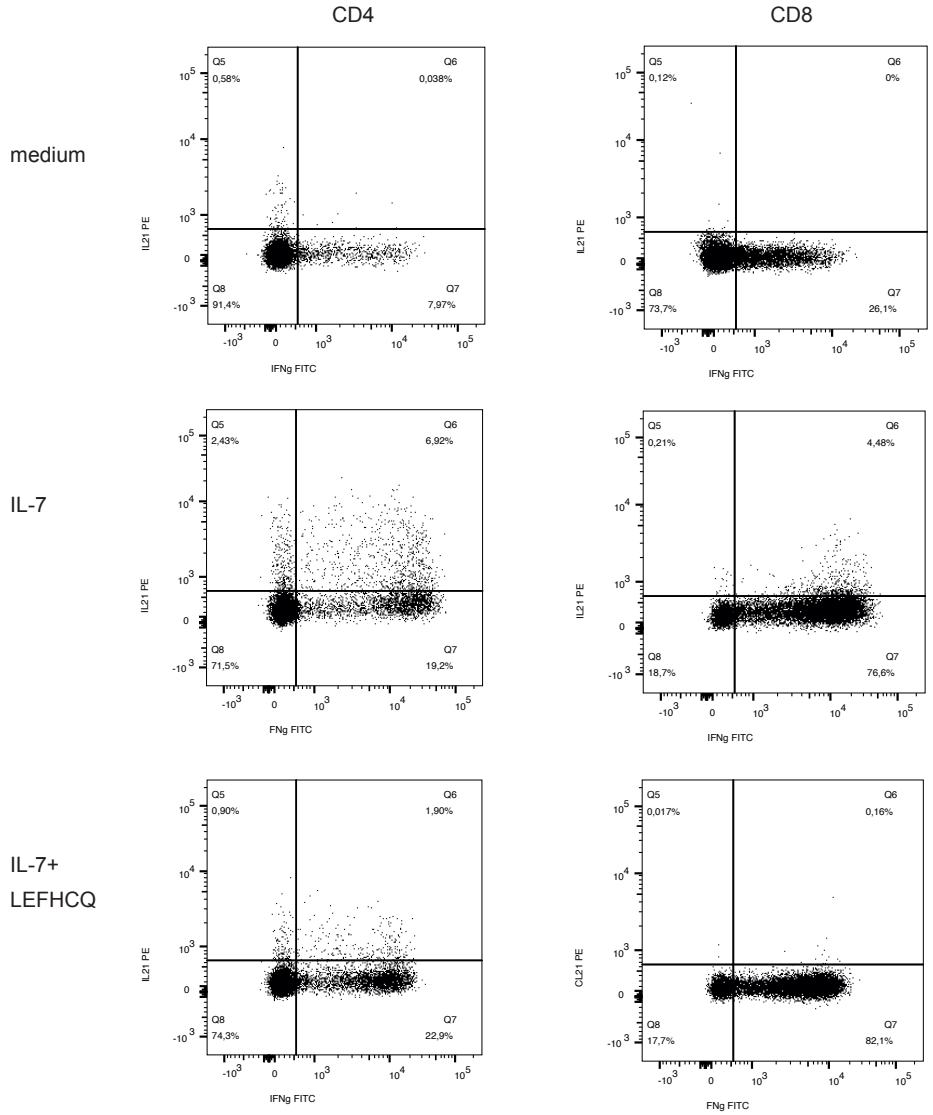
Supplementary figure 3 | Representative flow cytometry plots for CXCR5 staining of MAIT and non-MAIT T cells. Representative dot plots of one HC and one pSS patient. Staining in CD3+, CD4-CD8-, CD4+, CD8+, TCRVα7.2+CD161+, and TCRVα7.2+IL-18Rα+ populations. HC: healthy control; pSS: primary Sjögren's syndrome.



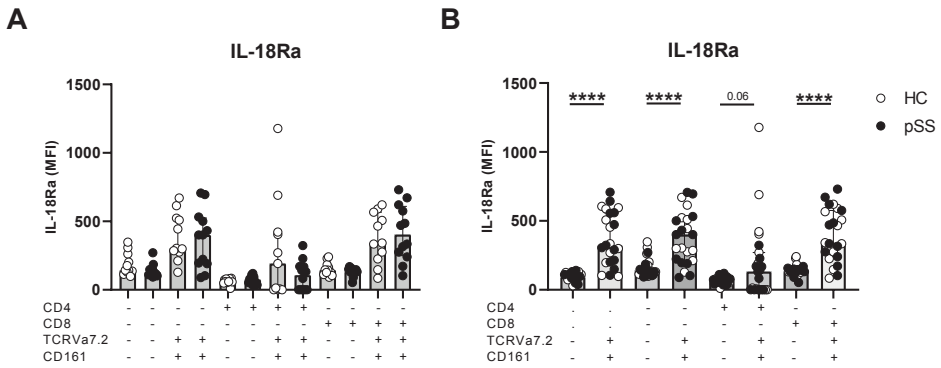
Supplementary figure 4 | Expression of IL-7R is increased in CD3, CD4-CD8- and CD4 MAIT cell subsets. Representative histograms of IL-7R expression on CD3+, CD4-CD8-, and CD4+ CD161+ MAIT cells, and IL-18Rα+ MAIT cells compared to IL-7R expression on CD3, CD4-CD8-, and CD4+ total populations. Bar plots showing medians (interquartile range) of IL-7R on CD3, CD4-CD8-, and CD4 cells, and related CCR9+ (MAIT) cell populations in pSS patients and controls. HC: healthy control; pSS: primary Sjögren's syndrome.* indicates statistical significance of $p<0.05$.



Supplementary figure 5 | Expression of IL-7R is similar in CCR9+ and CXCR5+ MAIT cells. Expression of IL-7R on CCR9-expressing cells in CD4/CD8-defined cell subsets, and in CD4/CD8-defined CD161+ and IL-18Rα+ MAIT cell subsets. Bar graphs show medians with interquartile ranges. HC: healthy control; pSS: primary Sjögren's syndrome.



Supplementary figure 6 | Expression of IL-21 and IFN-γ in CD4 and CD8 cells under different culture conditions. Dot plots of unstimulated cells, IL-7 stimulated cells and IL-7 stimulated cells in the presence of leflunomide and hydroxychloroquine are shown for CD4 and CD8 T cells.



Supplementary figure 7 | Expression of IL-18Rα is similar between pSS patients and controls, and elevated in MAIT cells compared to non-MAIT cells. (**A**) Expression of IL-18Rα on CD4-CD8-, CD4+, and CD8+ non-MAIT and CD161+ MAIT cells in pSS patients and HC. (**B**) Expression of IL-18Rα on CD3+, CD4-CD8-, CD4+, and CD8+ non-MAIT and CD161+MAIT cells, data from pSS patients and HC pooled. Bar graphs show medians with interquartile ranges. HC: healthy control; pSS: primary Sjögren's syndrome. **** indicates statistical significance of p<0.0001.

Chapter 7

Untargeted metabolomics analysis of sebum: a non-invasive strategy that shows potential to identify new biomarkers in primary Sjögren's Syndrome and systemic sclerosis

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Manuscript in preparation

Abstract

Objectives. To evaluate if metabolome analysis of sebum can be used as a non-invasive method to identify skin metabolic signatures in patients with primary Sjogren's syndrome (pSS) as compared to healthy controls and other systemic autoimmune diseases.

Methods. Untargeted metabolomics of sebum samples collected from sebutapes placed on the forehead was performed using mass spectrometry. Sebum metabolomes of healthy controls (HC) and pSS patients were compared. Additionally, the metabolomes of two other systemic autoimmune diseases, i.e. systemic sclerosis (SSc) and systemic lupus erythematosus (SLE), were compared to pSS and HC.

Results. Modest significant differences were observed between the sebum metabolome of pSS patients as compared to HC. Unsupervised dimensionality reduction using UMAP showed no strong differences between metabolic signatures of pSS patients and HC. However, correlation analyses of metabolic changes and disease activity markers did identify several metabolite ions with a good correlation with disease activity. More robust differences, associated with metabolites involved in neurotransmission, were identified in patients with systemic sclerosis. Interestingly, when analyzing differentially expressed metabolite ions (DEMs) of pSS and SSc patients relative to HC robust and strong correlations were observed.

Conclusion. This pilot study demonstrates that sebum metabolomics might be a novel strategy to identify biomarkers in pSS and other (systemic) autoimmune diseases. Larger follow-up studies using more targeted metabolomics strategies that take into account potential confounding factors and optimize sebum collection should demonstrate the feasibility of this novel non-invasive method in molecular monitoring of disease.

Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by exocrine gland inflammation with lymphocytic infiltrates.(1,2) Subsequently, typical symptoms include ocular and oral sicca symptoms due to lacrimal and salivary gland dysfunction.(1) Another hallmark feature of pSS is B cell hyperactivity, which is associated with an increased risk of lymphoma development.(1,3,4)

In recent decades the use of multiple technological platforms has provided helpful insights into the immunopathology of primary Sjögren's syndrome. Phenotypic, proteomic, transcriptomic and metabolomic analyses of the immune cells from the inflamed tissue and blood have shown to be of value to determine the immunopathological mechanisms that underly pSS.(5–12) A downside of these techniques is that invasive procedures such as venipuncture and tissue biopsies are necessary for cellular and molecular analyses. Saliva and (to a lesser extent) tear collection are considered less invasive. Using metabolomics on saliva has shown potential in establishing metabolic signatures of pSS.(13,14) However, saliva and tears have as a major drawback in their collection that a substantial proportion of pSS patients suffers from severe dryness, implicating that the patients with most sicca complaints cannot be studied. Thus, novel non-invasive strategies applying state-of-the-art technologies should be welcomed.

Sebaceous glands are exocrine glands producing sebum. With its mixture of lipids sebum moisturises the skin and provides a layer of protection for the skins barrier function.(15) Studies using sebum have been performed in dermatological conditions, e.g. determining sebum lipid consistency in atopic dermatitis or juvenile acne.(16,17) Recently one study using sebum metabolomics has shown lipid dysregulation in Parkinson's disease and identified potential biomarkers for this disease.(18) Sebum or sebaceous glands have barely been studied in pSS. To our knowledge only a few publications studied this so far, demonstrating that no pathological alterations were detected in a small group of pSS patients compared to healthy controls (HC) and that corneometry and evaporimetry measures in pSS and HC were not associated with morphological alterations or reduction in number of sebaceous glands as compared to HC.(19,20) Nonetheless, as sebum offers potential for molecular analyses and can be easily sampled it might represent an opportunity to identify disease biomarkers, without using invasive procedures. In this study we set out to evaluate if sebum metabolomics can be used as a non-invasive method to identify a skin metabolic signature for pSS. To study whether we could identify a pSS-specific signature we compared metabolic signatures from pSS patients with those from systemic sclerosis (SSc) and systemic lupus erythematosus (SLE) patients.

Methods

Patients and healthy controls

We included 32 pSS patients and 17 HC. In addition, disease specificity was assessed by comparing pSS sebum metabolome with those from SSc patients (n=21) and SLE patients (n=8). All patients were diagnosed by a rheumatologist and met the disease specific classification criteria, i.e. 2016 ACR/EULAR criteria, 2013 ACR/EULAR criteria and 2019 EULAR/ACR criteria for pSS, SSc and SLE, respectively.(21–23) Inclusion occurred between March and June 2019. All patients and healthy controls were included at the University Medical Center (UMC) Utrecht, the Netherlands. Demographics and clinical data are shown in Table 1. The UMC Utrecht Medical Research Ethics Committee ("METC") evaluated the study as low-risk, without requiring further evaluation according to the Dutch Medical Research Involving Human Subjects Act ("WMO") by the committee (study number 19/092). All participants gave written informed consent.

Sebum collection

All participants avoided using facial products for 24 hours and did not wash their forehead up to 3 hours prior to sample collection. Before sebum collection the participant's forehead was systematically cleaned with soap (Dove™ Deeply Nourishing) and water. After which 2 Sebutape Adhesive Patches for collecting sebum (Clinical & Derm) were applied to the forehead using tweezers (sebutapes were applied simultaneously). After 5 minutes the sebutapes were removed with tweezers and collected on a microscope slide, covered with another microscope slide and packed in parafilm. This package was kept on dry ice until storage at -80 °C. The overall workflow is schematically presented in Figure 1.

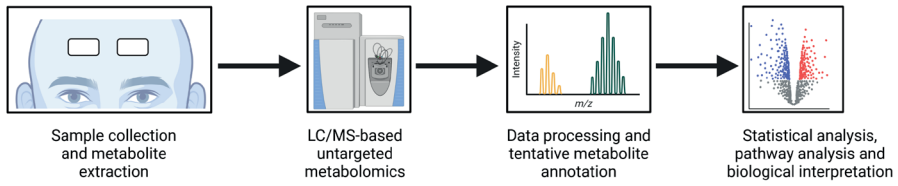


Figure 1 | Sebum metabolomics workflow. Schematic overview of study workflow. LC/MS: liquid chromatography mass spectrometry; m/z: mass/charge. *Figure created with Biorender.com*

Sample extraction, LC/MS and data quality control

Detailed methods on sample extraction, LC/MS and data quality control measures are described in Supplementary File 1. In summary, untargeted metabolomics analysis of extracted sebum was performed using a previously described platform.(24) Spectral data including peak detection and alignment was performed using an automated pipeline in R that was designed analogously to the previously published MATLAB version(25), and several data quality control measures were taken. Importantly, detected ions were tentatively annotated as metabolites based on matching accurate mass within a tolerance of 5 mDa using the Human Metabolome database as reference.(26) Summary statistics on the acquired datasets are provided in Supplementary Table 1.

Data analysis

Data analysis was performed in Rstudio version 2021.09.1 running R version 4.1.2 using functions embedded in the readxl, xlsx, pracma, mzR, MALDIquant, MALDIquantForeign, tcltk2, snow, rhdf5, preprocessCore, robustbase, gplots, VennDiagram, SIBER, venneuler, qvalue, RColorBrewer, ellipse, scales, rgl, mixOmics, viridis and umap packages. Differential testing was performed using unpaired two-sided t-tests ("Welch's t-test"). In some cases, statistically significant hits were filtered based on additional criteria such as fold change as indicated. Metabolic pathway enrichment (PWE) analysis was performed based on KEGG pathway definitions, with isobaric pathway metabolites collapsed to reflect the inability of our metabolomics approach to distinguish between isomers.(27) Using these pathway definitions, hypergeometric testing based on significant differential annotated metabolite ions was performed, with resulting enrichment p-values corrected for multiple hypothesis testing using Benjamini's and Hochberg's approach. For further evaluation of metabolite ions contributing to enrichment of pathways, we used a threshold of a minimum of two involved metabolite ions, with at least 20% contribution to the pathway. To quantify correlations between metabolite ion intensities and clinical variables, Spearman's rho was calculated.

To capture as much of the biochemical diversity of the polar sebum metabolome as possible, we analyzed samples in both negative and positive ionization mode. Due of the propensity of many metabolites to preferentially ionize in one of the two modes, the two resulting datasets are expected to provide partially overlapping and partially complementary insights into the sebum metabolome. In order to highlight findings and avoid repetition of information, we have chosen to describe results from negative ionization mode in the main text of this paper and refer to results from positive ionization mode in Supplementary Figures and Supplementary Files 2-10 unless stated otherwise.

		HC (n=17)	pSS (n=32)	SSc (n=21)	SLE (n=8)
General	Female gender, n (%)	17 (100)	32 (100)	16 (76)	6 (75)
	Age (years), mean ± SD	53 ± 8	59 ± 11	53 ± 12	46 ± 11
	Immunosuppressant use, n	-	9	11	7
pSS specific characteristics	Anti-Ro/SSA positive, n (%)	-	27 (84)	-	-
	Lymphocytic focus score (foci/4mm2), median (IQR)	-	2.0 (1.3 – 3.0)	-	-
	Schirmer positive (≤5mm/5min, ≥1 eye), n (%)	-	17 (94)	-	-
	Serum IgG (g/L), median (IQR)	-	14.1 (12.0 – 17.6)	-	-
	ESSDAI score, median (IQR)	-	6.5 (2.0 – 8.8)	-	-
	ESSPRI score, median (IQR)	-	6.5 (5.7 – 7.3)	-	-
SSc specific characteristics	Clinical domains for SSc:	-	-		-
	Raynaud's phenomenon, n (%)			20 (95)	
	Skin thickening of the fingers (puffy fingers or sclerodactyly), n (%)			18 (86)	
	Fingertip lesions (digital tip ulcers or fingertip pitting scars), n (%)			7 (33)	
	Telangiectasia, n (%)			9 (43)	
	Pulmonary arterial hypertension and/or Interstitial Lung Disease, n (%)			13 (62)	
	Disease phenotype:	-	-		-
	Early SSc, n (%)			4 (19)	
	Limited cutaneous SSc (lcSSc), n (%)			13 (62)	
	Diffuse cutaneous SSc (dcSSc), n (%)			4 (19)	
	Modified Rodnan Skin Score, median (IQR)			3 (0 – 5)	
	Abnormal nailfold capillaroscopy, n (%)	-	-	12 (57)	-
	Scleroderma related antibodies (anti-centromere, anti-topoisomerase-1, anti-RNA-polymerase-III), n (%)	-	-	13 (62)	-
SLE specific characteristics	Immunological domains for SLE:	-	-	-	
	Anti-ds-DNA positive, n (%)				4 (50)
	Anti-phospholipid antibodies, n (%)				1 (20)
	Low C3 and/or C4, n (%)				3 (38)
	Clinical domains for SLE:	-	-	-	
	Constitutional, n (%)				1 (13)
	Hematologic, n (%)				3 (38)
	Neuropsychiatric, n (%)				1 (13)
	Mucocutaneous, n (%)				5 (62)
	Serosal, n (%)				0 (0)
	Musculoskeletal, n (%)				0 (0)
	Renal, n (%)				5 (62)

Table 1 | Patient characteristics and demographic data. Medians with interquartile ranges (Q1-Q3) are shown unless otherwise specified. HC: healthy controls; pSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; IQR: interquartile range.

Results

Differential analysis identifies metabolic profiles associated with disease activity in pSS patients

Unsupervised dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP) showed no clear separation between pSS patients and HC, both in negative (Figure 2A and positive Suppl. Figure 1A) ionization mode. Nevertheless, in both modes differential analysis between pSS and HC identified several significant metabolic changes (Figure 2B, Suppl. Figure 1B and Suppl. File 2). These changes included metabolites from several distinct classes, such as steroid metabolism (e.g. cortisol), fatty acid metabolism (e.g. various acyl carnitines), arachidonic acid metabolism (e.g. leukotrienes) and vitamins (e.g. niacinamide) (Figure 2C and Suppl. Figure 1C). Next we performed a metabolic pathway enrichment (PWE) analysis to estimate whether identified differentially expressed metabolite ions (DEMs) could be categorized into one or more particular metabolite pathways. In negative ionization mode no significantly enriched pathways were found, and in positive mode five significantly enriched pathways were found (Suppl. Figure 2, Suppl. File 3). In pathways that were significantly enriched between pSS patients and HC the difference was driven by only few contributing metabolite ions: m/z=498.268 (leukotriene D4), m/z=403.178 (cortisol), as well as m/z=123.055 and m/z 124.059 (both niacinamide).

Identification of metabolite ions significantly correlating to clinical parameters

Since in our cohort patients with a broad spectrum of disease activity are included, we next performed correlation analysis between all measured metabolite intensities and the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI), serum IgG (sIgG) and lymphocytic focus score (LFS) from labial salivary gland biopsies.

For negative ionization mode a total of 335 metabolite ions significantly correlated to the ESSDAI score (Figure 3A), 13 metabolite ions correlated with sIgG (Figure 3B) and 41 metabolite ions with LFS (Figure 3C). In positive ionization mode similar results were found (Suppl. Figure 3ABC). Correlation plots of the top annotated metabolite ions correlating with each parameter are shown in Figure 3DEF (Suppl. Figure 3DEF for positive mode). Several metabolite ions corresponding to the classes described in the differential analysis between pSS patients and HC correlated with more than one clinical parameter. Examples from negative ionization mode include ions such as steroid tetrahydrocortisol, and fatty acid derivatives hydroxyvalerylcarnitine and N-heptanoylglycine. From positive mode annotated ions such as 18-oxocortisol, aminonicotinic acid/nicotinamide, 20-hydroxy-leukotriene E4, valerylcarnitine and spermidine were found to be significantly correlated to several clinical parameters. Two metabolite ions from negative mode correlated with all

three parameters (m/z=162.093, a pyridine or pyrrolizine derivative, and m/z=173.082, e.g. suberic acid, a fatty acid derivative) and from positive mode data one metabolite ion (m/z=461.249, e.g. 3-Sulfodeoxycholic acid, a type of bile acid which could be produced by skin microbiota) (Suppl. File 4 and Suppl. Figure 4).

To understand if pathways in disease activity associated metabolites may underlie the observed changes we performed PWE analyses to investigate potential group function of the metabolite ions identified in these comparisons. Two significantly enriched pathways were identified correlating to the ESSDAI score: alpha-linolenic acid metabolism and phenylalanine metabolism (Figure 3G). In the alpha-linolenic acid metabolism pathway 4 ions which can be summarized to 2 fatty acid derivatives are found: m/z=211.134 and m/z=212.137 (both annotated as 3-Ethenyl-4-hydroxy-2,5-dimethylhex-5-en-2-yl acetate) and m/z=171.103 and m/z=172.106 (both annotated as 9-Oxo-nonanoic acid) (Figure 3H). Of note, m/z=212.137 was the annotated metabolite ion with the highest correlation to a clinical parameter (i.e. ESSDAI) and was differentially expressed between pSS patients and HC. From the 11 metabolite ions identified in the enriched phenylalanine metabolism pathway several are compounds directly involved in this pathway, such as m/z=166.059 (L-3-Phenyllactic acid) and m/z=179.035 (4-Hydroxyphenylpyruvic acid) (Figure 3I).
In the PWE analyses for sIgG and LFS significantly enriched pathways were based on few individual metabolite differences (Suppl. Figure 5). Full details of identified metabolite ions and their correlation to clinical parameters can be found in Suppl. File 5. Of note, despite not being formally enriched in individual pathways, multiple metabolite ions that significantly correlated with clinical parameters could be annotated as chemically related metabolite classes such as steroids, leukotrienes, acyl carnitines as well as fatty acid derivatives.

Pathway enrichment analysis reveals metabolic signatures for SSc and SLE patients and metabolites that relate to significantly enriched pathways

Since skin involvement is not considered a hallmark disease feature of pSS, we also included patients with the systemic autoimmune diseases systemic sclerosis (SSc) and systemic lupus erythematosus (SLE). Phenotypically patients with these diseases more often show skin involvement, therefore we expected to find a stronger metabolic signature when performing sebum metabolomics and included these patients as a potential positive control.
Firstly, we repeated the UMAP analysis including SSc and SLE patients, which similarly to pSS patients showed no clear separation of the SSc and SLE metabolome as compared to HC on group level (Figure 4A negative mode and Suppl. Figure 6A positive mode). Differential analysis between SSc and HC and between SLE and HC showed several metabolite ions with trending metabolic changes in both modes (Figure 4B SSc vs HC and Figure 4C SLE vs HC, negative mode. Suppl. Figures 6BC positive mode).

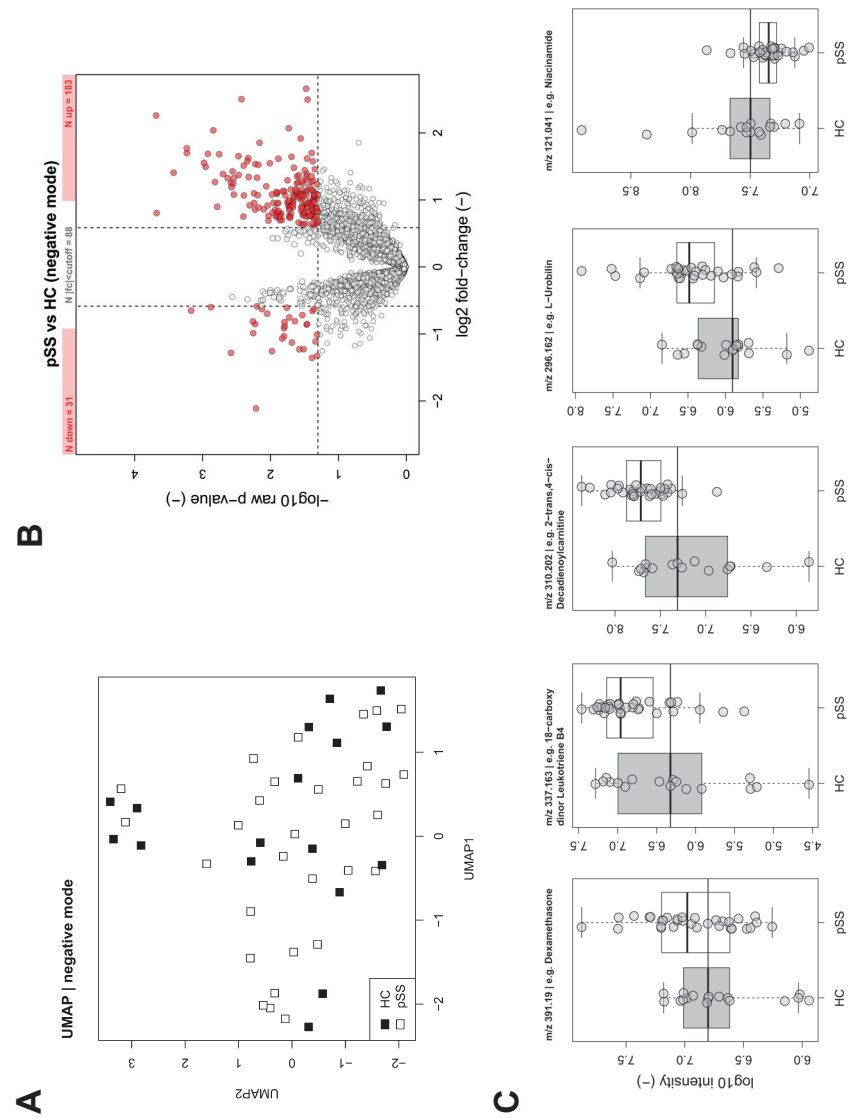
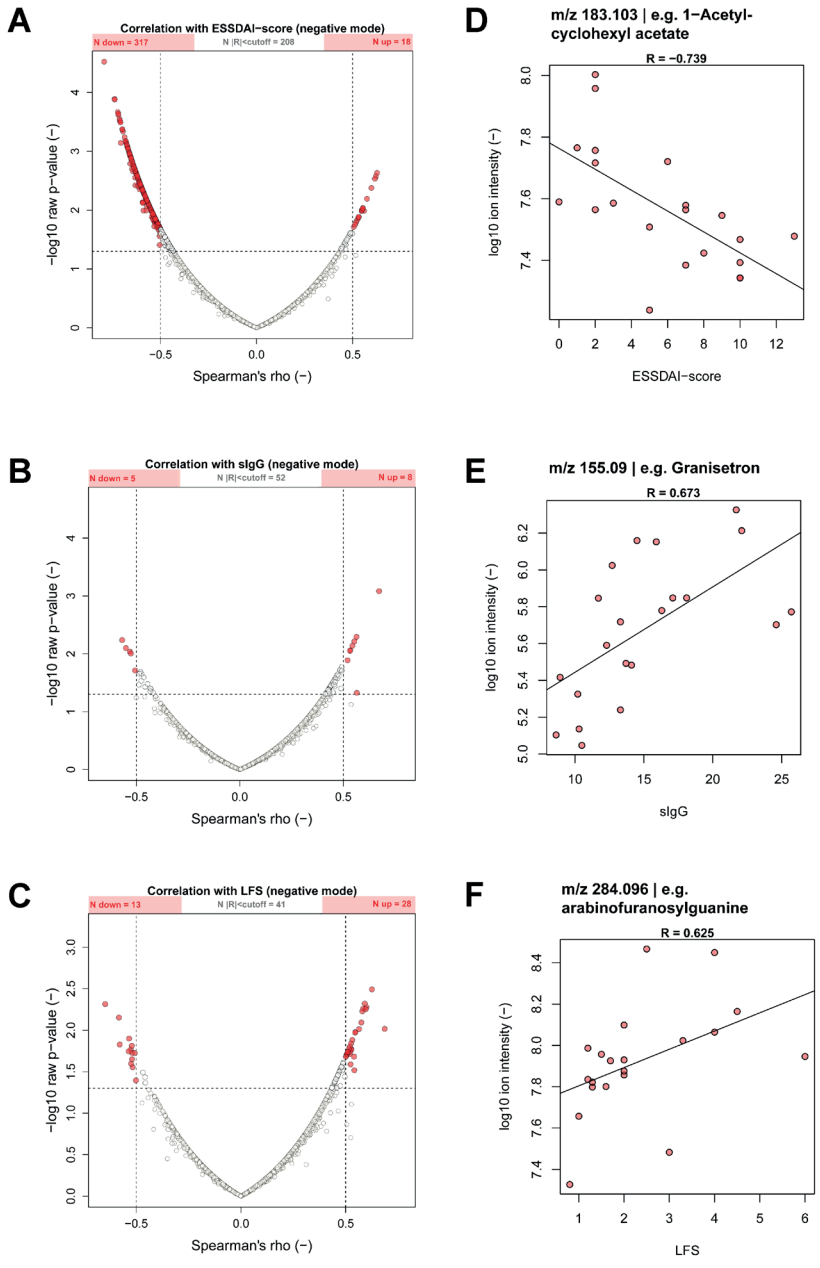


Figure 2 | Differential expression between patients with pSS and HC. (A) Unsupervised dimension reduction by Uniform Manifold Approximation and Projection (UMAP) was performed for all identified metabolite ions of pSS patients and HC. (B) Volcano plot using all identified metabolite ions for differential analysis between pSS patients and HC. Each dot represents a single metabolite ion. Red metabolites are considered differential between HC and pSS patients based on log2(1.5)-fold change (vertical lines) and p-value<0.05 (horizontal line). (C) Boxplot examples of identified annotated DEMs from different metabolite classes. All plots show results from negative ionization mode. HC: healthy controls; pSS: primary Sjögren's syndrome; UMAP: Uniform Manifold Approximation and Projection; m/z: mass/charge; DEMs: differentially expressed metabolites.



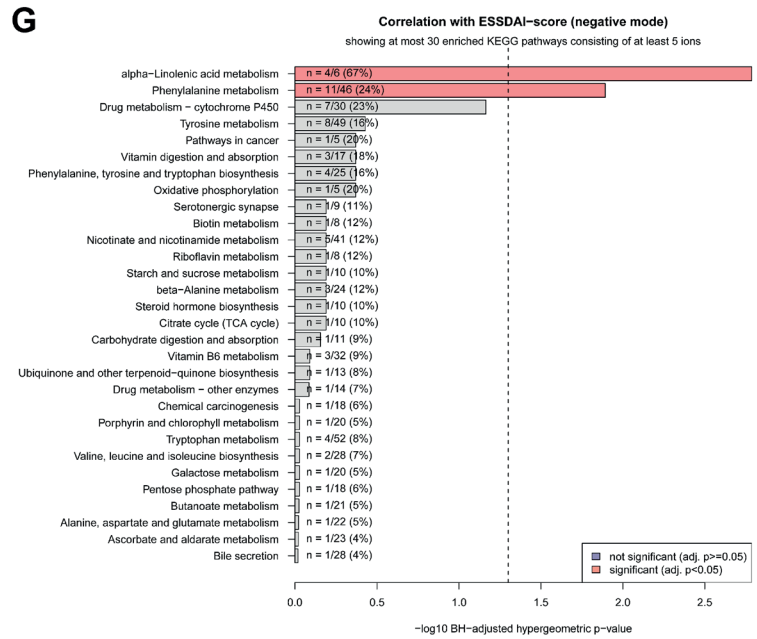


Figure 3 | Correlation of individual metabolite ions with clinical parameters. Spearman's rho correlation of metabolite ions with (A) ESSDAI, (B) serum IgG, and (C) lymphocytic focus score (LFS). Correlation plots of the annotated metabolite ions with strongest significant Spearman rho correlations for ESSDAI, sIgG and LFS (D, E, F, respectively). (G) Result of pathway enrichment analysis with significant correlation to ESSDAI. Correlation plots of metabolite ions from found pathways, alpha-linolenic pathway (H) and phenylalanine pathway (I). All data shown is from negative ionization mode. HC: healthy controls; pSS: primary Sjögren's syndrome; ESSDAI: EULAR Sjögren's syndrome disease activity index; sIgG: serum IgG; LFS: lymphocytic focus score; m/z: mass/charge.

Next we performed PWE analyses for DEMs between SSc vs HC and SLE vs HC. For SSc vs HC this demonstrated pathways associated with gap junction, synaptic vesicle cycle, morphine addiction and Parkinson's disease, all significantly enriched in negative mode (Figure 4D). No pathways were significantly enriched in positive mode between SSc vs HC (Suppl. Figure 7A). For the comparison of SLE vs HC no significantly enriched pathways were identified (Suppl. Figure 7B). When looking at which metabolite ions are involved in the identified significantly enriched pathways, metabolite ions with annotations as L-glutamic acid, noradrenaline, dopamine and 3'-AMP were found (Figure 4E).

Interestingly, when performing a subanalysis for differential expression between HC and SSc patients with the most severe clinical phenotype, diffuse cutaneous SSc (dcSSc), the difference in metabolic signature consists of most DEMs. In negative mode 642 DEMs and in positive mode 666 were found, as compared to 382 and 590 DEMs in SSc overall, for negative and positive mode respectively (Suppl. figure 8 and Suppl. Files 6, 7), identifying similar metabolites (e.g. glutamines and noradrenaline) and enriched pathways as in all SSc patients (all pathways enriched with over 24%, see Suppl. Figure 9 and Suppl. File 8).

Metabolic signatures from pSS and SSc are strongly correlated in contrast to the metabolic signatures of pSS and SLE

To identify commonalities and differences between pSS, SSc and SLE we performed correlations of their metabolic signatures (Figure 5, Suppl. Files 9, 10). Surprisingly, the metabolic signatures of pSS and SSc had a high correlation in both ionization modes (R=0.78 in negative (Figure 5A), and R=0.75 in positive ionization mode (Suppl. Figure 10A)), indicating that these diseases might have a common metabolic dissimilarity as compared to HC. The metabolic signature of SLE patients, had a poor correlation with the metabolic signatures of both pSS and SSc patients (R=0.35 and R=0.03 for pSS and R=0.28 and R=-0.16 for SSc, in negative and positive modes respectively, Figure 5BC and Suppl. Figures 10BC), which might be due to the fact that SLE has little DEMs compared to HC, based on low fold change difference. The top annotated metabolites (based on fold change differences) are shown in Figure 5D. Of note, the two annotated metabolite ions with the biggest negative fold change difference between pSS and SSc metabolic signatures (disease vs HC) are the same in both signatures (m/z=346.237 and m/z=153.019). Few metabolite ions were differentially expressed in all three diseases versus HC: 17 in negative ionization mode and 10 in positive ionization mode. These included metabolites as gentisic acid; 2-trans,4-cis-decadienoylcarnitine and N-heptanoylglycine.

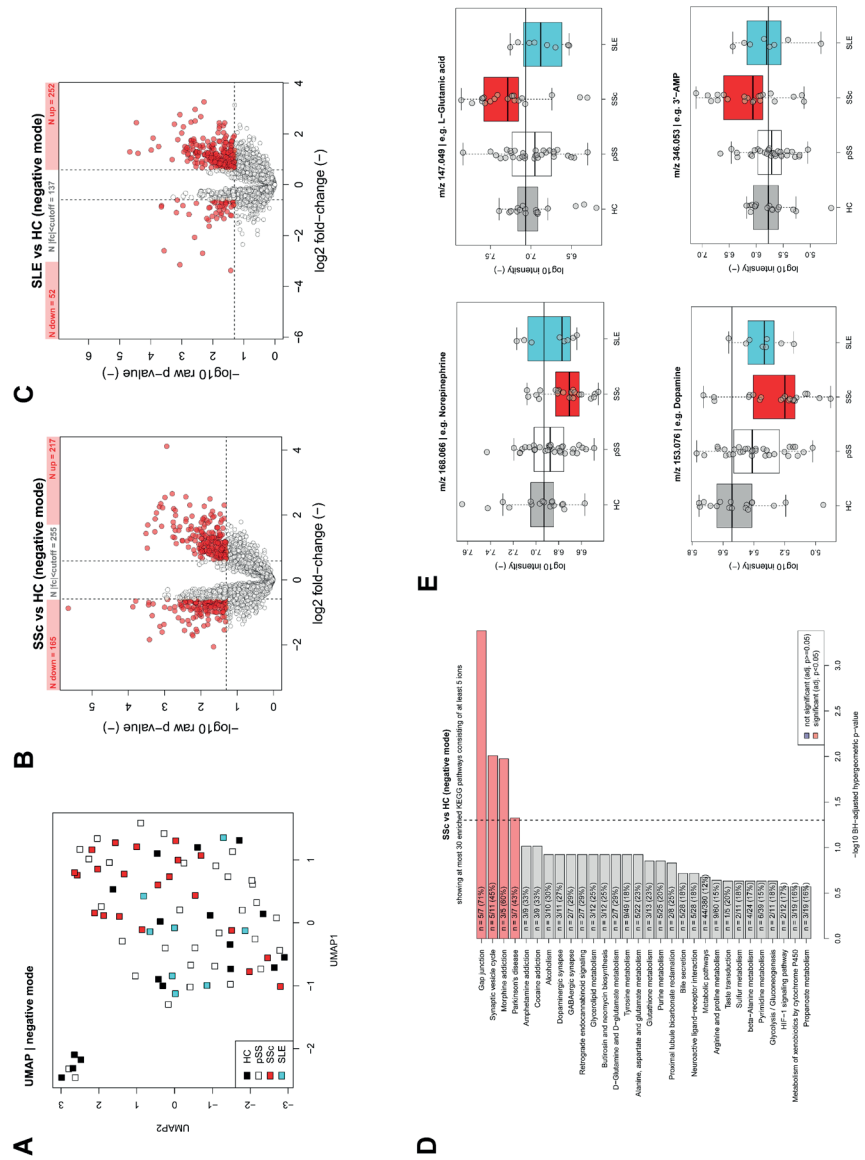


Figure 4 | Metabolomic analysis of sebum from patients with SSc or SLE. (A) Unsupervised dimension reduction by UMAP is shown for all identified metabolite ions of HC, pSS patients, SSc patients and SLE patients. Volcano plots showing differential metabolite ion expression between (B) SSc and HC and (C) SLE vs HC. Each dot represents a single metabolite ion. Ions in red are considered differential between HC and pSS patients based on log2(1.5)-fold change (vertical lines) and p-value<0.05 (horizontal line). (D) Significantly enriched pathways identified with pathway enrichment analysis in SSc are depicted in red. (E) Boxplots of metabolite ions contributing to identified significant pathways. All data shown is from negative ionization mode. HC: healthy controls; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; UMAP: Uniform Manifold Approximation and Projection; m/z: mass/charge.

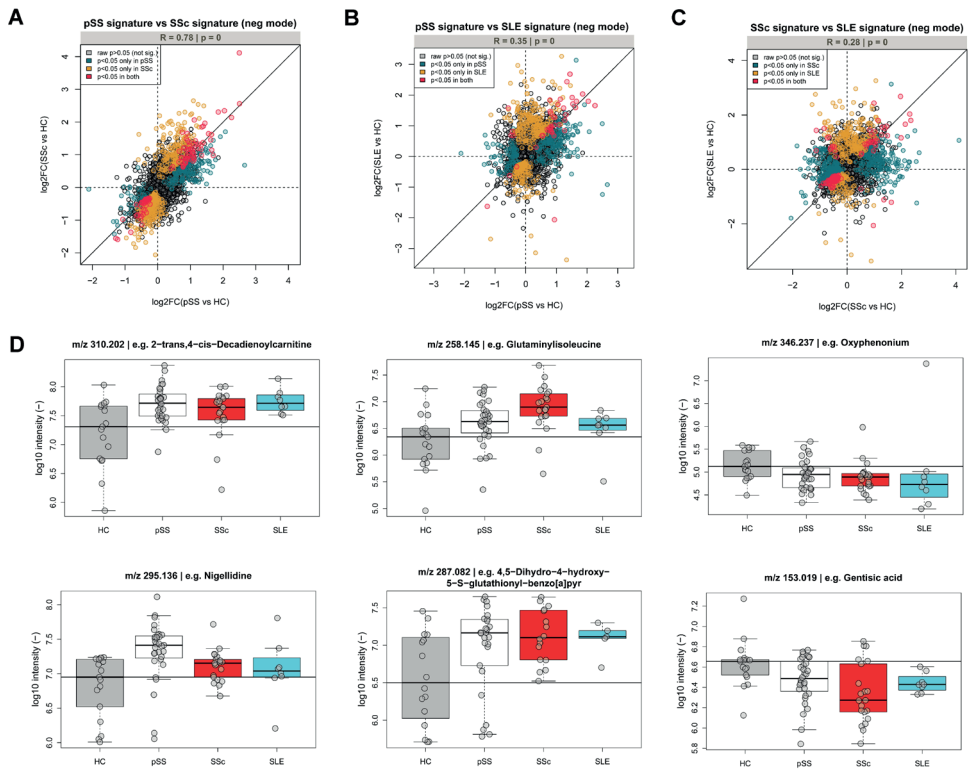


Figure 5 | Coherence of metabolic signatures from patients with pSS, SSc and SLE. Correlation of metabolic signatures between (A) pSS and SSc, (B) pSS and SLE and (C) SSc and SLE. Each disease signature consists of DEMs between the disease and HC. (D) Boxplots of top DEMs with p<0.05 in both pSS vs HC and SSc vs HC signatures (left column top 2 DEMs for pSS vs HC; middle top 2 DEMs for SSc vs HC; right column lowest 2 DEMs, which are similar in both signatures). All data shown is from negative ionization mode. HC: healthy controls; pSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; m/z: mass/charge; DEMs: differentially expressed metabolites.

Discussion

Non-invasive strategies to perform molecular analyses that could help in understanding, delineating, predicting and targeting of disease such as systemic autoimmune disease could be very valuable. Metabolomics of sebum could potentially be such a novel strategy. To our knowledge this is the first study to demonstrate the feasibility of performing metabolomics analysis of sebum in systemic autoimmune diseases. Using an untargeted metabolomics approach we picked up metabolic signatures for pSS, SSc and SLE patients.

Correlation analyses of metabolomic changes and disease activity markers indicated that several metabolites were associated with disease activity. Interestingly, when analyzing DEMs of pSS and SSc patients relative to HC we observed robust and strong correlations between metabolic signatures of pSS and SSc patients.

In our exploratory pilot study we used untargeted metabolomics to capture the full metabolome. This approach ensures that no selection bias was made in our metabolite data and relevant metabolites for these diseases would not be missed. On the other hand the resolution of the mass spectrometry is lower, due to putative annotation variability, leaving some uncertainties as to what would be the correct biological equivalent. In this paper we highlighted only annotated metabolites, but in all comparisons also significant unannotated metabolite ions were found, indicating the further potential of targeted metabolomics. For our exploratory analyses we used nominal p-values to not lose potentially relevant molecular and biological information, at the expense of having potentially false positive observations. Nonetheless, we found confirmation of many metabolites analyzing the data from both ionization modes. In addition, we found multiple metabolites that contributed to enriched pathways and in support of a potential role in the disease we found robust correlations of numerous metabolites with systemic and local clinical parameters. Also, DEMs observed in pSS patients were also detected in SSc patients, thus providing confirmation of observed DEMs in the sebum of these two separate patient groups. Furthermore, we found abundant expression of metabolites that were unique to SSc sebum, strongly enriched for pathways involved in neurotransmission.

Many metabolites can be derived from food intake or environmental factors such as microbes or smoking, potentially influencing disease parameters. It is anticipated that more targeted approaches may map some of these metabolites to factors such as diet, smoking status, or microbiome. In this respect it is interesting that levels of essential fatty acids and amino acids were identified in this study. Using the DEMs between pSS patients as compared to HC several significantly enriched pathways were identified. This included the alpha-linolenic acid metabolism pathway and the phenylalanine pathway. Reduced levels of metabolites in these two pathways have been described earlier in metabolomics analysis of urine and plasma of pSS patients, corroborating our sebum data.(28) Here we for the first time also demonstrate that both pathways correlate to disease activity as measured by ESSDAI scores.
Alpha-linolenic acid (ALA) is an omega-3 essential fatty acid, which enters the human metabolism by eating e.g. seeds and vegetable oils.(29) A recent review has pointed out that higher ALA dietary intake and corresponding elevated ALA blood levels are associated with a reduced mortality rate due to cardiovascular disease.(30) Also as a group omega-3 polyunsaturated fatty acids (PUFAs) are described as anti-inflammatory, with associations between e.g. Western (omega-3 PUFA low) diet and exacerbations of inflammatory bowel

disease, in which a role through activation of PPARγ and downregulation of NF-κB is suggested.(31,32). Hence, the reduced levels of ALA correlating with increased disease activity may point to a role for the ALA pathway in regulation of disease activity in pSS.
Phenylalanine is an essential amino acid and a precursor for several neurotransmitters, such as dopamine and norepinephrine.(33) Previously, dysregulation of the phenylalanine metabolism pathway, and reduced levels of phenylalanine, were found in serum and urine of pSS patients.(28) Dysregulation in this pathway was also found in saliva of pSS patients, but in contrast to serum and urine, phenylalanine concentrations were found to be elevated in the saliva of pSS patients.(34,35) High levels of phenylalanine in saliva were found to be associated with dysbiosis of oral microbiota in periodontitis.(36) Several amino acids, including phenylalanine, were associated to muscarinic M3 receptors, which upon activation may trigger salivary gland secretion.(37) However, in both studies describing elevated phenylalanine in saliva of pSS patients clinical characteristics were not defined and no correlations were found with salivary flow rate.(34,35,38) Hence, it is unclear how the discrepancy between reduced systemic and sebum levels and increased saliva levels can be explained, but this could be related to dryness-associated (immuno)pathology.

In SSc patients the metabolites that were enriched for pathways associated with neurotransmission were related to L-glutamic acid, dopamine, noradrenaline, which are neurotransmitters, and 3'-AMP, a metabolite related to adenosine, which is also a neurotransmitter.(39,40) The finding of neurotransmitters may reflect disturbances in vasculature, like vasoconstriction that is common to SSc patients.(41,42)
L-Glutamic acid, a derivative of glutamate, which we found to be increased in sebum of SSc patients, is considered an excitatory amino acid with effects on vasoconstriction through vasopressin release.(43,44) Glutamate-induced vasoconstriction in the skin was shown, corroborating a potential contribution of glutamic acid.(45)
3'-AMP is part of the 2',3'-cAMP-adenosine pathway, in which extracellular cAMP can be converted to adenosine, that is an inhibitory neurotransmitter.(40) This pathway was previously shown to have a neuroprotective role in mice.(39,40) In addition, 2',3'-cAMP, 2'-AMP and 3'-AMP were shown to have an effect on vascular smooth muscle cells.(46) Under stress these mediators may play important regulatory roles in the skin.Dopamine and norepinephrine levels were decreased in sebum of SSc patients.
Dopamine and norepinephrine are typically considered neurotransmitters mediating vasoconstriction, however, the balance between these neurotransmitters and their receptors is critical for their biological effect, which e.g. can be vasoconstriction or vasodilatation.(47) In line with this suggestion it has been shown that e.g. dopaminergic stimulation can cause relaxation of cutaneous arteries.(48) The concentrations of none of the significantly contributing metabolites in the identified neurotransmission pathways was associated with the mRSS score. This might reflect the differential expression of the metabolites and the complex regulation of the skin vasculature and fibrosis in SSc. Our data suggest that metabolomics analysis of the sebum may provide a valuable tool to understand the immunopathology of the skin of SSc patients.

Our untargeted metabolomics approach yields a broad range of metabolites that highlight some metabolite classes with differences between patients and controls. Metabolism of unsaturated fatty acids (alpha-linolenic acid metabolism), acylcarnitines and metabolites directly involved in the phenylalanine pathway were differentially expressed between pSS patients and controls. Interestingly, metabolites associated with neurotransmission and possibly vasoconstriction were identified in SSc patients.

In conclusion this study demonstrates the potential of sebum as an easily accessible product of the sebaceous glands, to determine metabolite ion abundances in systemic autoimmune diseases such as pSS, SSc and SLE. Future research, using targeted metabolomics focusing on metabolite classes that we have identified, such as carnitines, vitamins, neurotransmitters and metabolites involved in unsaturated fatty acids metabolism might be used to validate the results from this first study. Such research could focus on larger patient groups taking into account patient heterogeneity and potential confounding factors. Finally, sebum metabolomics analysis in systemic autoimmune diseases, that can have overlapping features such as pSS and SSc, may identify shared (immuno)pathological pathways.

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Supplementary Material

Supplementary File 1: Methods and Supplementary Table 1

Sample extraction

Sebutapes were transferred to uncoated 6-well plates (Sigma CLS3516-50EA) kept on wet ice using forceps. The two sebutapes collected from the same donor were combined in the same well. Polar metabolites were extracted by applying 2 mL of a 70/30%-v/v EtOH/H2O solution pre-heated to 75°C followed by a 3 min incubation in a water bath (Heating Bath Circulator S21, Haake) at 75°C with occasional gentle shaking. Plates were subsequently allowed to cool down on wet ice for 5 min and finally incubated at 4°C for another 2 h to complete the extraction process. Supernatants containing extracted metabolites were dried under vacuum in a SpeedVac (UNIVAPO 150 ECH, Uniequip) overnight, before resuspension in 120 µL of LC/MS-grade 30/70%-v/v ACN/H2O solution. To remove any insoluble debris, extracts were filtered using a 96-well filter plate with 1.2 µm pore size (Millipore MSBVN1250) and centrifugation for 2 min at 1,000 g (Multifuge X3R, Heraeus). Filtered extracts were collected in 96-well plates (Thermo Scientific 249944) and sealed with adhesive film (VWR 60941-090) to prevent evaporation. Samples were stored at -80°C for no longer than 1 week until performing metabolomics analysis.

LC/MS

Untargeted metabolomics analysis was performed using a previously described platform. (Vappiani et al., 2021) Briefly, samples were analyzed on a LC/MS system consisting of a Thermo Scientific Ultimate 3000 liquid chromatography system with autosampler temperature set to 10°C coupled to a Thermo Scientific Q-Exactive Plus Fourier transform mass spectrometer equipped with a heated electrospray ion source and operated in negative or positive ionization mode, generating two distinct datasets. For negative ionization mode, the isocratic flow rate was 250 µL/min of mobile phase consisting of 60:40% (v/v) isopropanol: water buffered with 1 mM ammonium fluoride at pH 9 containing 10 nM taurocholic acid and 20 nM homotaurine as lock masses. For positive ionization mode, the isocratic flow rate was 250 µL/min of mobile phase consisting of 60:40% (v/v) methanol: water with 0.1% (v/v) formic acid containing 10 nM taurocholic acid and 20 nM homotaurine as lock masses. For negative mode, mass spectra were recorded in profile mode from 50 to 1000 m/z with the following instrument settings: sheath gas, 15 a.u.; aux gas, 5 a.u.; aux gas heater, 400°C; sweep gas, 0 a.u.; spray voltage, –3 kV; capillary temperature, 250° C; S-lens RF level, 50 a.u; resolution, 70 k @ 200 m/z; AGC target, 3x106 ions, max. Inject time, 120 ms; acquisition duration, 60 s. For positive mode, instrument settings were identical to negative mode except inverted signs of voltages, a spray voltage of 3.5 kV and reduced aux gas heater temperature of 300°C. Spectral data processing including peak detection and alignment was performed using an automated

pipeline in R that was designed analogously to the previously published MATLAB version. (Fuhrer et al., 2011) In brief, the pipeline aligns and averages scans of each individual file, then aligns spectra across all files in the dataset, performs centroiding and peak binning, re-calibrates the mass axis based on the above lock masses and finally assembles a consolidated matrix of ion intensities. Detected ions were tentatively annotated as metabolites based on matching accurate mass within a tolerance of 5 mDa using the Human Metabolome database as reference considering [M-H] and [M-2H] (negative mode) or [M+], [M+H], [M+Na] and [M+K] (positive mode) ions and up to two 12C to 13C substitutions.(Wishart et al., 2018) Importantly, this approach does not enable to conclusively identify metabolites, as for instance metabolites with identical chemical formula, with different adducts or within the 5 mDa annotation window can map to the same ion. Therefore, we aimed to interpret our data conservatively by focusing on most likely annotations, for instance known highly abundant metabolites in conserved metabolic pathways.

Data quality control

To minimize and compensate technical artifacts in the metabolomics dataset, we applied several preventive and corrective measures. First, we randomized the sample injection sequence to prevent technical artifacts from impacting statistical outcomes. Second, we injected each sample twice to improve the accuracy of metabolite ion quantification. Third, we injected at regular intervals three different QC standards (a solvent blank, a defined mixture of highly pure chemicals, a pooled aliquot of all biological samples referred to as "CMATRIX") to control for changes in instrument performance over the course of data acquisition. Fourth, we corrected for systematic trends in metabolite ion intensities over the course of (randomized) acquisition by subtracting residuals obtained by fitting a robust linear model. Fifth, we confirmed that, for both acquired metabolomics datasets, good separation between the above QC samples and biological samples was achieved using unsupervised hierarchical clustering, and that data from analytical replicates of each sample were strongly correlated. Sixth, we calculated the coefficient of variation (CV) of each ion in CMATRIX and biological samples, and removed ions whose CV in biological samples did not exceed the CV in CMATRIX samples. Since the CV in CMATRIX samples estimates only technical variability, any biologically informative ion would be expected to have larger variability as it would be subjected to technical as well as other sources of variability. Seventh, we applied a PCA-based approach to detect and remove outlier samples that were found to lie outside of the 95% confidence interval containing all biological samples in the space spanned by principal components 1 and 2. Summary statistics on the acquired datasets are provided in Supplementary Table 1.

	Negative mode	Positive mode
Number of ions detected	7573	13105
Number of ions remaining after curation (c.f. methods)	4689	6076
Number of ions annotated after curation (c.f. methods)	1400	2303
Number of unique tentatively annotated metabolites	3742	4670
Median metabolite ion variability in CMATRIX (coefficient of variation)	24.2%	18.0%
Median metabolite ion variability in biological samples (coefficient of variation)	71.6%	74.5%
Outlier samples	none	6 (n=2 pSS, n=1 SSc, n=1 SLE, n=2 HC)

Supplementary Table 1 | Statistics on the acquired sebum metabolomics datasets. pSS: primary Sjögren's syndrome, SSc: systemic sclerosis, SLE: systemic lupus erythematosus, HC: healthy controls.

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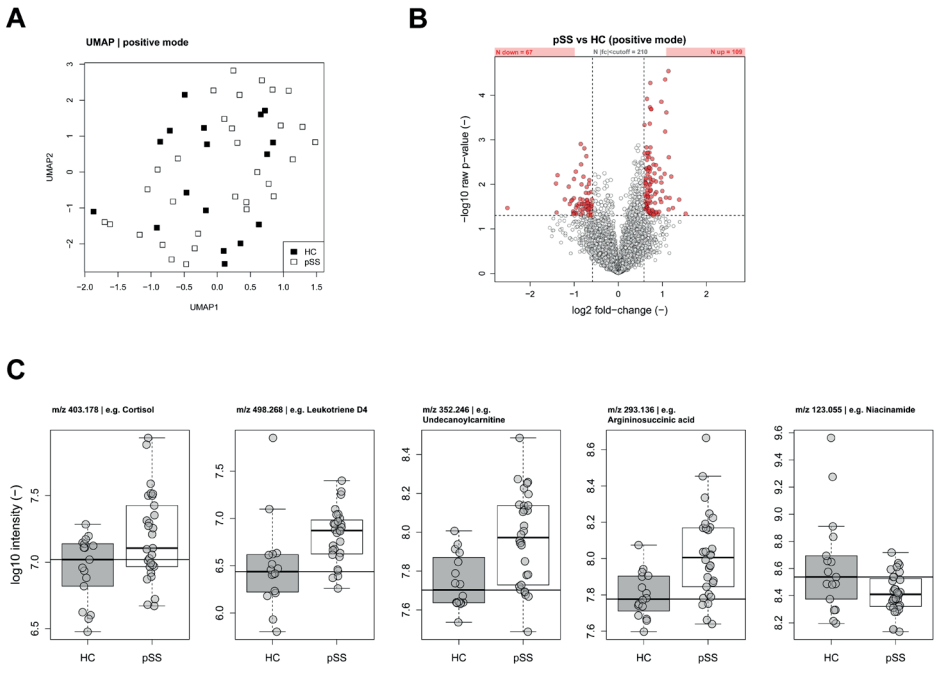
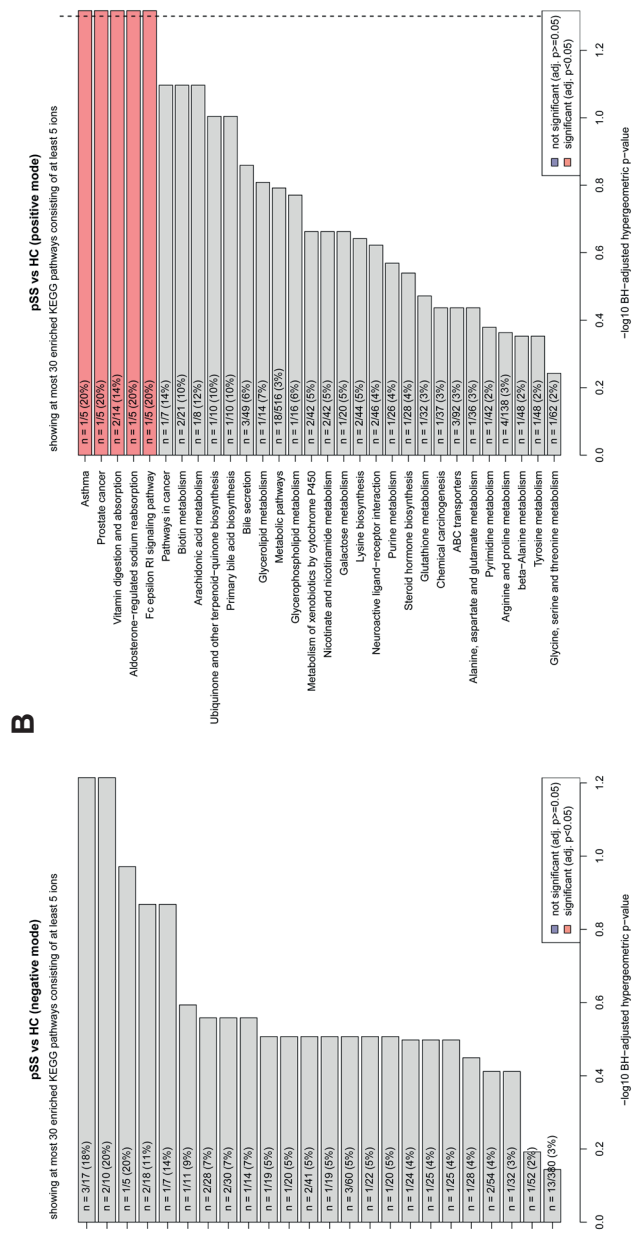
Supplementary Files 2-10 will be available online after publication of the article. Before that time the files are available on request, and will be made available by the authors without undue reservation.

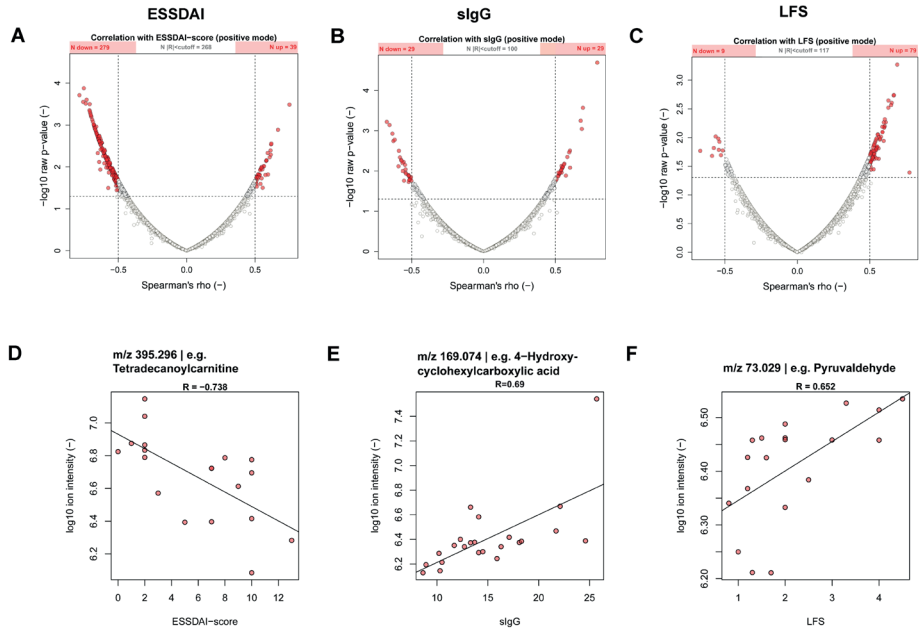
Supplementary Figures



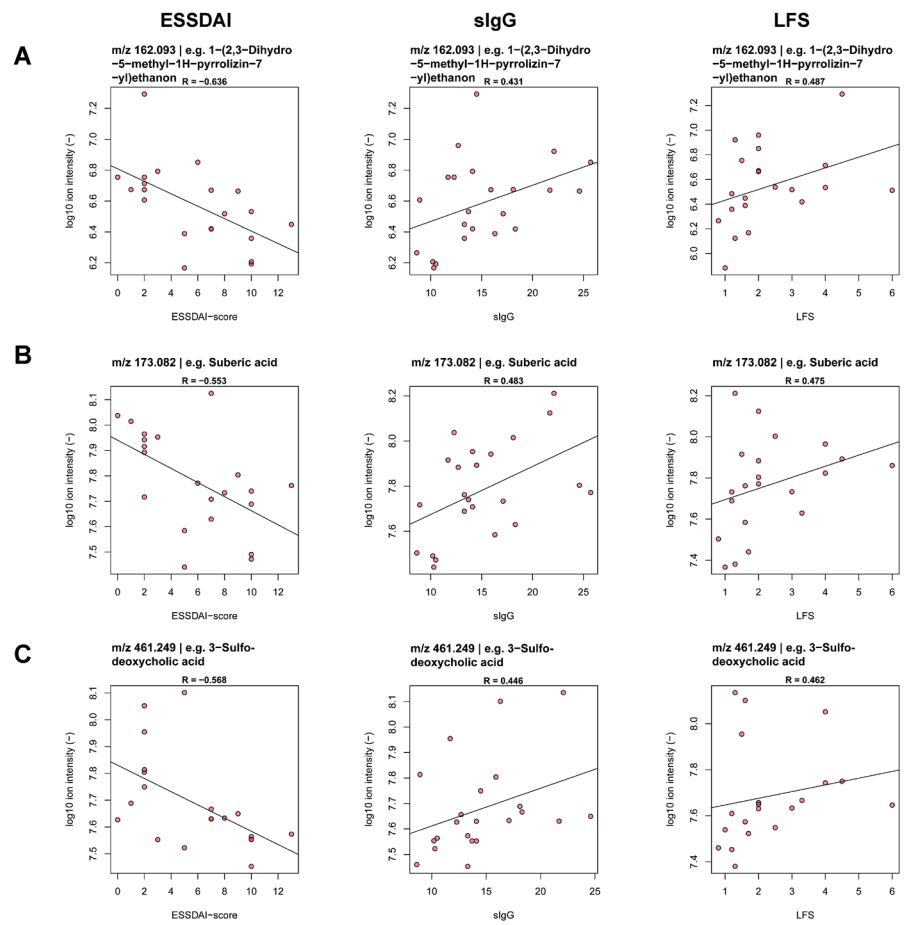
Supplementary Figure 1 | Differential expression between patients with pSS and HC. (A) Unsupervised dimension reduction by Uniform Manifold Approximation and Projection (UMAP) was performed for all identified metabolite ions of pSS patients and HC. (B) Volcano plot using all identified metabolite ions for differential analysis between pSS patients and HC. Each dot represents a single metabolite ion. Red metabolites are considered differential between HC and pSS patients based on log2(1.5)-fold change (vertical lines) and p-value<0.05 (horizontal line). (C) Boxplot examples of identified annotated DEMs from different metabolite classes. All plots show results from positive ionization mode. HC: healthy controls; pSS: primary Sjögren's syndrome; UMAP: Uniform Manifold Approximation and Projection; m/z: mass/charge; DEMs: differentially expressed metabolites.



Supplementary Figure 2 | Pathway enrichment analyses of differentially expressed metabolite ions (between pSS vs HC). Results from pathway enrichment analysis in both (A) negative and (B) positive ionization mode. Significantly enriched pathways are shown in red. HC: healthy controls; pSS: primary Sjögren's syndrome.

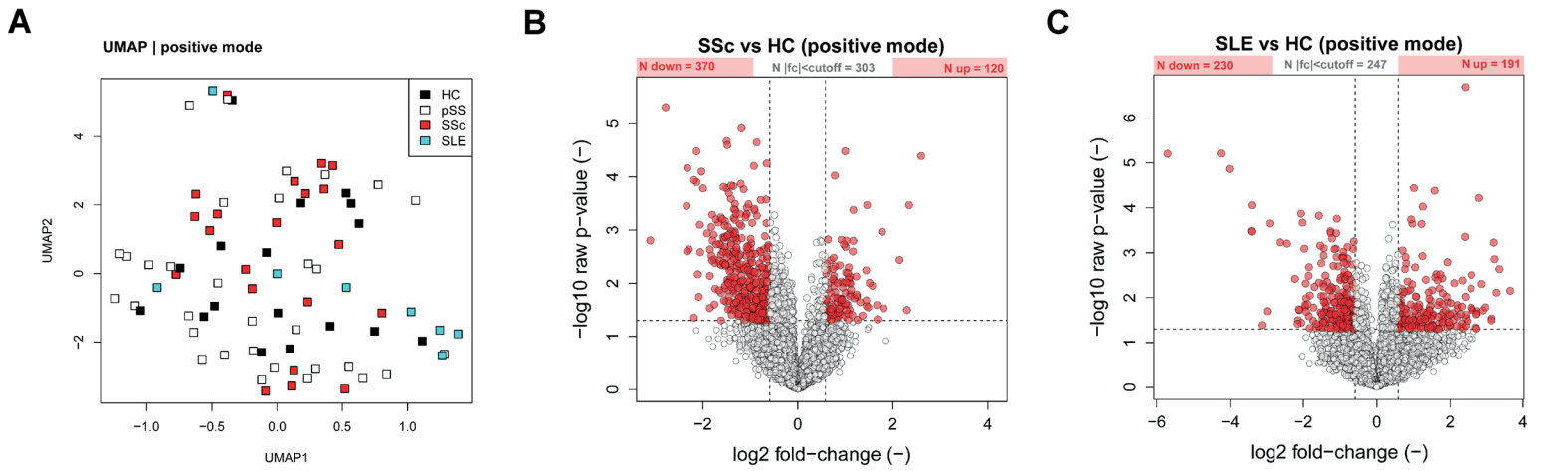


Supplementary Figure 3 | Correlation of individual metabolite ions with clinical parameters. Spearman's rho correlation of metabolite ions in positive ionization mode with (A) ESSDAI, (B) sIgG and (C) LFS. Correlation plot of the annotated metabolite with strongest Spearman rho value for these parameters (DEF). ESSDAI: EULAR Sjögren's syndrome disease activity index; sIgG: serum IgG; LFS: lymphocytic focus score; m/z: mass/charge.

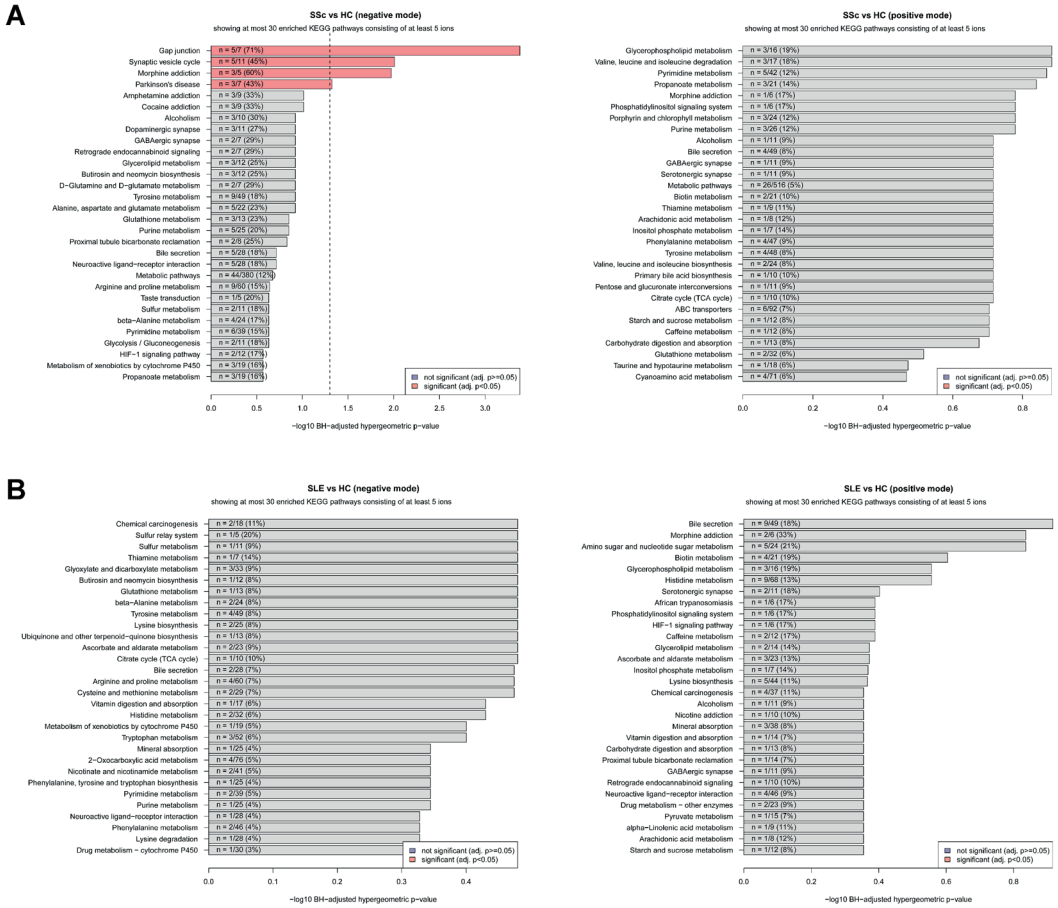


Supplementary Figure 4 | Individual metabolite ions which correlate with all three clinical parameters. Spearman's rho correlation of metabolite ions that correlate with ESSDAI, sIgG and LFS. (A) m/z=162.093; (B) m/z=173.082; (C) m/z=461.249. ESSDAI: EULAR Sjögren's syndrome disease activity index; sIgG: serum IgG; LFS: lymphocytic focus score; m/z: mass/charge.

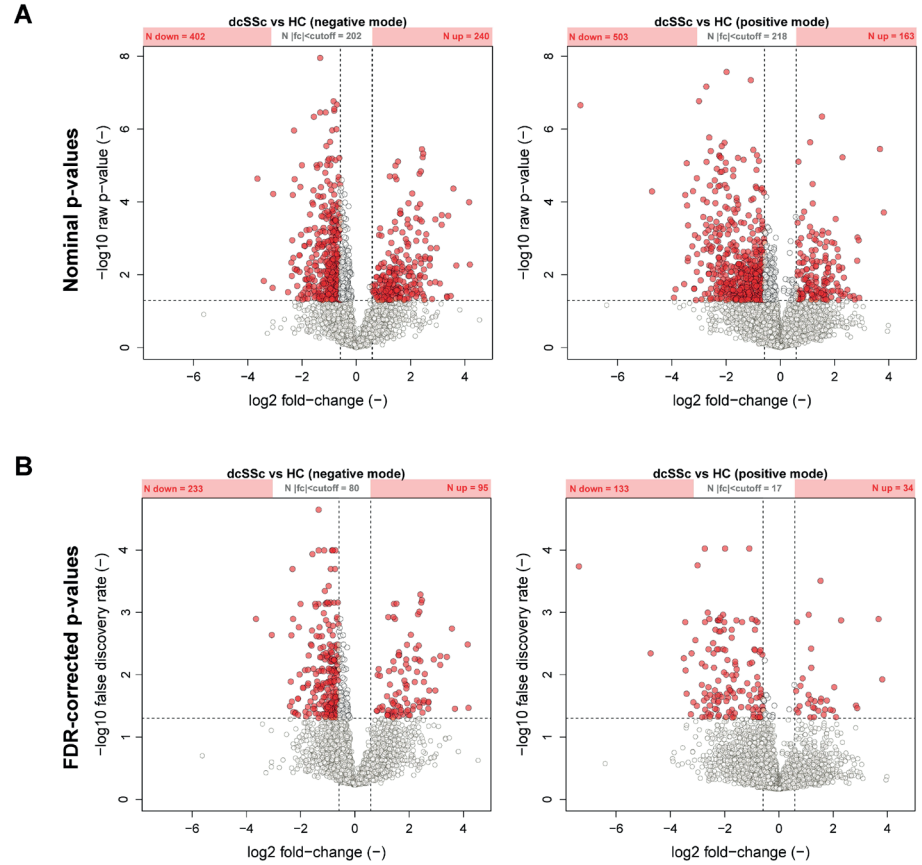
Supplementary Figure 5 | Pathway enrichment analyses of metabolite ions with correlation to clinical parameters. Results from pathway enrichment analysis in both (A) negative and (B) positive ionization mode for ESSDAI, sIgG and LFS. Significantly enriched pathways are shown in red. ESSDAI: EULAR Sjögren's syndrome disease activity index; sIgG: serum IgG; LFS: lymphocytic focus score.



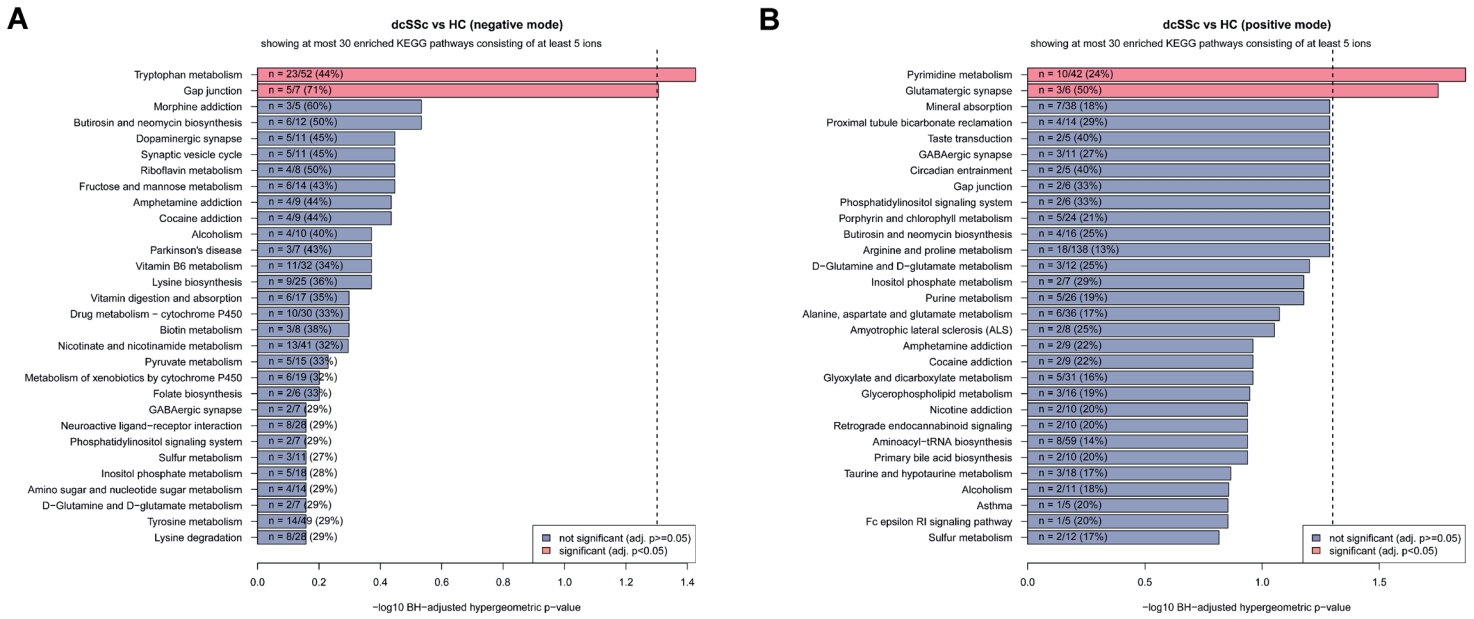
Supplementary Figure 6 | Exploratory analysis of SSc and SLE. (A) For positive ionization mode unsupervised dimension reduction by UMAP is shown. Volcano plots showing differential metabolite ion expression between (B) SSc and HC and (C) SLE vs HC in positive ionization mode. Red metabolites are considered differential between HC and patients with SSc (in B) or SLE (in C) based on log2(1.5)-fold change (vertical lines) and p-value<0.05 (horizontal line). HC: healthy controls; pSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; UMAP: Uniform Manifold Approximation and Projection.



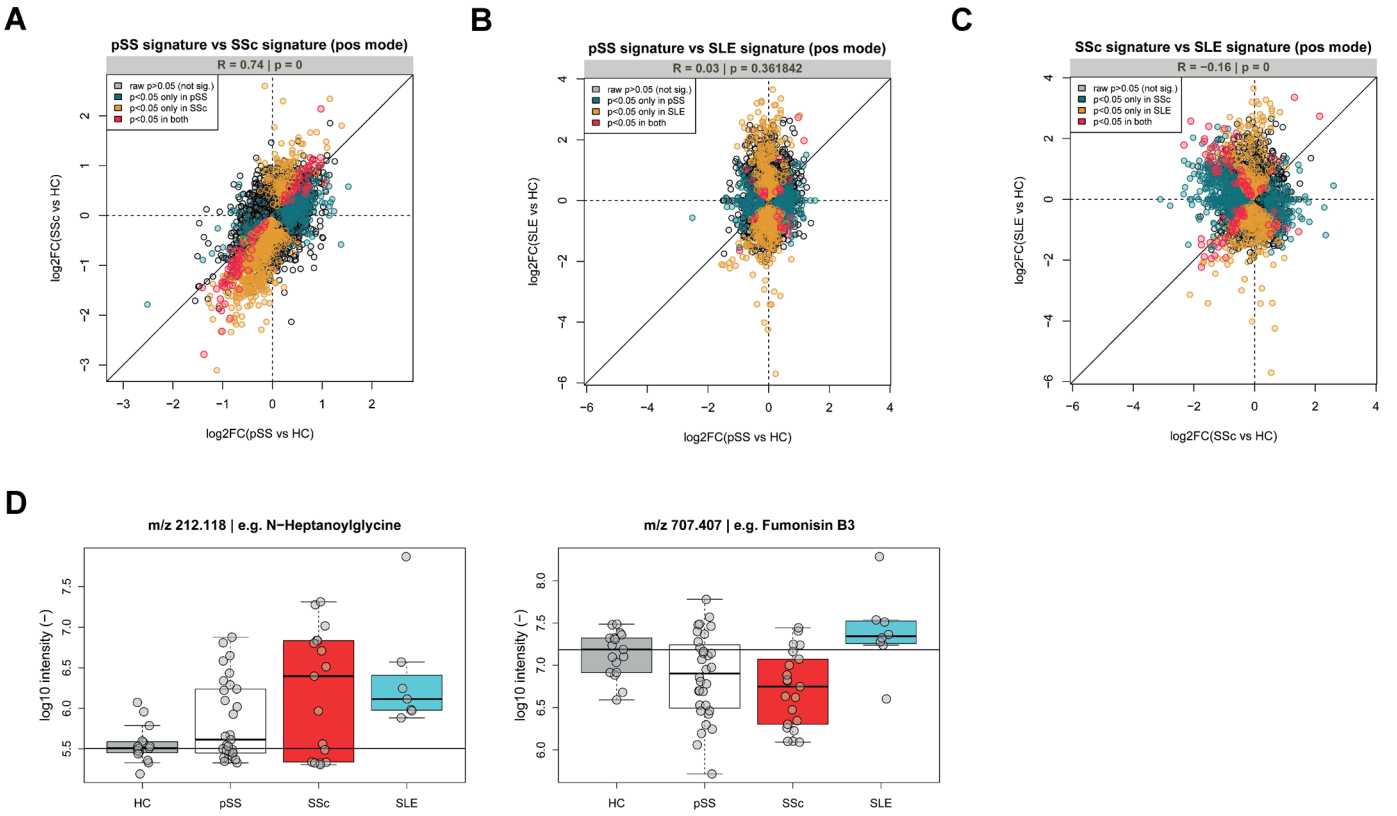
Supplementary Figure 7 | Pathway enrichment analyses using metabolite ions that are differentially expressed in SSc or SLE versus HC. Results from pathway enrichment analysis in both negative and positive ionization mode for (A) SSc and (B) SLE. Significantly enriched pathways are shown in red. HC: healthy controls; SLE: systemic lupus erythematosus; SSc: systemic sclerosis.



Supplementary Figure 8 | Differential analysis between diffuse cutaneous systemic sclerosis (dcSSc) patients and healthy controls. Comparing the most severe SSc disease subtype (dcSSc) to HC, quite some differentially expressed metabolite ions were identified. (A) Nominal p-values in top row for both negative (n=642 metabolites) and positive (n=666 metabolites) ionization mode. (B) After FDR-correction using Storey and Tibshirani n=328 metabolite ions are found to be differentially expressed between dcSSc and HC in negative ionization mode and n=167 in positive ionization mode. HC: healthy controls; dcSSc: diffuse cutaneous systemic sclerosis; FDR: false discovery rate.



Supplementary Figure 9 | Pathway enrichment analyses of metabolite ions from diffuse cutaneous SSc (dcSSc) patients' metabolic signature. Results from pathway enrichment analysis in both (A) negative and (B) positive ionization mode using metabolite ions with differential expression between dcSSc and HC. Significantly enriched pathways are shown in red. HC: healthy controls; dcSSc: diffuse cutaneous systemic sclerosis.



Supplementary Figure 10 | Coherence of metabolic signatures between pSS, SSc and SLE. Spearman's rho correlation of metabolic signatures between (A) pSS and SSc, (B) pSS and SLE and (C) SSc and SLE from positive ionization mode. Metabolic signature of disease consists of differential expression analysis between disease (pSS, SSc, or SLE) versus HC. (D) Boxplots of the top annotated metabolites from the combination of pSS and SSc signatures. HC: healthy controls; pSS: primary Sjögren's syndrome; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; m/z: mass/charge.

Chapter 8

Summary and discussion

Summary

The main aim of this thesis was to gain a better understanding of the role of CCR9-expressing T cells in pSS. So far in pSS patients CCR9+ CD4 T cells had been studied, but other CCR9-expressing cell subsets that could play a role in pSS immunopathology had not been considered. Using novel techniques the molecular, phenotypic, and functional features of several CCR9+ T cell subsets were studied, as described in **chapters 2-6**. Additionally, in **chapter 7** a new minimal invasive method using sebum metabolomics was investigated to evaluate its potential to monitor immunopathology and to discover novel biomarkers in pSS.
The main findings reported in this thesis are summarized below.

In **chapter 2** the transcriptome of CCR9+ Tfh-like cells was studied. CCR9+, CXCR5+, and CXCR5-CCR9- CD4 T cells from pSS patients and healthy controls (HC) were sorted using flow cytometry, and RNA sequencing was performed. Computational analysis was used to identify differentially expressed genes (DEGs), coherent gene expression networks (modules), and differentially regulated pathways. From the three subsets CCR9+ CD4 T cells showed most DEGs between pSS patients and HC. For further analysis one module was selected that showed high eigengene expression in CCR9+ CD4 T cells, and pathways enriched for genes involved in Th1 function (cytokine and chemokine production), and migration and adhesion. Target genes were validated in additional cohorts by qPCR or on protein level by flow cytometry. However, although the findings in CCR9+ CD4 T cells as compared to CXCR5+, and CXCR5-CCR9- CD4 T cells were validated, the differences between HC and pSS patients were not.
One gene of interest was *CCL5*. CCR9+ CD4 T cells were shown to express higher levels of the chemokine CCL5 than CXCR5+ and CXCR5-CCR9- CD4 T cells, with the highest expression of CCL5 found in effector CCR9+ CD4 T cells. Antigenic triggering and stimulation with IL-7 of the Th cell subsets co-cultured with monocytes strongly induced CCL5 secretion in CCR9+ CD4 T cell co-cultures. Additionally, effector CCR9+ CD4 T cells rapidly released CCL5 and secreted the highest CCL5 levels upon stimulation. Given the increased numbers of CCR9+ CD4 T cells in the blood and inflamed glands of pSS patients, and presence of inflammatory stimuli to activate these cells, this suggests that CCR9-specific functions such as cell recruitment upon CCL5 secretion, could significantly contribute to immunopathology in pSS.

CD8 T cells previously were shown to secrete more CCL5 than CD4 T cells. Furthermore, using epigenetic cell counting we found that CD8 T cell presence in labial salivary gland tissue was associated with the LFS and presence of Tfh cells, implicating that CD8 T cells can play a role in lymphocytic infiltration/organization and ectopic follicular responses. The finding that CCL5 is highly expressed by CCR9+ effector CD4 T cells, led to the hypothesis that CCL5 expression by CCR9+ CD8 T cells might also be elevated in pSS patients and contribute more to total circulating CCL5 levels. In **chapter 3** we were the first to show that CCR9-expressing CD8 T cells are more abundant in pSS patients as compared to HC. Antigen-experienced CD8 T cells, and especially CCR9+ effector CD8 T cells, expressed the highest CCL5 levels, and released the highest levels of CCL5 upon stimulation. CCR9+ CD8 T cells rapidly released CCL5, and released significantly more CCL5 than CCR9+ CD4 T cells. pSS patients expressed less CCL5 in their effector and memory CD8 T cells compared to HC, and subsequently released lower levels of CCL5 upon stimulation, which could point to increased release in vivo. Together these findings suggest a role for CCL5-releasing (CCR9+) CD8 T cells in pSS immunopathology.

Tph cells and CCR9+ Tfh-like cells are two CXCR5- Tfh-like cell subsets with B cell activating potential and higher frequencies in the circulation of pSS patients compared to controls. In **chapter 4** the question was answered if in fact these Tfh-like cell subsets are the same or separate subsets. It was demonstrated that the two populations of cells are largely distinct and only have limited overlap. Only a small percentage of CXCR5-PD-1^{hi} memory cells expressed CCR9. Using flow cytometry the expression of activation markers PD-1 and ICOS was compared between both CXCR5- cell subsets and CXCR5+ cells, taking the differentiation status of the cells into account. PD-1/ICOS expression was higher in memory cells expressing CXCR5 or CCR9. The highest expression of PD-1/ICOS was found in CXCR5/CCR9 co-expressing T cells, which were enriched in the circulation of pSS patients. Since the ligands for CCR9 and CXCR5 (CCL25 and CXCL13, respectively) are upregulated in pSS salivary gland tissue, cells expressing either or both chemokine receptors will migrate to the inflammatory site, and may contribute to the ongoing immune responses.

Previously we have demonstrated that CCR9-expressing CD4 T cells have increased expression of IL-7R and subsequently strongly respond to IL-7. In recent years it has become clear that besides adaptive immune cell subsets such as CD4 T cells, also innate and innate-like cell subsets can play a role in IL-7-related immunopathology, including B cell hyperactivity and local inflammation as are seen in patients with pSS. In **chapter 5** the potential of IL-7 to activate IL-7R-expressing innate(-like) cells is summarized.
As pSS is characterized by inflammation in mucosa-associated tissues, in **chapter 6** innate-like mucosal-associated invariant T (MAIT) cells were studied for their expression of CCR9, CXCR5, IL-18Rα and IL-7R in pSS. Both CD161+ and IL-18Rα+ MAIT cell numbers were decreased in pSS patients as compared to HC. pSS patients showed a significantly increased CD4/CD8 MAIT cell ratio, due to elevated CD4 MAIT cell frequencies compared to controls. The number of CCR9+ and CXCR5+ MAIT cells was significantly higher in pSS patients. Furthermore, expression of IL-7R, IFN-γ, and IL-21 was higher in MAIT cells as compared to

non-MAIT cells. IL-7R expression on CD8 CD161+ MAIT cells correlated to lymphocytic focus scores, percentage of IgA-expressing plasma cells in salivary gland tissue, and serum IgG levels. Production of IL-21 by MAIT cells was significantly inhibited by LEF/HCQ exposure. Given the enriched CCR9/CXCR5 expression of MAIT cells, this suggests that overexpressed CCL25 and CXCL13 in the inflamed salivary glands of pSS patients could facilitate increased migration of these cells. Given the pivotal role of IL-7 and IL-21 in inflammation in pSS this also indicates a potential role for MAIT cells in driving pSS immunopathology.

Although many new technologies require limited amounts of biological material to study disease, most still do require invasive procedures to obtain the material. Therefore, the development of new minimal invasive technologies might be of interest to patients, physicians and researchers. In **chapter 7** a new minimal invasive method of high throughput metabolomics technology of sebum, the product of exocrine sebaceous glands, was investigated. Sebum was easily collected from the forehead of individuals using sebutapes, and the extracted sebum was analyzed. Using untargeted mass spectrometry several thousands of metabolite ions were found in all samples. Modest differences were observed between the sebum metabolome of pSS patients as compared to controls. However, correlation analyses of metabolic changes and disease activity markers identified a few metabolite ions with a good correlation with disease activity. Sebum metabolome analysis of SSc patients showed a more outspoken difference compared to controls, with several identified metabolites involved in neurotransmission. When analyzing differentially expressed metabolite ions of pSS and SSc patients (relative to controls) strong correlations between the metabolites were observed. Altogether, this pilot study demonstrated that sebum metabolomics might be a novel strategy to identify biomarkers in pSS and other (systemic) autoimmune diseases.

Discussion

Derailment of B cell activation is a hallmark feature in pSS, reflected by increased production of autoantibodies, elevated serum IgG levels, and elevated numbers of IgM/IgG+ plasma cells in the salivary glands, which are associated with increased lymphocytic focus scores and T follicular helper cell numbers.(1) Help from T cells in the organized lymphocyte aggregates (either GC-like or not) can come from different T cell subsets that have B cell activating properties. Considering the fact that B cell hyperactivity is strongly associated with increased disease activity and an increased risk for developing lymphoma, understanding the mechanisms of B cell hyperactivity in pSS is of pivotal importance.(2) The data presented in this thesis contribute to increased understanding of CCR9+ T cell-driven immunopathology in pSS.

CCR9+ T cell-driven immunopathology in pSS: potential role in initiation and perpetuation of glandular inflammation

Based on the results from this thesis it is hypothesized that multiple CCR9-expressing T cells including CD4 and CD8 T cells and subsets of these cells such as PD-1-expressing CXCR5- CD4 T cells, innate-like MAIT cells, and CCR9/CXCR5 co-expressing CD4 T cells could play significant roles in immunopathology of pSS.

Previously and in this thesis CCR9-expressing CD4 Tfh-like cells were demonstrated in elevated frequencies in salivary gland tissue and the circulation of pSS patients.(3,4) The majority of CCR9+ Tfh-like cells are antigen-experienced memory/effector T cells, that express elevated levels of PD-1, ICOS, and IL-7R and secrete high amounts of IL-21, IFN-γ, and CCL5 (**chapter 2, 4**).(3,4) In **chapter 4** also CCR9/CXCR5-coexpressing CD4 T cells are described, which are also increased in the circulation of pSS patients compared to controls, and express high levels of PD-1 and ICOS. Furthermore, the frequency of circulating CCR9-expressing CD8 T cells was also found to be elevated in pSS patients compared to controls (**chapter 3, 6**). CCR9-expressing CD8 T cells show high expression of IFN-γ, TNF-α, and CCL5, particularly antigen-experienced CCR9+ CD8 T cells. Also, CCR9-expressing MAIT cells were identified and found in higher frequencies in the circulation of pSS patients compared to controls (**chapter 6**). MAIT cells express high levels of IL-18Rα and IL-7R, indicating that once migrated to pSS salivary glands these cells locally can become activated by elevated levels of IL-18 and IL-7 which previously have been demonstrated.(5–8) This could explain the reduction of MAIT cells in the circulation of pSS patients compared to controls.
Importantly, CCL25, the ligand for CCR9, is overexpressed in pSS salivary gland tissue, indicating that CCR9-expressing cells, often co-expressing other chemokine receptors (e.g. CXCR5, CCR5, CXCR3) are addressed to migrate to the inflamed glands.(4) CCL25 levels in secretomes of salivary gland tissue were associated with presence of anti-SSA-autoantibodies, elevated serum IgG levels, and levels of IL-21 and soluble IL-7R.(9)

In **Figure 1** the anticipated role of the cells studied in this thesis is summarized in a schematic representation.

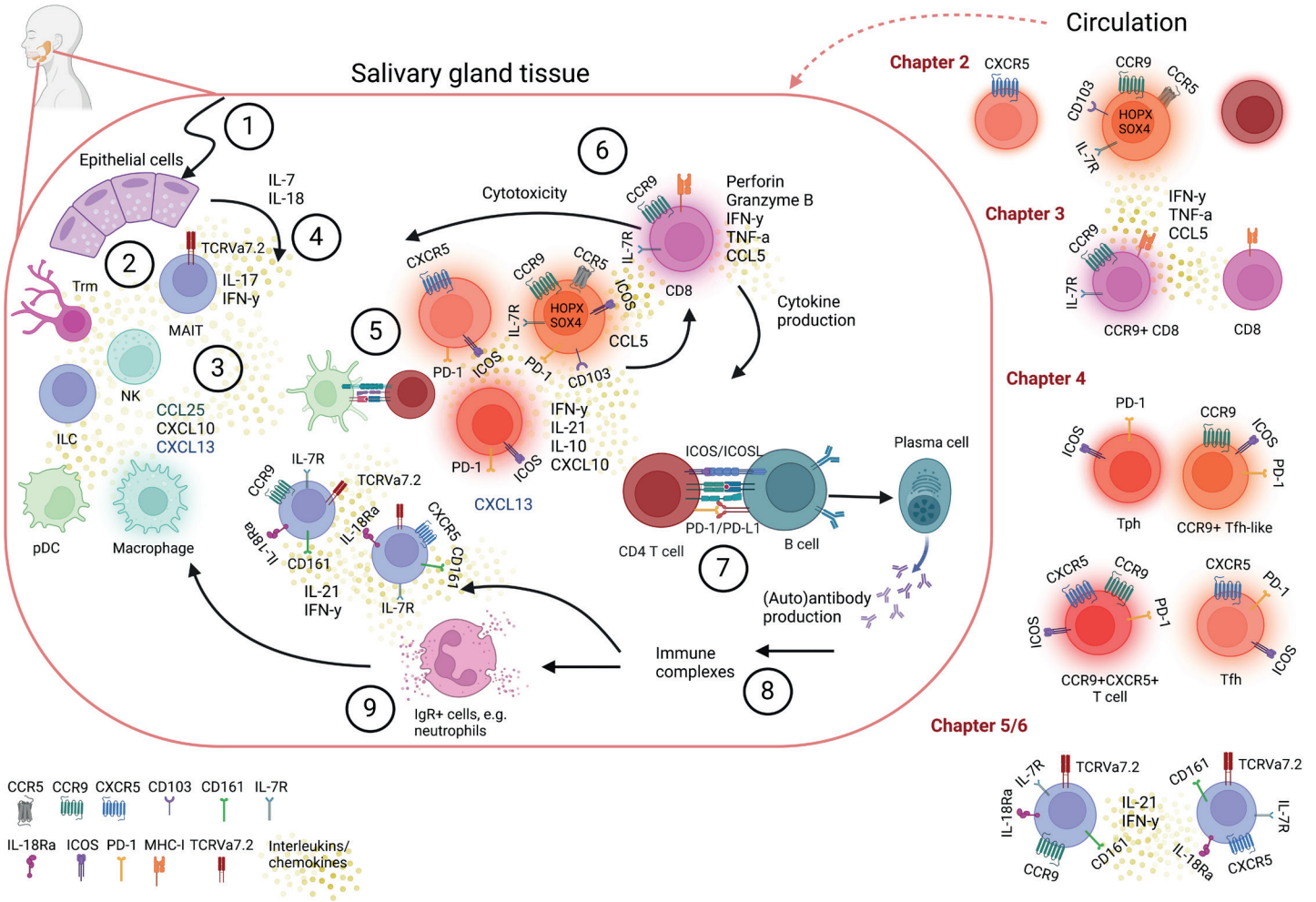


Figure 1 | Proposed role of CCR9-expressing T cells in immunopathology in inflamed salivary gland tissue of pSS patients. In pSS (1) exposure to infectious triggers such as viruses and other environmental factors may cause disruption of tissue homeostasis in the mucosa-associated salivary gland tissue of pSS patients. Tissue cells, such as epithelial cells, endothelial cells, and fibroblasts become activated, and upregulate MHC class I and II molecules and costimulatory molecules preparing for T cell activation and inflammatory responses. (2) Alternatively, these triggers can also directly and indirectly (through tissue cell response) activate innate cells (such as dendritic cells (DCs), macrophages, innate lymphoid cells (ILCs), and natural killer (NK) cells), innate-like MAIT cells, and tissue resident memory T (Trm) cells. Just like tissue cells, these immune cells upregulate MHC and costimulatory molecules, and some present antigen (as antigen presenting cells, APCs) to lymphocytes. (3) Activated tissue and innate(-like) cells also produce chemokines such as C-C motif ligands (CCL19, CCL20, CCL21, and CCL25), and C-X-C motif ligands (CXCL9, CXCL10, CXCL13), causing chemotaxis of specific immune cells such as CCR9 and CXCR5-expressing T cells (via CCL25 and CXCL13), and Tph cells towards the tissue. (4) Activated tissue cells and innate(-like) cells produce cytokines such as IL-7 and IL-18 that activate IL-7R-expressing T cells and IL-7R/IL-18Rα-expressing MAIT cells, to stimulate their proliferation and differentiation, e.g. to a Tfh phenotype in the context of APCs. (5) Activated APCs interact with and activate T cells inducing subsequent T cell-dependent activation of APCs and B cells. (6) The activation of T cells by APCs allows cross presentation of antigens to CD8 T cells, including CCR9-expressing T cells. This activates CD8 T cells, which release cytokines (IFN-γ, TNF-α, and CCL5) and cytotoxic molecules (perforins, granzyme B), contributing to immunopathology. (7) Activated T cells stimulate B cells via co-stimulation (e.g. ICOS/ICOSL), and B cell activating cytokines such as IFN-γ, IL-10 and IL-21. In addition, T cell produced chemokines, such as CXCL10, CXCL13, and CCL5 can further contribute to cell influx of numerous inflammatory cells. CCR9 and CXCR5-expressing CD4 T cells, innate-like MAIT cells, and Tph cells thus can contribute to B cell hyperactivity and autoantibody production. (8) This triggers increased immune complex formation and complement activation/consumption and (9) activation of immune cells expressing immunoglobulin-receptors (IgR), such as neutrophils and macrophages contributing to inflammation and immunopathology. *Figure created with BioRender.com.*

Initiation and perpetuation of immunopathology

Although the findings in this thesis indicate that multiple CCR9-expressing T cells can find their way to CCL25 expression in the inflamed salivary glands, it is unknown whether and to what extent the CCL25/CCR9 axis and the different CCR9-expressing cell types contribute to initiation and/or perpetuation of the inflammation. The CCR9-expressing T cells in the studies included in this thesis have demonstrated increased proinflammatory properties and effector functions as compared to T cells lacking CCR9 expression. Therefore, it is strongly suggestive that CCR9+ T cells could play a significant role in the perpetuation of inflammatory responses once attracted and activated.

With respect to the initiation of inflammation by CCR9+ T cells it is unclear which factors contribute to CCL25 induction. In general there is limited information on the triggers that can induce CCL25 expression. In studies on CCL25 expression in the small intestine LTβR signaling (LTα, LTβ) and TNFR1 signaling were shown to be unrelated to CCL25 mRNA expression, even though these are key regulators of homeostatic chemokine expression in secondary lymphoid organs and the small intestine.(10) Likewise, inflammatory chemokines as IFN-γ, TNF-α and LPS failed to induce CCL25 mRNA expression.(11) Also retinoic acid (a vitamin A metabolite), which enhances expression of CCR9 and integrin α4β7, does not enhance CCL25 expression.(12) In mice buccal epithelium CCL25 expression was upregulated upon local antigen challenge using hen-egg lysozyme (HEL) molecules or upon gingival wounding.(13,14) Yet, elevated expression of CCL25 that has been observed in salivary glands of pSS patients may cause CCR9+ effector T cells to initiate inflammation and contribute to maintaining an ongoing immune response. Alternatively, it is conceivable that CCR9-expressing cells are attracted to the salivary glands, e.g. by co-expressing chemokine receptors (CCR7, CXCR5, CCR5) and find their place as resident T cells. In that case these effector T cells might help in a first line of defense. Additionally, CCL25 might be involved in the organization of lymphoid aggregates in the salivary glands of pSS patients. The triggers for CCL25 induction remain unknown, as well as the timing and positioning of this in the disease course of pSS patients.

To what extent (CCR9+) MAIT cells contribute to initiation or perpetuation of glandular inflammation is unknown. Previously it has been demonstrated that a significant proportion of MAIT cells displays a tissue resident phenotype (CD103+CD69+) in buccal mucosa.(15) MAIT cell numbers are elevated in pSS salivary glands.(16,17) MAIT cells that provide a first line of defense may be triggered to initiate inflammation, which may subsequently cause CCL25 upregulation. Future studies will have to unravel this. Also, the ligand triggering MAIT cell activation in pSS patients is not known. In general, MAIT cells were shown to become activated by binding to glycolipid molecules, such as vitamin B2 derivatives, presented on MHC class I-like MR1 molecules.(18,19) In the presence of e.g. IL-18 or type I IFNs MAIT cells can recognize antigens from viruses upon viral infections.(20,21) Also, TCR-independent cytokine activation of MAIT cells was described.(22) Perhaps microbiome disbalance can contribute to altered ligand presence in pSS salivary gland tissue. However, results from studies on pSS buccal mucosa and salivary microbiota are inconsistent. Dysbiosis of buccal mucosa microbiome in pSS patients and Sjögren-like disease in mice was described, but the potential role in pSS immunopathology remains to be demonstrated.(23–30)

Another potential early trigger of pSS immunopathology is IL-7. Increased IL-7 expression and IL-7R-expressing CD4 and CD8 T cells in the salivary glands of pSS patients has been demonstrated.(31) Triggers such as TLR3 and poly I:C were found to induce IL-7 production in Sjögren-like disease in mice.(32,33) Furthermore, Poly I:C, IFN-α and IFN-γ induced IL-7 expression in human salivary gland epithelial cells.(33) It is unclear whether IL-7 triggered immune activation can cause CCL25 upregulation and stimulate attraction of CCR9-expressing T cells, facilitating CCR9+ T cell-associated immunopathology. Interestingly, it has been demonstrated that IL-7 can upregulate CCR9 expression.(34) Although speculative, in this way IL-7 might affect the lymphoid organization once CCL25 is produced. This indicates that IL-7, which is found in increased levels in the salivary glands of pSS patients and of mice with Sjögren-like disease, similarly to IL-7R-expressing cells, might play a role in pSS immunopathology from an early moment in the disease, potentially also activating CCR9-expressing T cells once attracted or present.(7,8,31,35)

CCR9+ T cells: effector cells that recirculate and reflect local inflammation?

Antigen-experienced memory and effector T cells are enriched at inflammatory sites and can drive immunopathology. Some of these cells remain in the tissues as resident T cells to warrant a first line of defense. Other cells recirculate to provide immunity at other sites of the body. Hence, circulating cells in pSS patients may to some extent reflect local inflammation. Several findings reported in this thesis support this hypothesis, such as the increased expression of ICOS and PD-1 on circulating CCR9-expressing cells, elevated IL-7R expression on circulating MAIT cells, and elevated CCL5 expression in circulating CCR9-expressing CD4 and CD8 T cells compared to their CCR9-lacking counterparts combined with decreased CCL5 expression on effector (memory) cells in pSS patients as compared to controls.

However, activation by inflammatory triggers at the site of inflammation is anticipated to cause specific alterations in the (inflammatory) phenotype of cells, that might not be reflected in the circulation, e.g. internalization of receptors. Therefore, matched samples from salivary gland tissue and peripheral blood taken at the same moment could be a valuable approach to compare local and peripheral cell subsets within an individual. However, this approach has certain challenges. Apart from the fact that the sampling of salivary gland

tissue is an invasive procedure, also the analysis of inflammatory cells from cell suspensions (and particularly those subsets that are present in low frequencies) after tissue digestion has proven a challenging procedure that can have some concerns, as cell viability and cell recovery. Also, longitudinal sampling is difficult, whereas this would provide most insight on the plasticity of expression patterns of cell subsets within individuals, and changes over time (in controls) or during the course of the disease (in patients).

New techniques to study immunopathology, such as single cell RNA sequencing (scRNA-seq) and mass cytometry, are emerging, and could be of use for further research on the role of CCR9-expressing T cells in pSS immunopathology. scRNA-seq allows comparison between transcriptomes of individual cells.(36) In pSS patients scRNA-seq of circulating mononuclear cells e.g. showed upregulation of IFN-related genes, and upregulated expression in CD4 T cells of chemokine *CCL5*.(37) At the moment, scRNA-seq is costly, and the depth of sequencing strongly determines the ability to detect transcriptional signatures, i.e. if a gene is expressed but at a low level it might not be detected by current scRNA-seq methods.(36) Also mass cytometry (or CyTOF) and image mass cytometry (IMC) are promising new tools for research allowing single cell analyses.(38) Combining elements from mass spectrometry and flow cytometry, DNA content and proteins can simultaneously be assessed.(39) Since antibodies bound to isotypically rare earth elements are used, no spectral overlap occurs as is the case in fluorescence activated measurements in flow cytometry (due to fluorescence emission profiles).(40) In pSS patients one study using CyTOF identified a disease signature that clustered pSS patients into subsets with distinct disease activity and glandular inflammation, using paired samples of salivary gland tissue and peripheral blood.(41) Although the complexity of this technique currently limits its application, it can be expected that these type of techniques will become more common practice, e.g. by development of automated research pipelines and commercialized ventures.

Studying the role of CCR9+ T cells in a heterogeneous disease as pSS

Although the studies reported in this thesis show differences on group level between pSS patients and controls, the data show there is heterogeneity in CCR9+ T cell frequencies and the activation markers of these cells. Multiple factors may contribute to this. pSS is a heterogeneous disease and patients with pSS present with a variety of symptoms reflected by immunological and clinical differences seen between patients, e.g. in autoantibody presence, the number of organized lymphocytic infiltrates and extraglandular manifestations due to inflammation in other organs. For some of these clinical manifestations immunological associations have been identified that help in prediction, such as the elevated risk of developing a lymphoma in pSS patients with GC-like structures or a high number of lymphocytic infiltrates (≥3 foci/4 mm^2) in the salivary glands.(42,43) However, clinical symptoms of pSS patients often do not relate to immunological findings. Specifically, correlations between the gold standard clinical disease parameters such as ESSDAI, the patient reported indices such as ESSPRI, and immunological parameters such as serum IgG or LFS scores are often weak or moderate. Possible explanations for this may be that our attempts to capture immunological events only focus on part of the inflamed glands, e.g. only on a limited number of minor labial salivary glands (less than ten glands out of hundreds present in the oral cavity(44)) and not on parotid, submandibular, or lacrimal glands. In addition, the frequency and timing of the sampling (e.g. by biopsies or blood collection) to investigate immune activation may be crucial. For example, cross-sectional analysis of B cell activity by measurement of long-lived immunoglobulins (e.g. through serum IgG) may not optimally reflect immune activation. In this respect, longitudinal analyses of e.g. IFN-associated proteins upon treatment were shown to tightly correlate with clinical parameters.(45) Lastly, in our attempt to analyze phenotypic and functional properties of CCR9+ T cells we were technically limited, unable to capture an extensive array of surface or intracellularly expressed proteins. In this respect we may have missed information that is associated with specific immunopathological processes. Future research, e.g. through single cell sequencing studying cells over time and using other inflammatory mediators to capture inflammation (e.g. circulating cytokines) may reveal more tight correlations with disease characteristics and immunopathological features.

PD-1: checkpoint or marker of proinflammatory (CCR9+) T cells?

In this thesis we have studied PD-1 expression on several T cell subsets. PD-1/PD-L interactions form a strong regulating mechanism for balance between T cell activation, tolerance and immune-mediated tissue damage.(46,47) As some malignancies use PD-1/PD-L interactions to control T cell responses, interfering in this balance by usage of PD-1/PD-L blockade (such as nivolumab or pembrolizumab) results in increased anti-tumor responses.(48) A downside of these therapeutic strategies affecting immune checkpoints is that increased immune responses are associated with induction of autoimmunity and clinical features resembling autoimmune disorders such as rheumatoid arthritis and pSS.(49,50) This illustrates the potential of regulation of T cell-driven responses by PD-1/PD-L interactions in pSS patients. As described in chapter 4 we found increased expression of PD-1 on CCR9/CXCR5-expressing T cell subsets. PD-1 expression in pSS patients was mostly found on antigen-experienced memory and effector T cells. Expression of PD-1 should serve as a checkpoint on these cells, however, for reasons still unknown this mechanism seems insufficient to control immune activation in pSS. A reason could be that factors like IL-7 override T cell control by PD-1/PD-L, as was previously shown for RA patients.(51) IL-7, despite upregulated PD-1, potently activated T cells, and in fact in the presence of IL-7 the expression of PD-1 was downregulated.(51) In addition, other surface costimulatory molecules may override activity of PD-1. In this respect we found a considerable number of ICOS/PD-1 co-expressing cells. ICOS, but also other molecules such as CD28, and CD40 may counteract the activity of PD-1. Finally, PD-1/PD-L interactions may be controlled by soluble

PD-1 levels. We recently demonstrated significantly increased levels of soluble PD-1 in pSS patients and normalization of these levels upon treatment with LEF/HCQ was associated with inhibition of disease activity (unpublished observations). Additionally, splice variants of soluble PD-1 may affect PD-1/PD-L signaling. In synovial fluid of RA patients a splice variant of soluble PD-1 was identified, which could block membrane-bound PD-1/PD-L interactions.(52) Overall, other inflammatory mechanisms seem to override checkpoint inhibition by PD-1/PD-L in pSS. Hence, PD-1 expression seems to represent a marker of activated T cells, including CCR9-expressing T cells. Given that PD-1 has an increased expression in salivary glands of pSS patients, and that blockade of PD-1 can result in Sjögren-like features, it is indicated that PD-1 expression on T cells from pSS patients is not sufficient to control the activation of effector T cells.(50,53,54)

Targeting options to halt CCR9+ T cell-driven immunopathology in pSS patients

In this thesis further support for the significant contribution of CCR9-expressing T cells and innate-like cells in the immunopathology of pSS has been provided. These findings and data from earlier studies suggest that targeting of CCR9-expressing T cells has potential for treatment of pSS. Several potential treatment options that can encompass direct and indirect strategies for targeting these cells in pSS are discussed below.

Targeting specific CCR9+ T cell-associated molecules

CCR9/CCL25

pSS patients have elevated frequencies of CCR9-expressing cells in their blood (**chapters 2, 3, 4, 6**) and both CCR9 and its ligand CCL25 are elevated in the salivary glands of pSS patients, whereas CCL25 is not detectable in healthy oral mucosa.(4,55)

Blockade of CCR9 has not been studied in pSS patients or mouse models of Sjögren-like disease. However, in recent years several CCR9 antagonists have been developed and tested in the context of inflammatory diseases, such as arthritis and inflammatory bowel disease (IBD). Inhibiting CCR9 reduced arthritic disease severity and migration of leukocytes to the synovium in an arthritis mouse model.(56) In ileitis mouse models inhibition of CCR9 decreased intestinal inflammation.(57–60) In patients with Crohn's disease treatment with CCR9 antagonists showed inconsistent results, with inhibition reported and lack of response.(57,61–63) The reasons for these discrepancies are unclear, but could be related to pharmacokinetic properties of the therapy. In a pilot study with patients with ulcerative colitis CCL25-tailored leukapheresis was used to extract CCR9-expressing cells, which had a positive clinical effect, leading to a currently ongoing follow-up trial (ClinicalTrials.gov Identifier NCT04550130).(64)

Integrins α4β7 and αEβ7

Another strategy to inhibit CCR9+ T cell activity may come from targeting specific integrins. CCR9 expression on T cells is often accompanied by expression of integrins α4β7 or αEβ7, as we confirmed in this thesis for HC and pSS patients (**chapter 2**).(65,66) Like for CCR9-expressing cells, most knowledge on α4β7 and αEβ7-expressing cells stems from studies in IBD. α4β7-expressing cells adhere to MAdCAM-1, and home to mucosal tissues.(67) In IBD patients cell numbers of α4β7-expressing cells and MAdCAM-1-expressing cells are increased in the intestinal tract.(68,69) Blocking α4β7/MAdCAM-1 results in an accumulation of T cells in high endothelial venules (HEVs) in the intestine.(70) Vedoluzimab, a selective α4β7 inhibitor, has shown good clinical results in IBD, and currently can be considered as a treatment option.(71) Although the overexpression of CCR9 and CCL25 in pSS patients has raised interest of pharmaceutical companies, treatment with vedoluzimab in pSS patients has not been studied yet.

Integrin αEβ7 is formed by the dimerization of CD103 (αE subunit) with subunit β7.(72) Its ligand is adhesion molecule E-cadherin, which is expressed on epithelial cells.(73) Whereas α4β7 is considered a homing molecule, αEβ7 is considered to retain immune cells to the mucosa.(72) Reports on expression and targeting αEβ7/E-cadherin in IBD are inconsistent.(72) In salivary gland tissue of pSS patients the levels of CD103 and E-cadherin were found to be elevated, and CD103-expressing CD8 T cells were shown to cluster around apoptotic acinar epithelial cells.(74,75)

Overall, these data suggest that specific targeting of the CCR9/CCL25 axis or specific CCR9+ T cell-associated molecules also may have potential in treatment of primary Sjögren's disease. However, future studies will need to demonstrate the efficacy of those treatment strategies as compared to other strategies.

Targeting functional properties of (CCR9+) T cells

IL-7 and IL-7R

In this thesis several IL-7R-expressing cell subsets were discussed (**chapters 2 - 6**). The IL-7/IL-7R axis is involved in ELS formation in pSS salivary glands, of which the presence is a predicting factor for lymphoma development in pSS patients.(31,43,76–78) IL-7R-expressing cells were found in elevated numbers in salivary gland tissue of pSS patients compared to nSS controls, and correlated to the number of T cells, B cells, and IgM/IgG+ plasma cells.(8) Previously and in this thesis we demonstrated that in controls and pSS patients the IL-7R is highly expressed on CCR9-expressing T cells and MAIT cells. Reduction of IL-7R expression on CD8 MAIT cells was associated to lymphocytic focus scores, serum IgG levels, and anti-SSA-autoantibodies, likely reflecting IL-7-induced activation, which causes receptor internalization (**chapter 6**). Previously, we demonstrated similar reduced IL-7R expression on salivary gland CD4 T cells, corroborating these results.(8) IL-7-induced T cell activation, whether it signals CD4, CD8 or MAIT cells, can induce vigorous production

of proinflammatory cytokines (including IFN-γ, TNF-α, IL-21, IL-17) and T cell-dependent activation of other immune cells as monocytes, dendritic cells and B cells.(31)
In Sjögren-like disease in mice blockade of IL-7R was associated with reduced leukocyte infiltration in the salivary glands and an increased saliva production.(79) In pSS patients and in murine Sjögren models IL-7 was found in increased levels in the salivary glands, saliva, and serum, and correlated to salivary output, lymphocytic infiltration, and autoantibody positivity.(7,8,31,35) Currently the efficacy and safety of a monoclonal antibody targeting IL-7Rα (CD127) is under investigation in pSS patients (ClinicalTrials.gov Identifier NCT04605978, EudraCT number 2020-001526-59). While less specific, blockade of the IL-7/IL-7R signaling pathway seems a good strategy to target effector functions of CCR9-expressing effector T cells that abundantly (over)express IL-7R. IL-7 is induced upon innate signaling early on in the disease process and it has a pivotal role in organization of lymphoid structures that are associated with disease outcome.(31) Considering the potential role of CCR9+ T cells and CCR9/CXCR5 co-expressing T cells in these processes IL-7R targeting also seems promising.

ICOS and ICOSL

Several T cell subsets described in this thesis show an increased expression of ICOS (**chapters 2, 4**) in pSS patients. Cells with ICOS expression interact with ICOSL-expressing cells, such as activated B cells, and blocking this interaction e.g. results in inhibition of IL-21 release, and can halt B cell hyperactivity, as the activity of Tph, Tfh, CCR9+ Tfh-like, and CXCR5/CCR9 co-expressing cells is inhibited halting their B cell help.(80–82) Because of its pivotal role in T cell-driven B cell activation targeting ICOS/ICOSL interaction was tested in pSS patients. However, in a phase 2a clinical trial of AMG557/ MEDI5872/Prezalumab (ICOSL) in 32 active patients with pSS (ESSDAI ≥6) the primary endpoint (change in ESSDAI of 3 or more) was not met (ClinicalTrials.gov Identifier NCT02334306, EudraCT Number 2014-003896-41).(83) In different hematological and solid tumors several treatment strategies combining ICOS/ICOSL pathway targeting with anti-CTLA-4 or anti-PD-1/PD-L agents are under investigation for safety and efficacy.(84)

CCL5 and CCR5

CCR9+ Tfh-like cells were shown to have increased expression of CCL5 and CCR5 (**chapter 2**). In particular, CCR9-expressing effector CD4 and CD8 T cells had significantly increased levels of CCL5 expression compared to controls, and released more CCL5 upon stimulation compared to CCR9- cells (**chapters 2, 3**). Expression of both CCL5 and CCR5 was found to be elevated in inflamed salivary glands of Sjögren-like disease, and blocking CCL5 reduced disease.(85,86). In pSS no clinical trials using anti-CCL5/CCR5 agents have been performed. However, for other autoimmune diseases several options are available such as maraviroc, or under clinical investigation such as cenecrivirox, anibamine, Met-CCL5, and vicrivirox.(87) Maraviroc is a CCR5 antagonist that competes with CCL5, and like most CCR5-antagonists is mainly studied in the context of HIV.(88) It was evaluated for proof of concept in a phase 2 trial in patients with RA undergoing methotrexate therapy, but showed no added clinical benefit.(89,90) In patients with primary sclerosing cholangitis treatment with oral CCR2/CCR5 antagonist cenecrivirox showed a modest improvement in an open-label phase 2 trial.(91)

Leflunomide and hydroxychloroquine: available DMARDs to target (CCR9+) T cell activity

The targets for treatment described in the above sections might be beneficial in the treatment of pSS due to their specificity and selectivity. Recently combination therapy with non-specific conventional disease-modifying antirheumatic drugs (cDMARDs) leflunomide and hydroxychloroquine, was shown to strongly halt B cell hyperactivity and disease activity in pSS patients.(45) Since these cDMARDs are already in use as treatment in several systemic rheumatological diseases, their safety profile is well-established and costs are low compared to newly developed treatments.(45)

Leflunomide (LEF) is a DMARD that inhibits T and B cell proliferation by inhibiting dihydro-orotate dehydrogenase (DHODH), the rate-limiting enzyme of pyrimidine de novo synthesis.(92) Also, in mice B cell antibody production is inhibited by LEF in a T cell dependent and T cell independent way by blocking cell cycle transition.(93)
Hydroxychloroquine (HCQ) is also a non-specific DMARD already in use as treatment in e.g. SLE and symptomatic treatment of pSS (e.g. in case of a vasculitis). The high intracellular concentrations of HCQ in APCs show that HCQ mainly targets APCs, and antigen presentation is decreased under the influence of HCQ.(94) Furthermore, activity of plasmacytoid DCs (pDCs), the main producers of type I IFN, and B cells are inhibited by HCQ, as HCQ interferes with the functional transformation of endosomal Toll-like receptors (TLRs) by raising endosomal pH.(94–96)
By combining these two DMARDS, different effector mechanisms are targeted. In vitro a synergistic effect was shown inhibiting T and B cell proliferation, resulting in decreased production of CXCL13, and type I and II IFNs.(97) Furthermore, in **chapter 6** LEF/HCQ was shown to inhibit IL-21 production in CD8 MAIT cells.
While at the moment the results from two clinical trials on LEF/HCQ combination therapy (EudraCT Number 2020-001933-11 and ClinicalTrials.gov Identifier NCT05113004) are awaited, it seems that one of the mechanisms that could contribute to the efficacy may comprise of inhibition of CCR9+ T cell activity. When proven effective LEF/HCQ combination therapy may be a good therapeutic cost-effective treatment option.

Sebum metabolomics as a new minimal invasive method to find biomarkers and monitor immunopathology in pSS

Metabolomics is a broad term for the analysis of metabolites in a biological sample.(98) The techniques used are quickly developing, e.g. the expected number of metabolites that can

be found in the human body, not only coming from the human genome, but also derived from external factors as food, medication and microbiota, was increased from approximately 19000 in 2013 to 80000 in 2018.(99,100) Metabolites can be of great interest for personalized medicine, as their abundance in biological samples have been shown to often be directly related to pathogenic mechanisms.(98,101) In general a metabolomics workflow after study design involves collection of the biological sample, sample treatment to extract metabolites, analysis using a form of mass spectrometry (e.g. liquid or gas chromatography), data processing and statistical analysis. Initially untargeted approaches (unbiased, i.e. without a preselected metabolite library) will be used in order to map the entire metabolome. However, more information is gained than currently can be annotated, which is e.g. reflected by the fact that in 2015 the proportion of human metabolites identified via untargeted mass spectrometry was less than 2% of identified MS peaks.(102) Based on the results from untargeted metabolomics, a targeted approach (using a prespecified library) can be used to confirm findings in additional cohorts in a (semi-) quantitative manner.(103)

In pSS metabolomics analysis of saliva has shown potential in establishing a metabolic signature of pSS.(104–106) One study using targeted metabolomics analysis of tear fluid was able to compose a pSS metabolomic signature distinguishing pSS from other dry eye syndromes.(107) Also, urine, plasma and serum metabolomics analyses indicated potentially altered metabolic pathways in pSS patients, e.g. in the metabolism of fatty acids, phospholipids, and amino acids as phenylalanine and proline.(108–110) The validation and potential application of the results from these studies might lead to new biomarkers to monitor disease, but the use of saliva and tear fluid do have the clear downside that pSS patients with more outspoken sicca complaints might not produce enough saliva or tear fluid to make this a feasible approach. In this respect no consistent metabolites of metabolite profiles from saliva have been implemented in the prognosis, diagnosis or monitoring of pSS patients. Hence, there is still an unmet need to identify (metabolome-based) biomarkers in pSS.

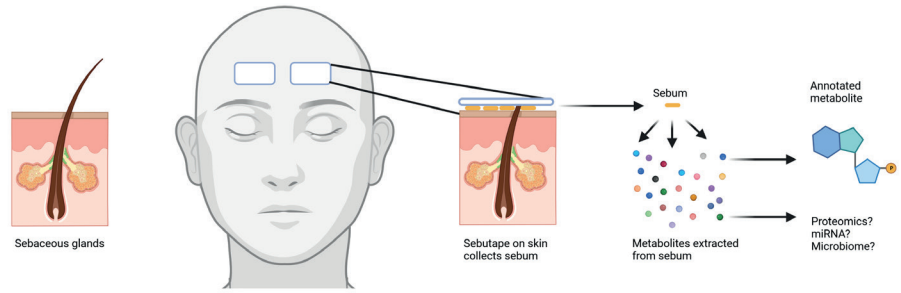


Figure 2 | Application of sebutapes to collect sebum from the skin of the forehead for metabolomics analysis.

As a product of sebaceous glands in the skin, sebum can be easily sampled. For the pilot study presented in **chapter 7** several alternative protocols for sebum collection were tested, which included different ways to clean the skin, alternative sample locations, and varying sample times (data GSK). Also, the desired amount of collected sebum was tested. For this the amount of sebutapes was tested and we have shown that increasing the number of used sebutapes from 1 to 2 tapes yielded an increase of approximately 30% more detected ions. Hence, in this thesis we show the practical approach to include 2 sebutapes from the skin of the forehead. Future studies may be focused on further improving the amount of collected sebum, e.g. from more sites of the (forehead) skin or for longer or consecutive time points. In this respect, it is conceivable that sebum can provide other molecular information, and e.g. can be used for proteomics, epigenetics (e.g. miRNA) or other analyses such as microbiome analyses (**Figure 2**). The exploratory analyses from this thesis (**chapter 7**) indicate good correlations of a number of metabolites with disease activity markers. Interestingly, sebum metabolome analysis of SSc patients identified multiple metabolites involved in neurotransmission. In addition, metabolic signatures of pSS and SSc patients (relative to controls) showed strong correlations. Altogether, this pilot study demonstrates the potential of sebum metabolomics, indicating this might be a novel strategy to identify biomarkers in pSS and other (systemic) autoimmune diseases.

Although sicca complaints resulting from salivary gland and tear gland dysfunction are undisputed clinical features of pSS, dryness of the skin (xerosis) is not. Moreso, xerosis is rarely studied in pSS. One study reported that 67% of pSS patients has xerosis. However, this was a retrospective study and the degree of xerosis was not objectified.(111) No morphological alterations or reduced numbers of sebaceous glands and sweat glands have been found, but instead changes in the epidermis (altered expression of skin cytokeratins and involucrin) were suggested as an explanation for xerosis in pSS.(112) Altogether, this could indicate that patients with a disease of more phenotypic skin involvement, such as SSc, might have a more robust sebum metabolome signature, as is also suggested in **chapter 7**.

Nevertheless, the results from our untargeted sebum metabolomics approach can be used to specify metabolite classes of interest, that can be measured using a targeted mass spectrometry approach in follow-up studies. Although this will not have the unbiased advantages of untargeted metabolomics it will provide improved resolution of metabolites. Potentially, this will also improve the associations with clinical activity. In fact, targeted approaches in larger cohorts to capture the heterogeneity of disease and environmental factors such as food intake may hold great promise.

Conclusion

In this thesis we have shown increased frequencies of several CCR9-expressing T cell subsets in the circulation of pSS patients. Based on the effector and migratory phenotype of these circulating cells it is hypothesized that CCR9-expressing T cell subsets play a role in pSS immunopathology by stimulating B cell hyperactivity and T cell-driven responses in the salivary glands of pSS patients. CCR9/CCL25, CCR9+ T cell-associated molecules, and/or molecules associated with the effector activity of CCR9-expressing cells, might be targets for treatment of pSS. The potential of such strategies should be established in future investigations.Furthermore, we have shown that metabolomics analysis of sebum can be used to identify metabolites (or metabolite classes), which might lead to the identification of new biomarkers in pSS and other systemic autoimmune diseases. Validation of the findings from this pilot study and potential for implementation of this low-invasive technique needs to be further investigated.

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Appendix

Nederlandse samenvatting

Het primair syndroom van Sjögren (pSS) is een chronische auto-immuunziekte waarbij de meeste patiënten last hebben van droge ogen en een droge mond, wat vaak gepaard gaat met vermoeidheid en spier -en gewrichtsklachten. De ziekte wordt het meest vastgesteld op een leeftijd tussen 30 en 60 jaar en komt vaker voor bij vrouwen (man:vrouw ratio circa 1:9). De klachten van droogte ontstaan door een verminderde productie van traanvocht en speeksel ten gevolge van ontstekingen in de traanvocht -en speeksel producerende klieren. Bij een deel van de patiënten ontstaan ook extraglandulaire ziektemanifestaties als gevolg van ontstekingen in andere organen, zoals de gewrichten, nieren of de longen.
Een ander kenmerk van pSS is B-celhyperactiviteit. Uitingen hiervan zijn o.a. de productie van autoantistoffen (anti-SSA/SSB en reumafactor antistoffen) en verhoogd serum IgG. Als gevolg van B-celhyperactiviteit hebben patiënten met pSS een verhoogd risico op het ontwikkelen van B-cel lymfomen (Non-Hodgkin). pSS wordt gezien als een multifactoriële ziekte, waarvan het precieze werkingsmechanisme onbekend is. Ook is er momenteel geen effectieve behandeling tegen pSS. Wetenschappelijk onderzoek is zowel gericht op het begrijpen van de immunopathologie en pathogenese, als op het vinden van een succesvolle behandeling.

In de exocriene klieren van pSS patiënten worden lymfocytaire infiltraten gevonden (lymfocyten foci, georganiseerde infiltratie met meer dan 50 lymfocyten per 4 mm^2), die zich kunnen organiseren in ectopische lymfoïde structuren (*ectopic lymphoid structures*, *ELS*). De organisatie van T -en B-cellen in de ELS lijken sterk op die van germinal centers (GCs) die in lymfoïde organen worden gevonden. De mate van aanwezigheid van foci, om precies te zijn 3 of meer foci, of de aanwezigheid van ELS in de speekselklieren is geassocieerd met het ontstaan van een lymfoom. T-cellen spelen een zeer belangrijke rol in de activatie van B-cellen. Een beter begrip van de rol van deze cellen is cruciaal om de ontsporing van B-cellen in het syndroom van Sjögren te begrijpen. Recent heeft onze groep aanwijzingen gevonden dat CCR9-positieve (CCR9+) T-cellen een belangrijke rol zouden kunnen spelen. In de afgelopen jaren zijn meerdere T-cel subsets genoemd als mogelijke aanjagers van de B-celhyperactiviteit in pSS, bijvoorbeeld *folliculaire helper* T-cellen (Tfh), CCR9+ CXCR5- Tfh-achtige (*CCR9+ Tfh-like*) cellen en PD-1^{hi} CXCR5- T *peripheral helper* (Tph) cellen.

Het hoofddoel van dit proefschrift was om meer inzicht te krijgen in de rol van CCR9+ T-cellen in pSS. Tot dusverre waren bij patiënten met pSS CCR9+ CD4 T-cellen bestudeerd, maar andere subsets van CCR9+ T-cellen die een rol zouden kunnen spelen in de immunopathologie van pSS waren niet onderzocht. Met behulp van nieuwe technieken werden de moleculaire, fenotypische en functionele kenmerken van verschillende CCR9+ T-cel subsets bestudeerd, zoals beschreven in hoofdstukken 2-6. Daarnaast werd in hoofdstuk 7 het potentieel van een nieuwe non-invasieve methode onderzocht om met *metabolomics* analyse van sebum (talg) immunopathologie te monitoren en nieuwe biomarkers voor pSS te vinden. De belangrijkste bevindingen uit de onderzoeken beschreven in dit proefschrift worden hieronder samengevat.

In hoofdstuk 2 is het transcriptoom van CCR9+ T folliculaire helper-achtige (*Tfh-like*) cellen bestudeerd. CCR9+, CXCR5+ en CXCR5-CCR9- CD4 T-cellen van patiënten met pSS en gezonde controles werden gesorteerd met behulp van flowcytometrie waarna RNA sequencing werd uitgevoerd. Computationele analyses werden gebruikt om differentieel tot expressie gebrachte genen (DEGs), coherente genexpressie netwerken (*modules*) en differentieel gereguleerde immuun pathways te identificeren. Van de drie subsets vertoonden CCR9+ CD4 T-cellen de meeste DEGs tussen pSS patiënten en controles. Een module werd geselecteerd voor verdere analyse, vanwege hoge eigengene expressie in CCR9+ CD4 T-cellen, en pathways met verrijking voor genen die betrokken zijn bij Th1-functie (cytokine- en chemokineproductie), migratie en adhesie. Geselecteerde genen werden gevalideerd in aanvullende cohorten met qPCR of op eiwitniveau met flowcytometrie. Hoewel de bevindingen tussen de verschillende CD4 T-cel subsets (CCR9+ CD4 T-cellen versus CXCR5+ -en CXCR5-CCR9- CD4 T-cellen) werden bevestigd, was dit niet het geval voor de verschillen tussen controles en patiënten met pSS.
Een van de geselecteerde genen was *CCL5*. CCR9+ CD4 T-cellen bleken hogere expressie van dit chemokine CCL5 te hebben dan CXCR5+ en CXCR5-CCR9- CD4 T-cellen, met de hoogste expressie van CCL5 in effector CCR9+ CD4 T-cellen. Antigeen-triggering en stimulatie met IL-7 van de CD4 T-cel subsets in co-cultures met monocyten, induceerde sterke CCL5-secretie in de CCR9+ CD4 T-cellen. Bovendien scheidden effector CCR9+ CD4 T-cellen snel en het meeste CCL5 uit na stimulatie. Gezien het toegenomen aantal CCR9+ CD4 T-cellen in zowel het bloed als in de ontstoken klieren van pSS patiënten, en de aanwezigheid van inflammatoire stimuli om deze cellen te activeren, zouden CCR9-specifieke functies, zoals rekrutering van andere immuuncellen na CCL5-secretie, significant kunnen bijdragen aan immunopathologie in pSS.

Eerder werd aangetoond dat CD8 T-cellen meer CCL5 uitscheiden dan CD4 T-cellen. We besloten verder in te gaan op de rol van CD8 T-cellen in pSS, aangezien we met behulp van DNA-gebaseerde cel kwantificatie (*epigenetic cell counting*) hadden vastgesteld dat de aanwezigheid van CD8 T-cellen in speekselklierweefsel geassocieerd was met de lymfocytaire focus score (*lymphocytic focus score*, LFS) en de aanwezigheid van Tfh-cellen. Dit impliceert dat CD8 T-cellen een rol kunnen spelen bij lymfocyten infiltratie/organisatie en ectopische folliculaire reacties. De bevinding dat CCL5 in hoge mate tot expressie wordt gebracht door CCR9+ effector CD4 T-cellen, leidde tot de hypothese dat CCL5-expressie door CCR9+ CD8 T-cellen ook verhoogd zou kunnen zijn bij pSS-patiënten en meer zou bijdragen aan het totaal circulerende CCL5. In hoofdstuk 3 toonden wij voor het eerst

aan dat CCR9+ CD8 T-cellen in hogere frequenties voorkomen bij pSS-patiënten dan bij controles. *Antigen-experienced* CD8 T-cellen, en vooral CCR9+ effector CD8 T-cellen, lieten de hoogste CCL5-expressie zien en scheidden het meeste CCL5 uit na stimulatie. Deze CCL5-uitscheiding was significant hoger door CCR9+ CD8 T-cellen ten opzichte van CCR9+ CD4 T-cellen. Patiënten met pSS hadden een lagere CCL5-expressie in hun effector/memory CD8 T-cellen in vergelijking met controles, en scheidden minder CCL5 uit na stimulatie, wat zou kunnen wijzen op een verhoogde afgifte in vivo. Deze bevindingen suggereren een rol voor CCL5-producerende (CCR9+) CD8 T-cellen in de immunopathologie van pSS.

Tph-cellen en CCR9+ Tfh-like cellen zijn twee CXCR5-negatieve (CXCR5-) Tfh-like cel subsets met B-cel-activerende eigenschappen en hogere frequenties zijn aangetoond in de circulatie van pSS-patiënten in vergelijking met controles. In hoofdstuk 4 werd de vraag beantwoord of deze Tfh-like cel subsets in feite dezelfde of afzonderlijke subsets zijn. Er werd aangetoond dat de twee cel subsets grotendeels uniek zijn en maar beperkt overlappen. Slechts een klein percentage van de CXCR5-PD-1^{hi} memory cellen bracht CCR9 tot expressie. Met behulp van flowcytometrie werd de expressie van activatiemarkers PD-1 en ICOS vergeleken tussen beide CXCR5- cel subsets en CXCR5+ T-cellen, waarbij rekening werd gehouden met de differentiatiestatus van de cellen. PD-1/ICOS-expressie was hoger in memory cellen die CXCR5 of CCR9 tot expressie brachten. De hoogste expressie van PD-1/ICOS werd gevonden in CCR9+CXCR5+ dubbelpositieve T-cellen, die tevens in hogere frequentie gevonden werden in de circulatie van pSS-patiënten. Aangezien de liganden voor CCR9 en CXCR5 (respectievelijk CCL25 en CXCL13) verhoogd zijn in speekselklierweefsel van pSS-patiënten, zullen cellen die een of beide chemokinereceptoren tot expressie brengen, naar de plek van ontsteking kunnen migreren waar ze kunnen bijdragen aan de immuunactiviteit.

Eerder werk uit onze groep had aangetoond dat CCR9+ CD4 T-cellen een verhoogde expressie van IL-7R hebben en sterk reageren op IL-7. In de afgelopen jaren is duidelijk geworden dat naast adaptieve immuuncellen zoals CD4 T-cellen, ook aangeboren (*innate*) en "innate-like" cellen een rol kunnen spelen in IL-7-gerelateerde immunopathologie, waaronder B-celhyperactiviteit en lokale ontsteking zoals gezien wordt in patiënten met pSS. In hoofdstuk 5 wordt het potentieel van IL-7 om IL-7R-positieve innate(-like) cellen te activeren beschreven.
Omdat pSS wordt gekenmerkt door ontsteking van mucosa-geassocieerde weefsels, werden in hoofdstuk 6 innate-like mucosa-geassocieerde invariante T (MAIT)-cellen onderzocht op hun expressie van CCR9, CXCR5, IL-18Rα en IL-7R in pSS. Zowel de aantallen CD161+ als IL-18Rα+ MAIT-cellen waren verlaagd bij pSS-patiënten in vergelijking met controles. pSS-patiënten vertoonden een significant verhoogde ratio tussen CD4/CD8 MAIT-cellen, als gevolg van verhoogde CD4 MAIT-celfrequenties in vergelijking met controles. Het aantal CCR9+ -en CXCR5+ MAIT-cellen was significant hoger bij pSS-patiënten. Bovendien was de expressie van IL-7R, IFN-γ en IL-21 hoger in MAIT-cellen vergeleken met non-MAIT-cellen. IL-7R-expressie op CD8 CD161+ MAIT-cellen correleerde met de LFS, het percentage IgA+ plasmacellen in speekselklierweefsel en serum IgG-spiegels. De productie van IL-21 door MAIT-cellen werd significant geremd door behandeling met LEF/HCQ. Gezien de verrijkte CCR9/CXCR5-expressie van MAIT-cellen, suggereert dit dat de verhoogde expressie van CCL25 en CXCL13 in de ontstoken speekselklieren van pSS-patiënten een verhoogde migratie van deze cellen zou kunnen vergemakkelijken. Gezien de cruciale rol van IL-7 en IL-21 bij ontstekingen in pSS, duidt dit ook op een mogelijke rol voor MAIT-cellen bij het aansturen van pSS-immunopathologie.

Hoewel veel nieuwe technologieën beperkte hoeveelheden biologisch materiaal nodig hebben om ziekten, zoals pSS, te bestuderen, vereisen de meeste technologieën nog steeds invasieve procedures om het materiaal te verkrijgen. Daarom is de ontwikkeling van nieuwe minimaal invasieve technologieën interessant voor patiënten, artsen en onderzoekers. In hoofdstuk 7 werd een nieuwe minimaal invasieve methode van *high-throughput metabolomics* technologie van sebum (talg), het product van exocriene talgklieren, onderzocht. Sebum kon makkelijk verkregen worden van het voorhoofd met behulp van sebutapes, waarna de sebum werd geanalyseerd. Met behulp van *untargeted* massaspectrometrie werden in alle verzamelde monsters enkele duizenden metaboliet-ionen gevonden. Er werden bescheiden verschillen waargenomen tussen het sebum metaboloom van pSS-patiënten in vergelijking met controles. Enkele metaboliet-ionen hadden ook een goede correlatie met ziekteactiviteit. Een andere interessante bevinding was dat sebum metaboloom analyse van SSc-patiënten een meer uitgesproken verschil toonde in vergelijking met controles. De verschillende geïdentificeerde metabolieten toonden verrijkte pathways die betrokken zijn bij neurotransmissie, een biologisch fenomeen dat van invloed zou kunnen zijn bij vasoconstrictie in deze patiënten. Tevens werd er tussen de differentieel tot expressie komende metabolieten van pSS -en SSc-patiënten (ten opzichte van controles, ook wel *metabolic signatures*) sterke correlaties waargenomen tussen de metabolieten van beide metabolic signatures. Al met al toonde deze pilotstudie aan dat sebum metabolomics een nieuwe strategie zou kunnen zijn om biomarkers in pSS en andere (systemische) auto-immuunziekten te identificeren.

Dankwoord

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Curriculum vitae

Anneline Catharina Hinrichs was born on the 25th of January 1991 in Oegstgeest, The Netherlands. Together with her younger sister Laura, she grew up in the Dutch-Portuguese household of their parents, Jule Hinrichs and Natividade Valente Proença.

In 2009 Anneline finished secondary school at the Stedelijk Gymnasium Leiden (Profile Nature and Health "N&G", with French, Latin and ancient-Greek) and moved to Utrecht to study medicine at the University Utrecht. During her bachelor's studies she also pursued her love of language and literature, completing several extracurricular courses, including a minor in comparative literature at the University Utrecht. In 2013 she was granted the opportunity to follow her opthalmology internship at Kasturba Medical College in Manipal, India, after which she could spend time pursuing another love: traveling. She spent 3,5 months in India and Nepal, and completed the Annapurna Circuit. Back in The Netherlands she became more interested in rheumatology, psychiatry, and teaching, and was able to follow several internships in these fields. In 2016 she obtained the students' educational qualification (StOK – studenten onderwijs kwalificatie), and she followed a research internship at the department of Rheumatology & Clinical Immunology of the University Medical Center Utrecht (UMCU) under supervision of dr. Aike A. Kruize and dr. Sofie L.M. Blokland. During this internship Anneline created an overview of all primary Sjögren's syndrome patients in care at the outpatient department of the UMCU, and was invited to join as one of the workshop presenters on behalf of the UMCU Sjögren team during the National Day of the Dutch Sjögren's Patients Society (NVSP – Nationale Vereniging Sjögrenpatiënten).

After her graduation in 2017 Anneline worked for a year as a medical doctor (resident not in training) at Tergooi Hilversum/Blaricum at the departments of Internal Medicine, Gastroenterology, Pulmonology, and Cardiology (supervisor: dr. Marjolein E.M. Rentinck). In april 2018 she started her PhD at the lab of prof. dr. Timothy R.D.J. (Tim) Radstake (Rheumatology & Clinical Immunology, and Center for Translational Immunology), under supervision of dr. Joel A.G. van Roon and dr. Aike A. Kruize. After the departure of prof. Radstake from the UMCU, prof. dr. Floris P.J.G. Lafeber joined her PhD team as a promotor. During her PhD her main focus was on CCR9-expressing T cells in primary Sjögren's syndrome and most time was spend in the lab. During her PhD she worked on the Sjögren Big Data Consortium project, was actively involved in patient participation (as one of the initiators of re-starting the UMCU Sjögren studypanel), taught immunology classes to medicine students, and was the tutor of a group of medicine students throughout their bachelor's studies. Furthermore, Anneline completed several PhD courses, e.g. on programming, statistics, and academic writing. Since September 2022 she is enrolled in the training to become a rheumatologist at the AmsterdamUMC/VUmc (supervisor prof. dr. Willem F. Lems), starting with her residency in Internal Medicine at Spaarne Gasthuis Haarlem/Hoofddorp (supervisor dr. W. (Pim) de Ronde). Anneline is married to Ralf Kohlhaas and together they live in Oegstgeest.

List of publications

This thesis

AC Hinrichs, SLM Blokland, AP Lopes, CGK Wichers, AA Kruize, A Pandit, TRDJ Radstake, JAG van Roon. Transcriptome analysis of CCR9+ T helper cells from primary Sjögren's syndrome patients identifies CCL5 as a novel effector molecule. Front Immunol. 2021 Jul 27;12:702733.

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AC Hinrichs, AA Kruize, FPJ Lafeber, HL Leavis, JAG van Roon. CCR9/CXCR5 co-expressing CD4 T cells are increased in primary Sjögren's syndrome and are enriched in PD-1/ICOS-expressing effector T cells. *Under review.*

AC Hinrichs, JAG van Roon. Do IL-7R+ innate cells orchestrate autoimmune pathology? Nat Rev Rheumatol. 2019 Nov;15(11):640-642.

AC Hinrichs, AA Kruize, HL Leavis, JAG van Roon. In patients with primary Sjögren's syndrome innate-like MAIT cells display upregulated IL-7R, IFN-γ, and IL-21 expression and have increased proportions of CCR9 and CXCR5-expressing cells. Front Immunol. 2022 Nov; 13:1017157.

AC Hinrichs, SLM Blokland, J Vappiani, A van Maurik, A Ottria, AA Kruize, HL Leavis, TRDJ Radstake, JAG van Roon*, DC Sévin*. Untargeted metabolomics analysis of sebum: a non-invasive strategy that shows potential to identify new biomarkers in primary Sjögren's Syndrome and Systemic Sclerosis. *Manuscript in preparation*. *Authors contributed equally.

Publications as contributor to Sjögren Big Data Consortium

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Other publications

AP Lopes, CPJ Bekker, MR Hillen, SLM Blokland, **AC Hinrichs**, A Pandit, AA Kruize, TRDJ Radstake, JAG van Roon. 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 Aug 3;12:701656.

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AP Lopes, MR Hillen, **AC Hinrichs**, SLM Blokland, CPJ Bekker, A Pandit, AA Kruize, TRDJ Radstake, JAG van Roon. Deciphering the role of cDC2s in Sjögren's Syndrome: transcriptomic profile links altered antigen processes with IFN-signature and autoimmunity. Ann Rheum Dis 2022. doi:10.1136/annrheumdis-2022-222728. E-pub ahead of print.

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NH Servaas, S Hiddingh, E Chouri, CGK Wichers, AJ Affandi, A Ottria, CPJ Bekker, M Cossu, M van der Kroef, **AC Hinrichs**, T Carvalheiro, N Vazirpanah, S Silva-Cardoso, L Beretta, M Rossato, F Bonte-Mineur, TRDJ Radstake, JJW Kuiper, M Boes, A Pandit. Nuclear receptor subfamily 4A signaling as a key disease pathway of CD1c+ dendritic cell dysregulation in systemic sclerosis. Accepted for publication in Arthritis & Rheumatology 2022. doi: 10.1002/art.42319.