Innovative techniques to investigate salivary gland immunopathology and exploration of the CCL25/CCR9+ T helper cell axis in Sjögren's syndrome

PhD thesis

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Innovatieve technieken om speekselklierweefsel te onderzoeken en de rol van CCL25 en CCR9-positieve T-helpercellen in het syndroom van Sjögren (met een samenvatting in het Nederlands)

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CHAPTER 1

Introduction

Sjögren's syndrome (SS) is a chronic, systemic autoimmune disorder characterized by dryness of mainly the eyes and mouth in association with lymphocytic infiltration of the exocrine glands. In addition to the dryness symptoms, most patients with SS suffer from fatigue, arthralgia and myalgia. Part of the patients develop extraglandular manifestations including involvement of for instance the lungs, nervous system, kidneys or skin. There is a strong female predominance (male to female ratio of 1:9) and most patients are diagnosed between the age of 40 and 60 years. Primary Sjögren's syndrome (pSS) occurs in the absence of other autoimmune diseases and secondary Sjögren's syndrome (sSS) presents along with other autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc).

Although after RA it is the most prevalent auto-immune disorder, SS is a relatively unknown disease with a large impact on daily life. The latter is best described by patients themselves and therefore Sjögren's syndrome patients were asked to write about their experiences living with the disease. In addition, all four of these patients are actively involved in research and/or care as a patient partner. Involving patients in research offers additional insights and has been shown to improve the quality of research. The patients were asked to write about their experiences in this field and their perspective on the importance of scientific research and on patient involvement in research and care. You can find their stories (in Dutch) after this Introduction section.

Immunopathology

In SS, like in most other autoimmune diseases, the process leading to loss of tolerance and the initiation and maintenance of immunity against self proteins (including anti-Ro/SSA, anti-La/SSB, anti-muscarinic receptor M3R) and tissue dysfunction and destruction has not yet been elucidated. pSS is considered to be a multifactorial disease, in which environmental factors can trigger inflammation in genetically predisposed individuals (1). Current models of the pathophysiology of this disease implicate the activation of mucosal epithelial cells, possibly by viral stimulation or by endogenous viral elements or other environmental factors. Genome Wide Association Studies (GWAS) in Sjögren's syndrome suggest also the involvement of multiple genetic components of both the innate and adaptive immune systems. Most strongly associated with pSS is the HLA-DR/MHC class II region, which indicates involvement of CD4+ T cells as these molecules present antigen to this subset of T cells. However, polymorphisms in six other, non-HLA loci were also associated with pSS. These loci are involved in the IFN pathway, TLR signaling, activation of B cells, CXCR5+ T-cells and dysregulation of the NFκB pathway(1,2), indicating that the pathological process in pSS is the resultant of the involvement of both the innate and the acquired immune system. Although the exact pathophysiological mechanisms of the disease have not been fully elucidated, significant progress has been made in the understanding of the complex interplay between the different cell types involved and the way they contribute to the immunopathology.

Triggering of epithelial cells and resident innate cells (macrophages, dendritic cells) in genetically susceptible hosts can lead to the activation of cells of the acquired immune system, e.g. surveilling T and B cells. In support of this, epithelial cells have been shown to express MHC class II and produce BAFF.(3-5) In addition to these early processes epithelial cells and cells of the innate immune system produce inflammatory cytokines that activate endothelial cells and stromal cells (e.g. IL-7) and produce chemokines including CXCL10 (interferon gamma-induced protein 10, IP-10) and CXCL13 (B lymphocyte chemoattractant, BLC) to attract and position other inflammatory cells.(6-8) This in turn may lead to the formation of lymphocytic infiltrates, which is one of the hallmark characteristics for diagnosis and classification of pSS.

The lymphocytic foci in pSS mainly consist of CD4+ T helper (Th) cells, B cells and plasma cells. Other immune cells including CD8+ cytotoxic T (Tc) cells, NK cells, dendritic cells and macrophages are present in smaller proportions. In a multifaceted interaction these cell-types contribute to another hallmark feature of pSS: B cell hyperactivity, reflected by presence of autoantibodies (characteristically anti-Ro/SSA in 60-80% of patients and anti-La/SSB in 30-40%), elevated serum IgG levels. The autoantibodies can form immune complexes that maintain and amplify the production of type I interferons, resulting in a cycle of immune system activation that leads to tissue damage. Finally, B cell hyperactivity is associated with an increased risk of lymphoma development (in ~10% of patients). In part of the patients the lymphocytic infiltrates in the exocrine glands develop into germinal center (GC)-like or ectopic lymphoid structures (ELS). Both the presence of germinal center-like structures and a high number of lymphocytic infiltrates (≥3 foci/4 mm2) in the salivary glands are associated with lymphoma development.(9) Hence, detailed understanding of the role of the key factors that drive lymphoid activation, in particular B cell activation, and organization into ectopic lymphoid structures (ELS) is of pivotal importance.

Ectopic lymphoid structures in Sjögren's syndrome

A large percentage of activated CD4+ T cells in the salivary gland can potently stimulate B cell activity. Formation of ELS occurs in part of the patients, but a large proportion of the patients that seem to lack ELS have high numbers of lymphocytic aggregates and are characterized by prominent B cell activation, both systemically and locally, as is evident from increased serum IgM and IgG and increased numbers of IgM and IgG-producing plasma cells at the site of inflammation. This has also been observed in other systemic autoimmune diseases such as rheumatoid arthritis.(10) The B cell stimulating capacity of T cells in SS SG comes from robust cytokine secretion that directly stimulate B cells or indirectly activate myeloid cells to secrete B cell activating factors (e.g. BAFF).(3,11,12) In addition, activated T cells have strongly upregulated levels of costimulatory molecules such as CD40L and ICOS and many more that induce B cell activation including class switching and affinity maturation.(13-17)

Nonetheless, foci within the inflamed exocrine glands can exist in more sophisticated structures that resemble secondary lymphoid organs. In around 25-30% of SS patients some of the periductal infiltrates develop into highly organized ELS containing high endothelial venules (HEV), stromal follicular dendritic cells (FDCs) and segregation of T and B cell areas. This spatial organization into specialized B cell and T cell areas together with CD21+ FDCs is called ectopic lymphoid neogenesis. It is believed that these structures direct T and B cell responses more optimally and result in increased antibody generation, affinity maturation, class switching and clonal expansion. The exact initiating steps in ELS formation in pSS are not shown. Induction of ELS within inflamed tissue has been shown to be critically dependent on lymphotoxin (LT) α and LTβ which can induce stromal lymphoid tissue organizer (LTO) cells to secrete cytokines such as IL-7, RANKL, CXCL13 and CCL21. IL-7 and RANKL subsequently can activate LTi cells that sustain and activate LTO cells. However, triggering of glandular tissue with TLR ligands was also shown to induce IL-7 which can initiate activation of LTi cells to start the cascade.(18-20)

IL-7/IL-7R axis in Sjögren's syndrome and ELS formation

In SS and SS-like disease in mice, increased levels of IL-7 have been found in salivary glands and serum, correlating with clinical parameters including salivary output and immunopathology including lymphocytic infiltration and presence of autoantibodies.(8,21-23) At the site of inflammation, a number of cell subsets, including fibroblasts, endothelial cells, macrophages, and dendritic cells (DCs), were shown to secrete IL-7. IL-7 can contribute to the development and chronicity of the disease by enhancing T cell responses and T cell-dependent activation of epithelial cells and B cells. The role of the IL-7 axis in SS is further supported by the observation that IL-7R positive cells were highly increased in salivary glands of SS patients as compared to non-Sjögren's sicca controls, correlating with numbers of T cells, B cells and IgG and IgM producing plasma cells(21). IL-7 stimulates induction of pro-inflammatory T cells displaying a Th1, Th22, or Th17 phenotype. In addition it induces CXCL10 which is abundantly expressed in SS salivary glands and serum.(7,8) These latter observations are consistent with the predominance of Th1 and Th17 cell activity in primary SS patients.(8,22)

IL-7R-expressing LTi cells and stromal cells are critical not only in the development of lymph nodes in fetal stages, but also in the development of ELS, as seen in chronic inflammatory diseases such as RA . Moreover, the attraction of LTi cells to tumors by CCL21 results in the development of a ELS–like environment. Of note, IL-7 induces the expression of not only CCL21, but also LTα, LTβ and TNFα , all of which are critically involved in the development of ELS.(23) Finally, it was demonstrated that enhanced expression of IL-7 in transgenic mice caused accumulation of LTi cells by promoting their survival. Enhanced IL-7 availability induced a 5-fold increase in Peyer's patch numbers, resulting from LTi-stimulated de novo formation of stromal tissue organizer cells. Overexpressed IL-7 also induces the formation

of multiple organized ectopic lymph nodes, which after immunization, developed normal T cell-dependent B cell responses and GCs. Consistent with the formation of ELS in patients with autoimmune diseases, naive IL-7–transgenic mice spontaneously developed ELS in target sites for autoimmune reactions, such as the pancreas and salivary gland . Therefore, by controlling IL-7R–positive LTi cell numbers, IL-7 regulates the formation of both normal and ectopic lymphoid organs.(18,19,24)

T follicular helper cells in Sjögren's syndrome

Although all activated Th cells can stimulate B cell activity, CXCR5-expressing T follicular helper (Tfh) cells in lymphoid organs and inflamed tissue are considered one of the most potent T cells subsets inducing B cell hyperactivity. In addition, Tfh cells play an important role in formation and maintenance of tertiary or ectopic lymphoid structures (ELS)/germinal center (GC)-like structures in chronic autoimmune disorders. As indicated above in around 30% of SS patients some of the periductal infiltrates develop into highly organized ELS containing HEV, stromal FDCs and segregation of T and B cell areas, including Tfh cells. Accumulating evidence supports that ELS represent niches for B cells to undergo affinity maturation, clonal selection and differentiation into autoreactive plasma cells.(6,20,25,26) The chemokines CCL19, CCL21, CXCL12 and CXCL13 known to play a role in secondary lymphoid organs are expressed in ELS. All of these have been found to be elevated in SS salivary glands. CXCL13 tightly regulates movement of CXCR5+ Tfh cells and B cells, including in these structures. Stromal cells, antigen-experienced T cells, and Tfh cells secrete CXCL13. In serum and salivary gland, CXCL13 has been shown to correlate with disease progression in several SS murine models. Furthermore, Cxcl13 is elevated before disease becomes evident in a murine SS-like disease model, and neutralizing Cxcl13 diminishes the disease(27). Elevated CXCL13 levels are expressed by a large proportion of patients with SS in either serum, saliva or salivary gland tissue, correlate with B cell hyperactivity and disease activity and are elevated in patients with lymphoma(6,27,28). In summary, these findings indicate that Tfh cells and ELS formation play an important role in SS pathogenesis. Recently, a novel cell type with Tfh-like characteristics was discovered, expressing CCR9 instead of CXCR5. In the next section we will discuss its potential role in Sjögren's syndrome and the relevance of studying this subset.

CCL25 and Tfh-like CCR9 cells: a novel axis in autoimmune diseases

The CCL25/CCR9-axis is a well-known mechanism guiding immune cells to the intestine, where DCs imprint a "gut-homing address code" in T cells upregulating CCR9 and $\alpha 4\beta 7$ dependent on all-trans-retinoic acid and then migrate towards CCL25 and adhere to MAdCAM-1.(29,30) CCR9+ Th cells are important for maintenance of mucosal immune homeostasis but also may have a function in mucosal inflammation, potentially contributing to inflammatory bowel disease (IBD) and primary sclerosing cholangitis (PSC).(31-33) Increased numbers of CCR9-expressing cells have been found in the circulation and inflamed intestinal tissue of Crohn's disease patients as well as elevated CCL25 production at the inflammatory site.

(34,35) Inhibition of CCR9 decreased 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 therapy.(36-38) Still, clinical trials inhibiting migration of CCR9+ Th cells are conducted in IBD, using for instance anti-MAdCAM-1 or anti-$\alpha 4\beta 7$ agents (clinicaltrials.gov) and novel inhibitors are being developed.(39-42) Also in other inflammatory diseases there is potential involvement of CCR9+ Th cells: in ankylosing patients elevated frequencies of CCR9+ Th cells have been found(43) and inhibition of CCR9 led to improvement of disease in a collagen induced arthritis model.(44)
Recently, in non-obese diabetic (NOD) mice, CCR9+ Th cells were also shown to mediate immunopathology in mucosa-associated tissues in accessory organs of the digestive tract, including the pancreas and salivary glands. The NOD mice spontaneously developed pSS-like symptoms and had infiltration of IL-21 expressing CCR9+ Th cells in the salivary glands. (45)

CCL25 mRNA is not detectable in healthy human salivary gland tissue, but is upregulated during oral inflammation.(46) Reduced methylation of the CCL25 gene is found in gingival tissue of periodontitis patients.(47) In mice, CCL25 gene expression is upregulated in the oral mucosa upon antigenic triggering and during wound healing.(48,49) Apart from demonstrating a pivotal role for CCR9+ Th-cells in experimental Sjögren-like disease, McGuire et al. found that CCR9+ Th cells are enriched in the circulation of pSS patients, indicating that these cells might play a role in the disease.(45) In another study, no differences in CCR9+ B cell frequencies were found.(50) In summary, there is accumulating literature suggesting a role for CCR9+ Th cells in inflammatory disorders including SS. The current thesis further investigates the potential role of CCR9+ Th cells in SS, a disease in which immunopathology involves inflammation of mucosal tissues.

Thesis outline

Novel tools to study salivary gland immunopathology in Sjögren's syndrome

Salivary gland biopsy is valuable in SS diagnostics and prognostics. The lymphocytic focus score (LFS, a focus is a lymphocytic infiltrate of >50 lymphocytes, the number of these foci is scored per $4mm^2$) as enumerated in H&E-stained salivary gland tissue sections forms part of routine clinical diagnostics and has an important position in the classification criteria for SS. In addition, the decreased percentage of IgA-expressing plasma cells, indicating increased infiltration of IgG+ and IgM+ plasma cells can be used to aid in clinical decision-making. However, these methods of analyzing salivary gland inflammation have several drawbacks, including lack of standardization, laboriousness and poor correlation of the scored abnormalities with dryness and other important clinical features.

Apart from the prognostic value for lymphoma development, molecular markers associated with clinical parameters but also aberrances in function of immune cells or tissue cells including epithelial cells and fibroblasts are currently lacking. Quantification of the several types of immune cells in salivary glands is scattered through literature, where different cohorts and different techniques are used.

In **Chapter 2** and **Chapter 3** novel techniques to efficiently retrieve more information from salivary gland biopsies were explored. In **Chapter 2**, supernatants from short-term (1 hour ex vivo) salivary gland biopsy explant cultures - called 'secretomes' were collected. Subsequently using Luminex multi-analyte proteomics a wide range of cytokines and chemokines secreted by the tissue explants were evaluated and correlated to clinical parameters. In **Chapter 3** frequencies of immune cells are determined using a novel form of cell quantification termed epigenetic cell counting (ECC), which makes use of cell type specific demethylated regions (e.g. demethylation of a CD3 gene region for T cells).**Chapter 4** shows soluble IL-7R, an indicator of IL-7/IL-7R axis activation which potentiates its action, as an example of a molecular marker detectable in both serum and salivary gland supernatants (secretomes). The correlation of sIL-7R with clinical parameters was studied.

CCR9-expressing T helper cells and innate lymphoid cells are potential novel regulators of lymphoid neogenesis in Sjögren's syndrome

The IL-7/IL-7R axis has been shown to play an important role in SS pathogenesis and in the near future inhibition of this interaction will be studied in clinical trials in SS patients (fase II testing anti-IL-7R treatment are currently initiated).

Importantly, IL-7 is essential for formation of ectopic lymphoid structures (ELS), including in the salivary gland. A recently described cell subset playing an important role in epithelial homeostasis but also in lymphoid neogenesis, are innate lymphoid cells (ILCs), which express high levels of IL-7R and depend on IL-7 for their survival. In **Chapter 5** the frequency and phenotype of ILC subsets are studied in peripheral blood of pSS as well as systemic lupus erythematosus (SLE) patients.

T follicular helper (Tfh) cells are a more extensively studied cell subset in lymphocytic infiltrates in SS patients involved in lymphoid neogenesis. Tfh cells mainly reside in lymphoid organs and potently stimulate antigen specific B cell differentiation and antibody responses. They are characterized by expression of CXCR5, PD-1, ICOS, Bcl-6 and produce high levels of IL-21. In **Chapter 6** CCR9+ Th cells that have 'Tfh-like' characteristics were studied in SS patients. Their presence of these cells and their ligand in salivary glands of SS patients are assessed, as well as their phenotypic and functional characteristics *ex vivo* and in *vitro*. In **Chapter 7** these cells are studied more in depth, analyzing their transcriptome in comparison with CXCR5+ Th cells and Th cells not expressing CCR9 and CXCR5. In **Chapter 8** co-expression

of major effector Th subset-defining chemokine receptors is assessed on CCR9+ Th cells to study processes potentially involved in homing of these cells to the salivary glands of pSS patients.

In **Chapter 9** the mTOR pathway is studied in salivary glands and peripheral blood lymphocytes, including CCR9+ Th cells, of pSS patients. The mTOR pathway is important for lymphocyte function by integrating environmental triggers including T cell receptor (TCR) and B cell receptor (BCR) activation. The inhibition of this pathway is studied as a potential therapeutic target halting T and B cell activation in pSS. Finally, the capacity of mTOR inhibition to halt CCR9+ Th cell activation was studied.

In **Chapter 10** the literature on chemokines and chemokine receptors in SS is reviewed in which some of the main findings of this thesis are included.

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Ervaringen van patiënten:

leven met het syndroom van Sjögren en het belang van patiëntenparticipatie in wetenschappelijk onderzoek

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Leven met Sjögren

Sjögren, nooit van gehoord… is meestal het eerste dat mensen tegen mij zeggen als ik hen vertel dat ik het syndroom van Sjögren heb. Daarna is het een kwestie van uitleggen wat het inhoudt, tenminste als ik het gevoel heb dat men écht geïnteresseerd is, zo niet dan volsta ik met dat ik weinig speeksel en traanvocht heb.

Ik ben in de gelukkige omstandigheid dat in mijn (werk)omgeving iedereen weet wat Sjögren is en dat er heel veel ervaringsdeskundigheid is. Dat klinkt misschien alsof er alleen maar over Sjögren gesproken wordt, maar dat valt gelukkig mee. Het belangrijkste is het begrip dat ik ondervind.

De uitgebreidere versie van wat Sjögren voor mij betekent, is:
Het is een grillige ziekte, geen dag is hetzelfde qua energie en ongemakken. De ene dag heb ik heel veel last van (voornamelijk) één oog, dat ik met dat oog maar 20% kan zien en dat zo zeer doet dat ik mijn handpalm op mijn oog druk om het een beetje vol te houden. Uiteraard is dit heel vermoeiend en je bent vaak al moe. Dan druppel je toch, dat is de oplossing! Maar druppels (kunsttranen) zijn na een paar keer knipperen weg en geven niet echt verlichting. Dus moet er zwaarder geschut komen in de vorm van ciclosporine druppels.

'Gewone mensen' zullen het gevoel herkennen dat je een wimperhaartje in je oog krijgt en dat dan de hele dag door.

De andere dag heb ik hier minder last van, maar dan wel weer ergens anders van.
Gewoon iets proeven in de supermarkt? Daar moet ik goed over nadenken voordat ik iets neem. 'Heb ik mijn flesje water wel bij me?', anders kan ik het gevoel krijgen dat ik stik, want het blokje kaas/worst/chipje blijft gewoon in je keel hangen. Dat overkomt mijn 's nachts ook wel eens, dat ik wakker wordt en het idee hebben dat je door een droge mond stikt.

En dan heb ik het nog niet gehad over de – zoals men in folders over Sjögren beschrijft – invaliderende vermoeidheid. Een avondje vroeg naar bed gaan en je kunt de wereld weer aan. Helaas helpt dat dus niet. Van te voren proberen in te schatten dat je op een dag gaat werken en 's avonds uitgaat kan al te veel zijn. Een weekend weg? Klinkt erg leuk, maar hou ik dat wel vol? Het schuldgevoel dat je mensen dan belemmert in hun doen en laten, want men houdt rekening met jou, speelt dan ook een rol.

Mijn geliefde en onze zonen zien vaak al aan mij hoe het met mij gaat en nemen ongevraagd dingen van mij over, dat maakt dat ik ook aan 'de leuke dingen doen' tijd kan besteden. In plaats van alleen maar met het huishouden bezig zijn naast mijn werk en dan niets meer

kunnen door de vermoeidheid. (En uiteraard moet dat soms ook wel herhaaldelijk gevraagd worden ;) …).

Onbegrip is iets waar veel patiënten tegenaan lopen. 'Gisteren zag ik je vrolijk over de markt lopen, en nu moet je onze afspraak afzeggen omdat je moe bent.' Zelf heb ik hier gelukkig weinig problemen mee, omdat ik zelf bijna nooit afspraken afzeg want ik vind het veel te leuk om met iemand af te spreken. Dat ik het de dag(en) erna moet bekopen, dat heb ik er dan gewoon voor over. Ik moet er wel bijzeggen dat ik er wel voor moet waken dat ik niet te veel dingen achterelkaar probeer te plannen, want dan stort ik in. Een enkele keer geef ik zelfs een brochure aan iemand die écht heel erg geïnteresseerd is in mijn ziekte, maar dat komt zelden voor.

Kun je nog wel genieten? Niet als je je blijft verzetten en tegen je ziekte blijft vechten. Cognitieve therapie met beweging kan helpen, als je dat toelaat. Maar verwacht niet dat je ziekte dan over is en dat je dan weer alles kunt wat je voorheen ook kon. Realistische doelen stellen is belangrijk maar net zo goed over je grenzen heen gaan, ook al weet je dat je het daarna moet bezuren.

Leven met Sjögren, tuurlijk kan dat! Al is het niet altijd eenvoudig. Want toch blijft er die angst in je onderbewust zijn iedere keer dat je voor controle naar het ziekenhuis gaat. Gaat het nog goed met me? Hoe zijn de bloeduitslagen? Is er weer iets bijgekomen? Heb ik kans op een andere auto-immuunaandoening erbij? Hoe staat het met mijn nierfunctie? Longen? Non-Hodgkin? Op al deze vragen is NOG geen antwoord te geven, maar er is goed nieuws! Want overal ter wereld wordt heel veel onderzoek gedaan naar het syndroom van Sjögren.

Wij in Nederland zitten in een bevoorrechte positie dat men van onze expertise als Sjögrenpatiënten gebruik maakt bij wetenschappelijk onderzoek. Niet alleen als 'proefpersoon', maar ook als 'meedenker' voordat een onderzoek opgestart wordt en bijvoorbeeld als meelezer. Het UMCU is begonnen met patiënten te betrekken als patiëntpartners in onderzoek en ook bij het ontwikkelen van een Zorgpad bij Sjögren. Als patiënt heb je echt het gevoel dat jouw inbreng, hoe klein die in jouw ogen is, ertoe doet. Voorvechters in dit zijn de Nationale Vereniging Sjögrenpatiënten (NVSP) en de artsen en onderzoekers van dr. Aike Kruize geweest. Wie had er vroeger gedacht dat iemand als prof. dr. Tim Radstake een lezing op zowel de labdag van het UMCU als op de Landelijke Contact- en Informatiedag (LCID) van de NVSP vóór patiënten zou houden. Zoiets was ondenkbaar geweest.

Het feit dat het UMCU een labdag georganiseerd heeft voor patiënten met een rondleiding door het ziekenhuis om te kijken wat er met je lipbiopt en bloed gedaan wordt na afname, spreekt nu voor zich, maar enkele jaren geleden was dat not done geweest.

Het is echt heel leuk om te zien hoe de patiënten en onderzoekers steeds meer bij elkaar betrokken raken. De onderzoeker zag zelf geen patiënten, wist niet wat de ziekte voor een impact op de patiënt had, maar probeerde wel een oplossing voor de ziekte te vinden. Nu is de o-nderzoeker zichtbaarder voor de patiënt en anders om. We komen elkaar tegen bij posterpresentaties over het onderzoek op het Internationale Sjögren Syndroom Symposium of de EULAR (jaarlijks Europees Reumatologie congres), bespreken daar de uitkomsten (in lekentaal, die voor onderzoekers nogal moeilijk is) en opperen ideeën voor andere onderzoeken. De onderzoekers houden lezingen op de LCID, vertellen over hun onderzoeken d.m.v. een poster en de NVSP stimuleert op verschillende wijze wetenschappelijk onderzoek naar Sjögren. De betrokkenheid van deze onderzoeksgroep bij ons als patiënten is hartverwarmend!!!

Joyce Koelewijn-Tukker

Help ik heb Sjögren

In 2013 werd na een traject van drie maanden de diagnose Sjögren bij mij gesteld.Je gaat er niet aan dood, maar toen ik het boekje doorbladerde dat ik had mee gekregen, werd ik er ook niet echt vrolijk van.

Na jaren van vage klachten die zich voornamelijk kenmerkten in een chronische vermoeidheid, kreeg ik van de een op andere dag last van een enorm droge mond. Ik besloot hier op Google naar te zoeken. En deze gaf mij gelijk het antwoord. Mijn huisarts wist niets van Sjögren, maar gaf mij het voordeel van de twijfel en verwees mij naar het UMC Utrecht.

De drie maanden die aan de diagnose vooraf gingen waren intensief. Veel consulten bij mijn reumatoloog, bloedonderzoeken, uitslagen met elkaar bespreken en daar medicatie op afstemmen. Van staaltabletten en B12 injecties moest ik mij gelijk beter gaan voelen. Uiteindelijk besloot mijn reumatoloog op basis van mijn klachten en bloeduitslagen om een lipbiopt te laten uitvoeren. Door een stukje speekselklier uit mijn lip te verwijderen, kwam de definitieve diagnose Sjögren.

Langzaam begon ik mij beter te voelen, de bloeduitslagen gingen de goede kant op en de contacten met mijn reumatoloog gingen van maandelijks, naar vier maal per jaar. En uiteindelijk terug naar een keer per jaar.

Op een van deze consulten adviseerde hij mij om een keer naar de landelijke contact- en informatiedag van Sjögrenpatiënten te gaan. Een informatiedag? Zijn er nog meer patiënten dan? Je bent zo druk met jezelf dat je niet eens beseft dat er nog meer mensen kunnen zijn die Sjögren hebben. Op deze informatiedag is zonder dat ik het wist, bij mij de basis gelegd dat ik later actief zou worden in de patiëntenparticipatie.* Je spreekt met lotgenoten over de impact die Sjögren op je leven heeft. Als ik naar mijn klachten kijk en de invloed die de Primaire vorm van Sjögren op mijn leven heeft vind ik dit best behoorlijk. Om hier een klein beeld van te schetsen:

Altijd gebroken nachten. Ik word steeds wakker van een droge mond. Met het gevolg dat ik niet uitgerust opsta. Dit heeft invloed op mijn werk, maar ook op mijn privéleven. Ik ben een dag minder gaan werken zodat ik halverwege de week kan bijtanken. Mijn werkgever steunt mij en heeft samen met mij een aanpassing in mijn werk aangebracht zodat ik het met vier dagen werken red. De hele dag door mijn ogen druppelen tegen de droogte. Om mijn eten te kunnen doorslikken heb ik drinken nodig door mijn tekort aan speeksel. De informatiedag was een dag met gemengde gevoelens. Ik hoorde verhalen over onbegrip bij familie, vrienden en werkgever. Daarbij was er ook een patiëntengroep die bij ieder consult een andere reumatoloog krijgt. En hun verhaal steeds opnieuw moeten vertellen.

Een paar maanden na deze dag werd er een laboratoriummiddag in het UMC Utrecht georganiseerd. Wij, de patiënten, konden een kijkje achter de schermen nemen en zien wat er met ons bloed wordt gedaan. Bloed dat wij afstaan voor wetenschappelijk onderzoek. Het werd voor mij een middag van verbazing en ontroering.
Artsen, onderzoekers, wetenschappers allen kwamen op hun kostbare vrije dag naar het ziekenhuis om vol overgave en passie te vertellen hoe zij op zoek zijn naar een remedie tegen Sjögren. Je hebt als patiënt geen idee wat er zich achter deze schermen allemaal afspeelt.
Aan het eind van deze middag kon ik me opgeven als patiëntpartner. Ik wist op dat moment nog niet wat dit inhield. Maar ik dacht: Als deze mensen zo hard werken voor ons, dan mag ik daar best wat tegenover stellen.
*Patiëntenparticipatie is het inbrengen en benutten van de specifieke ervaringsdeskundigheid van patiënten met als doel onderzoek, beleid of kwaliteit van zorg te verbeteren en beter te laten aansluiten bij de behoeften van de patiënt.

Het belang van Patiëntenparticipatie
Nadat Ik het besluit had genomen om mij actief te gaan opstellen in de patiëntenparticipatie, heb ik als eerste een training patiëntenparticipatie in wetenschappelijk onderzoek gevolgd. Dit is een training voor mensen met een chronisch ziekte of beperking die betrokken willen worden bij onderzoekschema's en de uitvoering van onderzoeksprojecten. Je krijgt inzicht in de mogelijkheden en beperkingen om invloed uit te oefenen op deze projecten.
Kort na deze cursus heb ik meegeholpen een informatiedag voor patiënten te organiseren, zijn we nu van start gegaan om een goed zorgpad voor de patiënten op te stellen. Het belang om als patiënt actief te zijn in de patiëntenparticipatie is mij ondertussen heel duidelijk geworden.
Mijn reumatoloog juichte het toe toen ik hem vroeg hoe hij tegenover patiëntenparticipatie stond en mijn rol als patiëntpartner hierin. Ik wilde zijn goedkeuring omdat er altijd een kans is dat je als patiënt om je expertise wordt gevraagd en je met je eigen arts in aanraking kunt komen. Je moet er beiden heel bewust van zijn dat je in deze rol gaat samenwerken en je gelijkwaardige partners bent.
We zijn nu een kleine twee jaar verder en zowel mijn reumatoloog als ik zitten nu in de werkgroep voor het zorgpad. Met als doel dat de patiënt, een helder, doelmatig en kort traject doorloopt van diagnose tot behandeling. Allebei met onze eigen expertise. Een beter voorbeeld van patiëntenparticipatie kan ik niet geven. We willen allebei het beste.
Ik realiseer me terdege dat niet iedere patiënt dit wil of kan. Toch hoop ik dat ik door met andere patiënten en waar mogelijk ook met artsen, hierover te praten, het belang van patiëntenparticipatie kan uitdragen.

Lucienne Dekker

Mijn “Sjögren etiket”

Oorpijn?!?! Dat heb ik sinds mijn kinderjaren niet meer gehad. Flinke steken zijn het zo af en toe, en pijnlijk bij aanraken. Na een paar weken toch maar eens bij de huisarts langs. Die, professor, doctor, vermoedt een parotitis en heeft als gebruikelijk, zijn afwachtend beleid in de aanbieding. Napeinzend over dit gesprek komt bovendrijven, dat ik een overzicht van auto- immuunziektes, er een gezien heb, waar de speekselklier bij betrokken is. Mijn kans op een auto-immuunziekte is verhoogd, omdat al jarenlang) mijn schildklier niet meer goed werkt door een auto-immuunziekte. Al snel vind ik het syndroom van Sjögren en beginnen er zaken op zijn plaats te vallen. De vermoeidheid, zo snel de batterij helemaal leeg en lang tijd nodig om weer op te laden. Mijn droge keel, die maakt dat ik een vergadering alleen maar doorkom met een glas water onder handbereik. Mijn ogen die bij de laatste keer vliegen zo’n pijn deden. Een raar kuchje, als ik hard moest lachen. Ik was in al die jaren aan deze klachten gewend geraakt en weet het aan mijn slecht werkende schildklier. Maar nu had ik het idee dat er nog een ander etiket op mijn klachten zou kunnen passen.

Terug naar de huisarts om mijn vermoeden voor te leggen. Een verwijzing naar een internist volgt. Omdat er in mijn bloed geen aanwijzingen waren en mijn mond er helemaal niet zo droog uit zag, kon het volgens deze dokter toch geen Sjögren zijn en was mijn vermoeidheid toe te schrijven aan het harde werken , dat ik deed. Geen etiket dus om mijn klachten te verklaren. Opnieuw aan het zoeken geslagen. Vooral het forum van de Nationale Vereniging Sjögren Patiënten en de links van het Reumafonds, laten zien dat de diagnose Sjögren niet zo eenvoudig te stellen is. Er is duidelijkheid over het merendeel van de ingrediënten die op het etiket moeten staan, maar het opsporen van die ingrediënten en hun vereiste mix blijkt niet zo eenvoudig. Ik vraag een second opinion bij een specialist die grote bekendheid geniet in Sjögren kringen. Een paar maanden na het consult van de internist plakt deze reumatoloog het etiket er wel op en nog beter: mijn klachten worden behandeld. Ik word onmiddellijk lid van de NVSP.

In de jaren daarop probeer ik mijn eigen gebruiksaanwijzing te vinden. Dat lukt aardig met uitzondering van het energievraagstuk. Ik besluit minder te gaan werken in een minder zware functie. In die zelfde tijd sluit ik me aan bij de kennisgroep van de NVSP. Die heeft als doel : patiënten participatie in onderzoek te bevorderen en na te denken over kwaliteitscriteria e.d. Ik ben blij verrast dat patiënten mogen denken over de etiketten, hun ingrediënten en gebruiksaanwijzing. Bij de Universiteit Utrecht sluit ik aan bij het panel psycho-reumatologie, later opgevolgd door het patiëntenplatform van de afdeling Reumatologie van het UMC Utrecht. Ik volg jarenlang alle trainingen en studiedagen over onderzoeksparticipatie. Een paar jaar later, ik ben inmiddels met pensioen, word ik volwaardig partner in een onderzoeksgroep, die de ervaringen van vrouwen met Sjögren gaat onderzoeken en bundelen. Dat heeft grote gevolgen: ik mag een poster presenteren op de Eular (he `t

reumacongres); ik word onderzoekspartner voor Eular en lid van de patiëntencommissie van de Wetenschappelijke Advies Raad van het Reumafonds. Inmiddels heb ik vanuit patiëntenperspectief al vele onderzoeksaanvragen , lekensamenvattingen van artikelen en websites beoordeeld. Het inzetten van mijn ervaringskennis om kennis, behandeling en zorg van het syndroom van Sjögren te verbeteren geeft mij veel voldoening.

Persoonlijk kan ik inmiddels wat etiketten er bij plakken. Dit levert wel steeds gepuzzel op. Ik heb ook niet altijd behoefte aan nieuwe etiketten, vooral als ze toch niet leiden tot behandeling. Ik heb wel idee wat er op het etiket "ideale dokter" moet staan, maar vind het soms moeilijk die te vinden. Die heeft genoeg tijd , kennis (of bereidheid die te vergaren), geduld en gezond verstand om op basis van mijn mix aan ingrediënten met mij samen mijn gebruiksaanwijzing op mijn etiketten te plaatsen. Misschien dat het binnenkort gaat lukken, er komt een nieuwe kans.

Ruim 10 jaar heb ik nu dat Sjögren etiket, maar het is niet van buitenaf zichtbaar. Als ik wil dat er rekening met mijn ziekte wordt gehouden, moet ik zelf aangeven waar ik last van heb en/of hoe dit te voorkomen. Dus: geen volle agenda, activiteiten verdelen over de dag en de week met voldoende herstelmomenten; wakker worden een paar keer 's nachts vanwege een droge mond of pijnlijke spieren; altijd oogdruppels en water bij me als ik de deur uitga; niet te lang achterelkaar praten; me zorgen maken of mijn organen goed blijven werken en nog veel meer waarschijnlijk, maar … net als bij de schildklier raak je aan veel zaken gewend.

Marianne Visser

Leven met Sjögren

Het is half juni. Allerlei mensen om mij heen kunnen niets meer afspreken, want druk, druk, druk. De vakantie komt er aan en er moet nog zoveel gebeuren van tevoren. Ik zelf wil eigenlijk nog helemaal niet denken aan de vakantie, alhoewel ik op zich vakantie wel leuk vind, als ik op plaats van bestemming ben en geacclimatiseerd. Maar aan het hele proces van tevoren, het inpakken en de reis naar de vakantiebestemming toe, heb ik een hekel en dat komt grotendeels omdat ik het syndroom van Sjögren heb.

Tijdens het inpakken word ik altijd het meest zenuwachtig van het verzamelen van de medicatie die ik voor twee of drie weken nodig heb, want dat is nogal wat. Pijnmedicatie, oogdruppels, speciaal mondspoelmiddel, vette zalf voor mijn droge lippen zodat ze niet gaan scheuren bij mijn mondhoeken en de middeltjes voor mijn huid, die zich gedraagt alsof ik eindeloos in de puberteit zit.

Maar dan ben ik er nog niet. Sjögren komt zelden alleen. Het brengt in mijn geval ook andere, al dan niet gerelateerde, aandoeningen mee. Dus er gaat mee: medicatie voor de schildklier, voor de bloeddruk, voor mentale problemen en voor een migraineaanval. Uit voorzorg gaat er ook nog een antibioticakuur mee, want ik kan zomaar een ontsteking krijgen. Ooit moest ik in Toscane midden in de nacht naar een ziekenhuis en dat was geen leuke excursie. Mijn familie vraagt voor vertrek dan ook meermaals of ik alle medicatie bij me heb. Twee keer heb ik me vergist. In Engeland ging het om mijn schildkliermedicatie en dat was relatief snel opgelost, maar in de VS kostte me het een halve dag om vijf pilletjes antidepressiva te regelen. Tijdens de reis protesteert mijn lijf tegen de lange uren in één houding in vliegtuig, auto, trein of bus. Het zijn reumatische klachten die symptomatisch zijn voor Sjögren. Mijn huid, ogen en mond, die toch al droog en ontstoken zijn, protesteren tegen de droge en vieze lucht van airconditioning en ventilatie. Ik moet wennen aan de andere omgeving, aan het andere bed en het andere eten. En als ik pech heb, krijg ik als bonus ook nog een migraineaanval; een oorzakelijk verband tussen Sjögren en migraine is tot nu toe niet aangetoond, maar ze versterken elkaar wel degelijk.

Als ik een dag of vier op plaats van bestemming ben, is mijn lijf ook gearriveerd. Ik heb uiteraard nog steeds alle klachten van het syndroom van Sjögren, maar nu weer op het niveau van alledag en ik kan, op mijn manier, van de vakantie genieten.

Het is nu drieëntwintig jaar geleden dat ik de diagnose primair syndroom van Sjögren te horen kreeg. Ik had tijdens de zwangerschap van mijn oudste kind ineens peesontstekingen in mijn polsen en daarom werd ik doorgestuurd naar een reumatoloog. Ik was echter al jaren met mijn gezondheid aan het tobben en door deze diagnose vielen dingen op hun plek. Met

name waarom ik vaak zomaar heel moe was, terwijl ik echt geen gekke dingen had gedaan. Soms wilde ik op mijn werkplek wel op de grond gaan liggen slapen, want ik kon mijn ogen nauwelijks open houden. Ik was eindeloos grieperig was en koortsig, maar niet ziek genoeg om echt griep te hebben. En nu snapte ik ook waarom ik geen biscuitje meer kon eten zonder een hele kop thee en waarom een toastje met zalm tijdens een feestje geen feestje meer was. De eerste jaren na de diagnose heb ik mijn best gedaan om te leven zoals ik dat altijd had gedaan, ook toen ik twee kinderen had. Ik vond dat ik dat moest. Op een bepaald moment ging het echter niet meer. Uiteindelijk koos ik voor mijn gezin. Ik heb nog jaren geprobeerd om mijn proefschrift in de Middelnederlandse letterkunde af te ronden, maar op een zeker ogenblik ben ik ook daarmee gestopt. Toen mijn zoon tijdens een zware migraineaanval voor mij thuis bleef van school, omdat hij te ongerust was om mij alleen te laten, besloot ik dat de stress de eer van een doctorstitel niet waard was. Omdat het onderzoek toch mijn derde kindje is, zal ik de resultaten op enig moment nog op internet publiceren.

Toen ik gestopt was met werken, had ik lang het gevoel dat ik mezelf moest verantwoorden. Ik deed vrijwilligerswerk en allerlei andere klusjes, want de mensen dachten vast dat ik alleen maar vakantie aan het vieren was. Tot ik me realiseerde dat ík dat zelf vond en dat leven met Sjögren geen vakantie is. Bij alles wat ik doe, ben ik me altijd bewust van mijn droge mond, mijn droge ogen, mijn droge huid, mijn reumatische lijf en de niet weg te slapen vermoeidheid. Ik ben altijd ziek of onderweg en dat is niet op te lossen met meer rust, vitamines of hippe voedingsmiddelen. Het kost me veel moeite om mijn leven te leiden, zelfs als ik op vakantie ben, maar ik doe het toch. Nu geef ik les, ik speel toneel, ik doe vrijwilligerswerk, ik lees, ik naai en ik sport, en niet omdat ik dat moet, maar omdat ik het leuk vind. En ik participeer als patiënt bij de afdeling Reumatologie, omdat ik het belangrijk vind dat artsen en onderzoekers weten wat dat is, leven met Sjögren.

Wilma Wissink

CHAPTER 2

The Salivary Gland Secretome: a Novel Tool towards Molecular Stratification of Primary Sjögren's Syndrome and Non-Autoimmune Sicca Patients

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Abstract

Introduction. To explore the potential of salivary gland biopsy supernatants (the secretome) as a novel tool to aid in stratification of sicca patients and to study local immunopathology in Sjögren's syndrome.

Methods. Labial salivary gland biopsies were incubated in saline for one hour. In these tissue supernatants from a discovery cohort (n=16) of primary Sjögren's syndrome (pSS) and non-Sjögren's sicca (nSS) patients, 101 inflammatory mediators were measured by Luminex. Results were validated in a replication cohort (n=57) encompassing patients with pSS, incomplete SS (iSS) and nSS.

Results. The levels of 23 cytokines were significantly increased in pSS versus nSS patients in the discovery cohort. These 23 and three additional cytokines were measured in a second cohort. Elevated concentrations of 11 cytokines were validated and the majority correlated with clinical parameters. Classification tree analysis indicated that the concentrations of CXCL13, IL-21, sIL-2R and sIL-7Rα could be used to classify 95.8% of pSS patients correctly.

Conclusion. Labial salivary gland secretomes can be used to reliably assess mediators involved in immunopathology of pSS patients, potentially contributing to patient classification. As such, this method represents a novel tool to identify therapeutic targets and markers for diagnosis, prognosis and treatment response.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disorder characterized by dryness and lymphocytic infiltration of exocrine glands.(1) Salivary gland biopsy is a valuable tool for pSS diagnostics. The lymphocytic focus score (LFS) as enumerated in H&E stained salivary gland tissue slides is part of routine clinical diagnostics and has an important position in current and previous classification criteria.(2,3) However, this way of analyzing tissue has several limitations, including lack of standardization and poor correlation of the scored abnormalities with dryness.(4)

Optimization of existing histology protocols to determine salivary gland inflammation will help to improve diagnostics of pSS.(4) In parallel, exploration of other methodologies for analysis of local immunopathology could facilitate diagnostics, prognostics and patient-tailored treatment. Currently used techniques do not allow for high-throughput analyses of protein expression in tissues. Saliva proteomics is a promising tool, but has limitations including inapplicability to the driest patients and technical challenges such as degradation of cytokines by salivary enzymes.(5) We here explored whether multi-cytokine analysis of supernatants from whole minor salivary gland biopsy samples, which we refer to as the "secretome", can be used as a clinically relevant tool to classify patients and yield insights into immunopathology.

Patients and Methods

Patients

Primary Sjögren's syndrome (pSS) patients (n=8 and n=24 in discovery and validation cohort, respectively) were diagnosed by a rheumatologist and fulfilled the American European Consensus Group (AECG) classification criteria.(2) Non-Sjögren's sicca (nSS) patients (n=8 and n=17) were defined as patients with sicca complaints, without a connective tissue disease including pSS, without lymphocytic infiltration in the salivary gland biopsy (LFS=0), and without anti-Ro/SSA or anti-La/SSB autoantibodies. Patients with incomplete Sjögren's syndrome (iSS) (n=16) were defined as patients with sicca complaints, without a connective tissue disease including pSS, who do not fulfill the classification criteria for pSS, but do have signs of lymphocytic infiltration and/or the presence of anti-Ro/SSA or anti-La/SSB autoantibodies (clinical data are described in **Table 1**). Because the tissue supernatants are regarded as rest material and clinical data were provided pseudo-anonymously, no ethical approval or informed consent were required according to the guidelines of the hospital's ethical committee. Usage of left-over fresh tissue was approved by the hospital's ethical committee (document nrs. 09-011 and 14-589) and patients gave their written informed consent.

	Discovery cohort		Validation cohort		
	nSS /iSS (n=6/2)	pSS (n=8)	nSS (n=17)	iSS (n=16)	pSS (n=24)
Female gender, n (%)	8 (100)	7 (88)	15 (88)	14 (88)	21 (88)
Age, years (mean ± SD)	50 ± 17	51 ± 13	49 ± 16	54 ± 15	55 ± 12
Anti-Ro/SSA+, n (%)	2 (25)	6 (75)	0 (0)	10 (63)	16 (67)
Anti-La/SSB+, n (%)	0 (0)	2 (25)	0 (0)	0 (0)	4 (17)
ANA+, n (%)	3 (38)	6 (75)	9 (53)	7 (44)	17 (71)
RF+, n (%)	0 (0)	3 (37.5)	0 (0)	1 (6)	8 (33)
Lymphocytic focus score (foci/4mm2)	0 (0 – 0)	1.8 (1.0 – 2.4)	0 (0 – 0)	0.2 (0 – 0.7)	1.6 (1.0 – 2.6)
IgA+ plasma cells (%)	73 (70 – 80)	62 (45 – 67)	77 (71 – 80)	70 (68 – 76)	59 (49 – 64)
Schirmer (mm/5min)	2 (0 – 11)	7 (1- 25)	4 (1 – 7)	10 (2 – 13)	7 (3 – 11)
Serum IgG (g/L)	10.8 (7.6 – 14.2)	14.4 (11.8 – 19.0)	11.0 (6.5 – 12.3)	9.0 (8.0 – 13.6)	13.9 (10.1 – 18.3)
ESR (mm/h)	7 (5 – 12)	13 (7 – 50)	7 (6 – 10)	9 (5 – 15)	15 (7 – 29)
ESSDAI score (0-123)	NA	2 (0 – 6)	NA	NA	3 (1 – 7)
ESSPRI score (0-10)	NA	3.5 (1.6 – 5.4)	NA	NA	5.3 (2.3 – 6.0)
Immunosuppressants, n (%)	1 (12.5)	1 (12.5)	1 (6)	2 (13)	3 (12.5)

Table 1. Patients' characteristics.

Median with IQR are represented, unless specified otherwise. SSA: Sjögren's syndrome antigen A; SSB: Sjögren's syndrome antigen B; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor.

Salivary gland tissue supernatant preparation and multi-cytokine analysis

Fresh labial salivary gland tissues were thoroughly rinsed and incubated with 200µL of saline (0.9% NaCl) in a 500µL vial (Sarstedt) for 1 hour at room temperature. The vial with saline was weighed before and after adding the biopsy tissue to determine the weight of the salivary gland biopsy tissue. After 1 hour the biopsy tissue was transferred to the pathology department for diagnostic procedures. This introduced no changes to routine diagnostic procedures and all tissues could adequately be assessed by the pathologist. The remaining tissue supernatants were rendered cell-free by centrifugation at 500g for 5 minutes and stored at -80°C (**Figure 1A**). In tissue supernatants from pSS and nSS/iSS patients, 101 soluble mediators (**Supplementary Table 1**) were measured by Luminex as previously described.(6) Mediators that were significantly different ($p \leq 0.05$) between the two groups were measured in a larger validation cohort by Luminex. Total protein concentrations of the supernatants were measured using a BCA protein assay kit (Thermo Fisher Scientific).

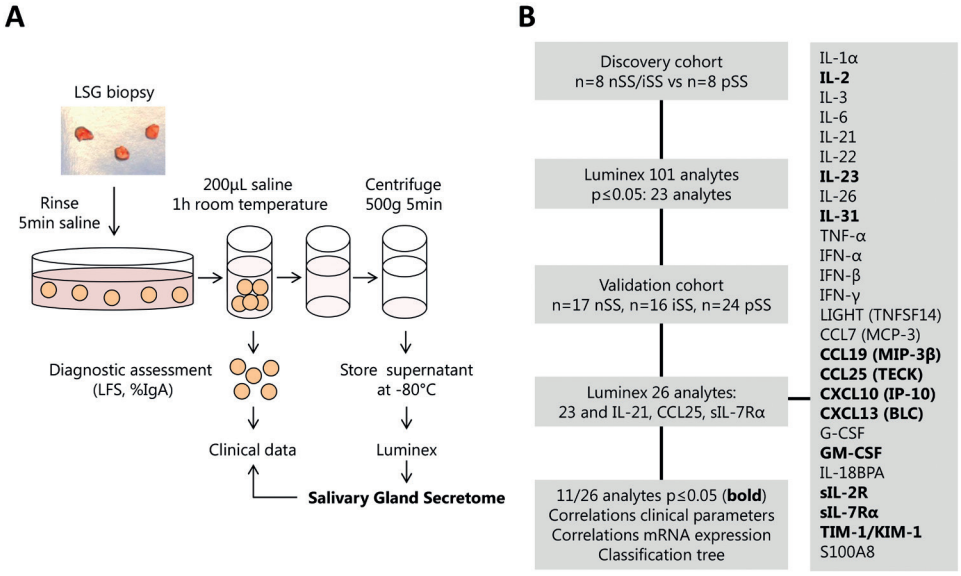


Figure 1. Laboratory procedure to obtain the salivary gland secretome and flow diagram of cytokine measurements.

(**A**) Labial salivary gland (LSG) tissues were rinsed directly after diagnostic biopsy procedures and incubated in 200µL of saline for 1 hour at room temperature. Tissue supernatants were rendered cell-free by centrifugation, frozen in liquid nitrogen and stored at -80°C until measurement by Luminex. Data retrieved from the salivary gland secretomes were correlated with the diagnostic biopsy characteristics and other clinical parameters. (**B**) In a discovery cohort of 8 pSS vs 8 nSS patients, 101 cytokines were measured in supernatants of weight-matched biopsies. The 23 cytokines that showed statistically significant differences and 3 additional cytokines were measured in a validation cohort of nSS, iSS and pSS patients. Cytokines detected at significantly different levels in pSS vs nSS in the validation cohort are indicated in bold.

RNA sequencing

Of biopsies of 6 nSS, 5 iSS and 12 pSS patients from this cohort in which the secretome was measured, RNA sequencing was performed, allowing for analyses of correlations between mRNA expression and protein concentrations in the secretome. Retrieval of stored, left-over, frozen tissue samples was approved by the hospital's ethical committee. Cryo-preserved salivary gland tissue was stored in Tissue-Tek at -80°C. Samples were selected based on availability. Sixty sections of 20µm were cut from each biopsy and lysed in RLT-plus (Qiagen) supplemented with beta-mercaptoethanol. RNA was isolated using the AllPrep Universal Kit (Qiagen). RNA sequencing was performed on an Illumina NextSeq500 sequencer providing approximately 20 million 75 bp single-ended trimmed reads for each sample. The sample qualities were assessed by FastQC and the reads were mapped to the human genome (GRCh38) using STAR aligner.(7) Since the samples were sequenced in two batches, the batch effect was corrected using RUVSeq package in R.(8) The normalized expression values (vsd normalized read counts) for the genes were calculated using the DESeq2 package in R.(9)

Statistical analyses

Statistical analyses were performed in SPSS (Version 21). Differences between the groups were assessed by Mann-Whitney U test. Differences between groups were considered statistically significant at $p \leq 0.05$. The cytokine concentrations in the validation cohort were used to perform correlations with all available clinical parameters, including LFS, percentage of IgG+/IgM+ plasma cells in the biopsy, Schirmer test results, serum IgG levels, ESR, EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) and EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI). Spearman's rank correlation coefficient was used for correlation analyses. In addition, the cytokine concentrations in the validation cohort were used to perform classification tree analysis using the CHAID (chi-squared automatic interaction detection) method.

Results

Salivary gland tissue supernatants allow for detection of a large amount of inflammatory mediators

To assess whether salivary gland supernatants can be used as a tool to study local inflammatory mediators, we measured 101 mediators in 8 pSS and 8 nSS patients with matched biopsy weights (mean±SD: 51.5±21.2mg versus 48.7±18.2mg) by Luminex. 95 of these mediators were detected in all samples and 6 were detected in the majority of the samples. 23 mediators were significantly increased in the pSS group compared to the nSS group (**Figure 1B**).

Robustly increased inflammatory mediators in the secretome of pSS patients correlate with clinical parameters

We next sought to validate the 23 mediators that were increased in pSS patients in a validation cohort of 24 pSS, 16 iSS, and 17 nSS patients. In addition, IL-21, soluble IL-7Rα and CCL25, which are increased in patients with pSS and play a role in salivary gland inflammation(10-12), were measured in this validation cohort. There were no significant differences between the groups in weights of the biopsies (mean±SD: 63.0±47.6mg in nSS, 72.7±45.2mg in iSS and 67.4±28.6mg in pSS) or in total secreted protein concentrations measured in the supernatants (mean±SD 0.42±0.28μg/μL in nSS, 0.53±0.24μg/μL in iSS and 0.55±0.31μg/μL in pSS).

Eleven mediators were significantly increased in pSS versus nSS patients: CXCL13, CCL19, CXCL10, TIM-1/KIM-1, IL-31, IL-2, GM-CSF, IL-23, sIL-2R, sIL-7Rα, and CCL25 (**Figure 1B, Figure 2** and **Supplementary Figure 1**).(10,11) In addition, CCL19 was increased in iSS versus nSS patients (**Figure 2B**). Although trends towards increased cytokine concentrations between pSS and iSS patients were observed, these did not reach statistical significance. The majority of the differentially expressed cytokines showed correlations with clinical parameters within the pSS patients including LFS and presence of anti-Ro/SSA auto-antibodies (**Table 2**). CXCL10, CXCL13, and CCL19 significantly correlated with LFS within the pSS group (**Figure 2B**).

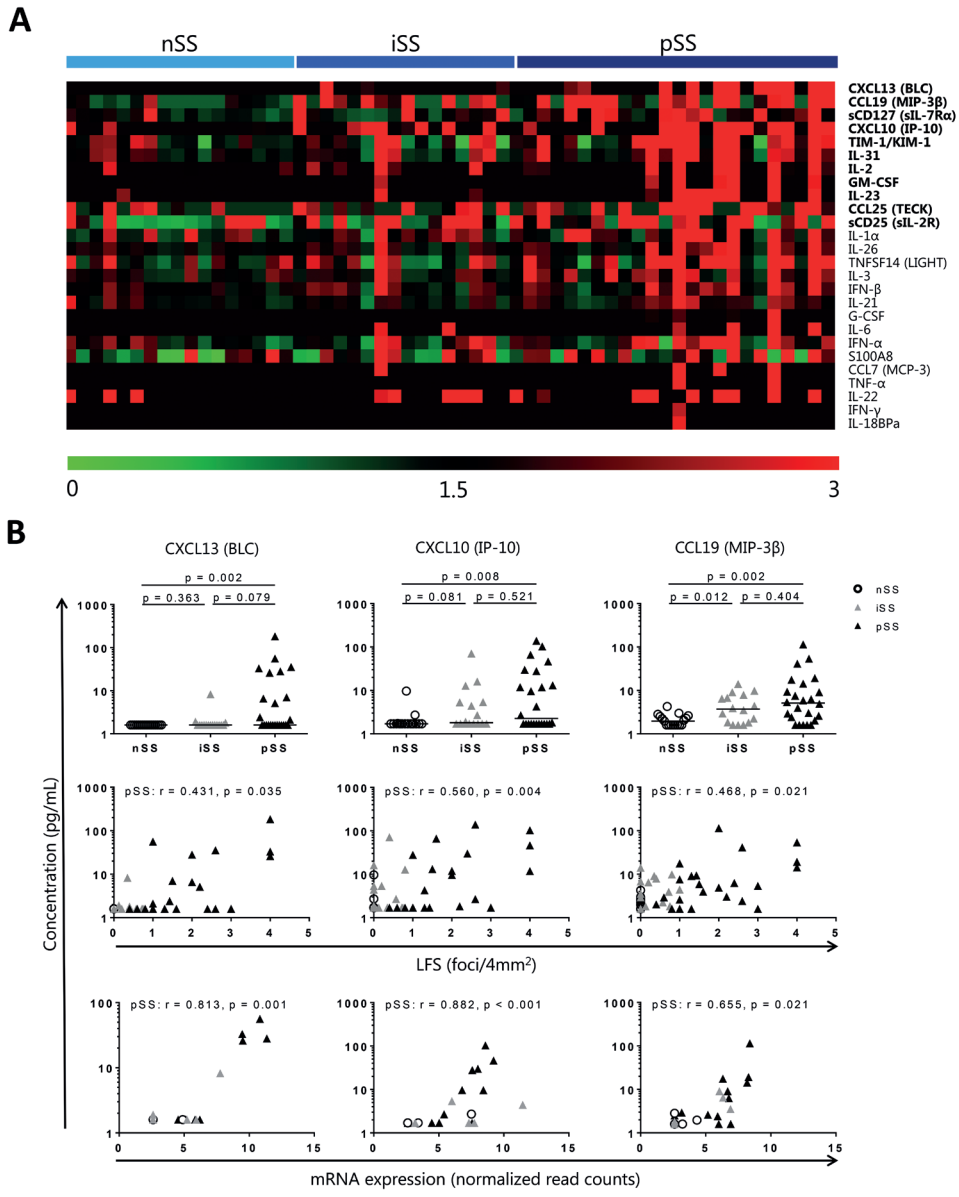


Figure 2. The secretome reveals multiple significantly upregulated cytokines in the salivary gland of pSS patients.

(**A**) Twenty-six cytokines were measured in the secretome of the validation cohort consisting of nSS, iSS and pSS patients using luminex. Cytokine expression data were quantile normalized around the median. Patients are sorted by subgroup: nSS (LFS 0 anti-SSAneg), iSS (anti-SSA+ and/or LFS>0), and pSS. The patients within the iSS and pSS groups are sorted by LFS (left to right: from low to high). Cytokines are sorted from low to high p-value (from top to bottom) in pSS vs nSS, significantly differing cytokines are indicated in bold. (**B**) The secretome levels of CXCL13, CXCL10 and CCL19 correlate with LFS and with the mRNA expression of their respective gene as measured by RNA sequencing within the pSS patients (Spearman r, p-value). Cytokine concentrations below the detection limit were converted to the lowest point on the calibration curve (lower limit of quantification) multiplied by 0.5 (lower limit of detection).

		IL-2	IL-23	IL-31	CCL19 (MIP-3β)	CXCL13 (BLC)	CXCL10 (IP-10)	TIM-1/ KIM-1	GM-CSF
Lymphocytic focus score	r	0.333	0.157	0.212	**0.468**	**0.431**	**0.568**	0.089	0.150
	p-value	0.111	0.463	0.321	**0.021**	**0.035**	**0.004**	0.680	0.484
% IgG+/IgM+ plasma cells	r	0.325	0.044	0.107	0.101	0.140	0.299	0.124	0.276
	p-value	0.151	0.850	0.643	0.663	0.546	0.188	0.592	0.226
Schirmer test	r	**-0.590**	-0.298	-0.451	-0.258	-0.314	-0.441	-0.338	-0.453
	p-value	**0.010**	0.230	0.060	0.301	0.204	0.067	0.171	0.059
ESSDAI	r	-0.005	0.215	0.286	0.084	0.138	0.330	0.392	0.118
	p-value	0.982	0.324	0.186	0.704	0.530	0.125	0.064	0.592
ESSPRI	r	0.118	-0.156	-0.186	0.022	-0.392	0.184	-0.11	0.244
	p-value	0.700	0.610	0.544	0.942	0.185	0.548	0.971	0.423
Serum IgG	r	0.259	**0.442**	0.411	-0.018	0.360	**0.427**	0.356	**0.427**
	p-value	0.232	**0.035**	0.052	0.934	0.092	**0.042**	0.096	**0.042**
ESR	r	0.150	0.154	**0.439**	0.410	0.331	0.131	0.400	0.081
	p-value	0.493	0.483	**0.036**	0.052	0.122	0.552	0.058	0.715
Anti-Ro/SSA pos vs neg	p-value	0.192	0.052	**0.027**	0.881	0.081	0.061	**0.032**	0.153

Table 2. Correlations of secretome-derived cytokine levels with clinical parameters.

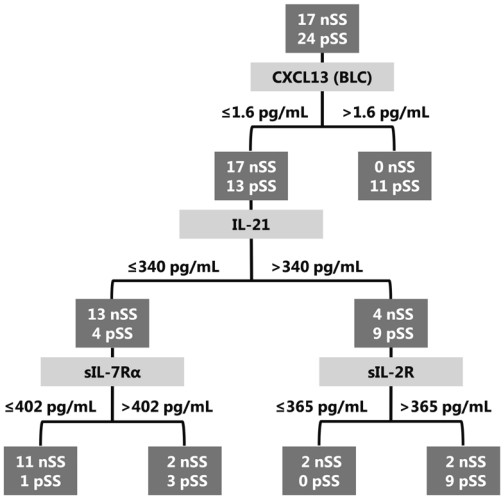
Correlations between cytokine concentrations and clinical parameters in the pSS patients are shown. Spearman r and p values are represented. Mann-Whitney U test was used to test if cytokine levels significantly differed between anti-SSA+ and anti-SSA- pSS patients. Significant correlations ($p<0.05$) are depicted in bold. Cytokines that did not significantly correlate with any clinical parameter (sIL-2R) or of which correlation have been published (sIL-7Rα, CCL25) are excluded from this table. No correlations with anti-La/SSB autoantibodies or C4 levels were found.

Although we anticipated that biopsies with larger total weights might secrete more protein, including cytokines and chemokines, of the cytokines and chemokines that showed significant differences between patients only CCL19 correlated with total tissue weight and total protein ($r=0.577$, $p=0.003$ and $r=0.497$, $p=0.013$, respectively). Due to the lack of linear correlations between tissue weight or total secreted protein and the levels of most cytokines and chemokines, normalization to either tissue weight or total secreted protein could not be generally applied. Correction of CCL19 levels for tissue weight (0.21±0.46 pg/mg/mL in pSS versus 0.03±0.04 pg/mg/mL in nSS, $p<0.001$) or total secreted protein (44.7±72.8 pg/ng/mL in pSS versus 13.1 ±28.6 pg/ng/mL in nSS, $p=0.002$) showed a similar significant differences between pSS and nSS patients. This indicates that the uncorrected data give comparable results to the corrected data. Given this substantiation and the lack of differences in biopsy weights and protein concentrations between the groups, we next confirmed whether the total secreted cytokines reflected actual production as measured by RNA levels of the tissue samples.

To assess whether the increased concentrations of the validated cytokines and chemokines reflect local production, we assessed the association of these cytokines with mRNA expression by RNA sequencing in paired labial salivary gland tissue samples. Indeed, the concentrations of CXCL10, CXCL13, and CCL19 measured in the tissue supernatants correlated with the tissue mRNA expression of these cytokines within the pSS patients (**Figure 2B**). In addition, *IL7R* mRNA expression correlated with sIL-7Rα concentrations in all donors (r=0.557, p=0.007). Messenger RNA expression of the other validated cytokines was detectable in <10% of patients, preventing us from drawing meaningful conclusions.

The salivary gland secretome accurately classifies pSS patients

To investigate whether the secretome can classify patients, decision tree analysis was performed using the expression of all 26 mediators measured in the validation cohort. In an automated and unsupervised manner, a combination of CXCL13, IL-21, sIL-7Rα and sIL-2R was identified to correctly classify 95.8% of pSS patients and 76.5% of nSS patients (**Figure 3**). The misclassified pSS patient had an LFS of 1, no anti-SSA/SSB autoantibodies and an ESSDAI of 1. There were no differences in clinical or laboratory parameters (Schirmer test, ESR, serum IgG levels) between the correctly and incorrectly classified nSS patients. Of the iSS patients, 11 were classified as pSS and 5 as nSS. The iSS patients who were classified as pSS had an elevated LFS (0.4±0.3 vs 0.0±0.0, p=0.009) compared to the other iSS patients.



	Predicted		Correct
Observed	nSS	pSS	
nSS	13	4	76.5 % (Specificity)
pSS	1	23	95.8 % (Sensitivity)
Overall correct			87.8 %

Figure 3. Classification tree analysis reveals that combinations of secretome-derived cytokines can be used to classify patients.

Classification tree analysis was performed using the CHAID method. Concentrations of CXCL13, IL-21, sIL-7Rα and sIL-2R were identified to distinguish nSS from pSS patients correctly overall in 87.8% of cases.

Discussion

In this study, we show that salivary gland tissue supernatants allow for measurement of a multitude of local pro-inflammatory mediators. Using this secretome, we demonstrate that pSS patients have a marked local increase in 11 inflammatory mediators compared to nSS patients, the majority correlating with clinical parameters. Furthermore, we show that levels of several identified mediators accurately classify pSS and nSS patients.

All mediators measured were detectable in the majority of patients, indicating that this technique can be used to study a broad range of targets. We observed increased levels of CXCL10, CXCL13 and CCL19 in pSS patients, all of which are described to be increased in pSS salivary glands according to literature.(13-15) Importantly, CXCL13 plays an essential role in germinal centers, and both CXCL13 and the presence of germinal center-like structures in pSS salivary glands are associated with the risk of lymphoma development.(13,16) In addition, we found strong correlations of released cytokine concentrations with gene expression in matched tissue explants, which confirms that the salivary gland tissue is the source of the mediators measured in the secretome. Moreover, the increase of sIL-7Rα and CCL25 in tissue supernatants, which we previously reported, was consistent with salivary gland tissue expression assessed by immunohistochemistry.(10,11,17) As such, the secretome is a promising novel tool to study broad panels of proteins and may be useful to assess other mediators including autoantibodies and proteins associated with the activity of tissue cells, such as epithelial cells and fibroblasts.

To properly measure low expressed mediators, the setup of this explant assay could be further optimized. For this study we used supernatants that were collected without changing standard clinical practice: biopsies consisting of multiple salivary glands that were kept for one hour in saline at room temperature. With this set up we mimicked protocols that were successfully applied in synovial tissue explants from rheumatoid arthritis patients(18) and mouse models of arthritis(19), but we did not apply optimal culture conditions. Interestingly, in more recent experiments we have shown that culturing only one single biopsy for one hour in culture medium at 37°C yields significant differences in the production of inflammatory mediators between nSS and pSS patients (CCL19 and CXCL10, **Supplementary Figure 2**). Hence, we believe that optimization of the culture protocols will increase the usability of this approach. In addition, to study which cells release the measured cytokines, and to more precisely quantify their production, culture experiments with isolated cells from biopsies should be performed. As a future perspective, the potential efficacy of therapeutics could be tested by assessment of alterations in the salivary gland secretome.

Using the salivary gland secretome, 95.8% of pSS patients and 76.5% of nSS patients could be classified correctly in an unsupervised manner. As such, the secretome could

aid in improvement of diagnostics of pSS in the future. The iSS group consists of patients, who are difficult to characterize in clinical practice. They show limited objective signs of Sjögren's syndrome, but are not diagnosed with pSS by the rheumatologist and do not fulfil the classification criteria. It is relevant to note that 10 out of 16 iSS patient might have met the 2016 AECG classification criteria if saliva production would have been quantified. Interestingly, the iSS patients who were molecularly categorized as pSS according to the decision tree analysis had a higher LFS compared to the rest of the group and were anti-Ro/SSA negative. There were no significant differences in cytokine levels between anti-Ro/SSA+ and anti-Ro/SSA- iSS patients. This indicates that molecular profiling using secretome detects inflammatory activity that was underscored by LFS assessment, which may be of added value in Sjögren's syndrome diagnosis. However, follow-up of these patients and longitudinal analyses in larger cohorts are needed to show whether the classification on the basis of the salivary gland secretome is clinically relevant for this group of patients.
In conclusion, the salivary gland secretome represents a valuable novel tool to measure many local soluble mediators, to provide future insights in immunopathology and potentially aid in diagnostics.. This method could be of use to identify therapeutic targets and to develop markers for stratification, prognosis and treatment response in sicca patients.

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Supplementary material

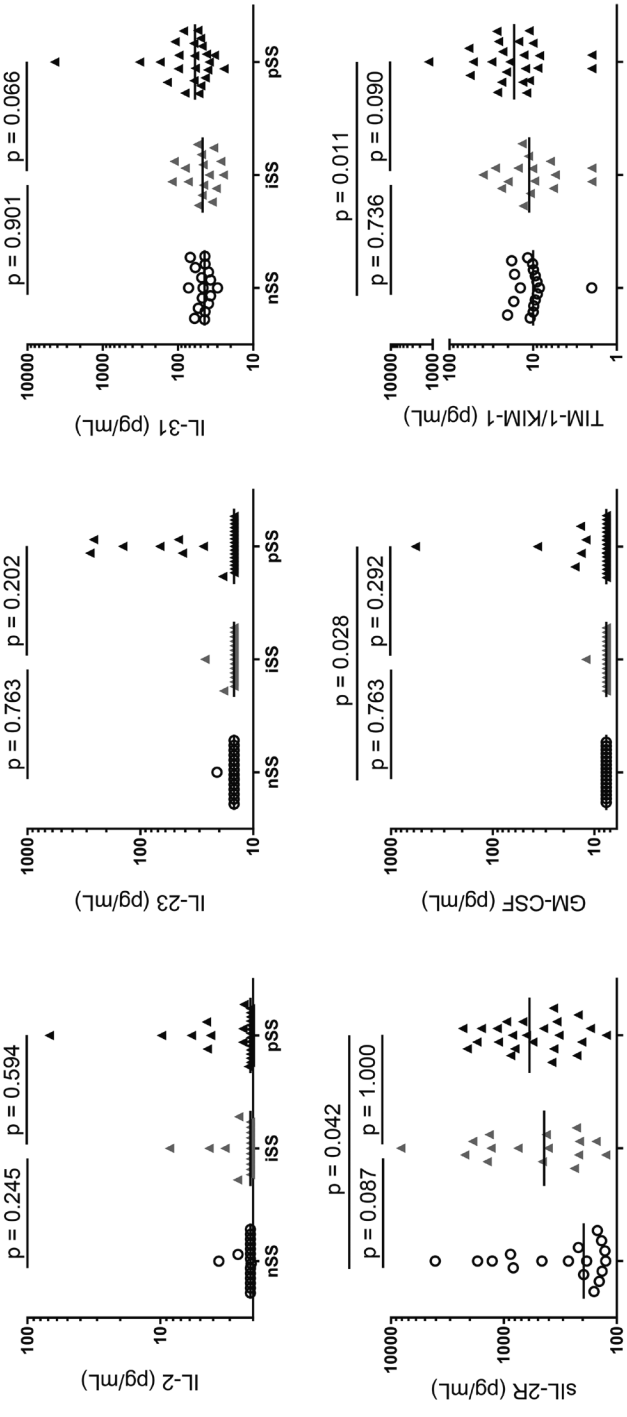
Supplementary table

BDCA-3	IL-15	IL-6	OPN	XCL1
BLC (CXCL13)	IL-16	IL-7	OSM	
BRAK (CXCL14)	IL-17	IL-8 (CXCL8)	PF-4 (CXCL4)	
Cathepsin	IL-18	IL-9	PIGF	
CTACK (CCL27)	IL-18BPA	IP-10 (CXCL10)	S100A8	
EGF	IL-1A	ITAC (CXCL9)	sCD14	
ENA-78 (CXCL5)	IL-1B	LAIR-1	sCD163	
Endoglin	IL-1R1	LIF	SCF	
Eotaxin-1 (CCL11)	IL-1R2	LIGHT	sIL-2R (sCD25)	
FAS	IL-1R4	MCP-1 (CCL2)	SLP-1	
FASL	IL-1RA	MCP-2 (CCL8)	SOST	
FGF	IL-2	MCP-3 (CCL7)	SRPSOX (CXCL16)	
Galectin-1	IL-20	MCP-4 (CCL13)	sSCFR	
Galectin-9	IL-22	M-CSF	sVEGFR1	
G-CSF	IL-23	MDC	TARC (CCL17)	
GM-CSF	IL-24	MIF	TIM-1/KIM-1	
GRO-1α (CXCL1)	IL-26	MIG (CXCL11)	TIMP-1	
I-309 (CCL1)	IL-27	MIP-1α (CCL3)	TNF-α	
IFN-α	IL-29	MIP-1β (CCL4)	TNF-β	
IFN-β	IL-3	MIP-3α (CCL20)	TNFR1	
IFN-γ	IL-31	MIP-3β (CCL19)	TNFR2	
IL-10	IL-33	MMP-9	TREM1	
IL-11	IL-37	MPIF-1	TSLP	
IL-12	IL-4	NGF	TWEAK	
IL-13	IL-5	OPG	VEGF	

Supplementary Table 1. All analytes measured in the discovery cohort.

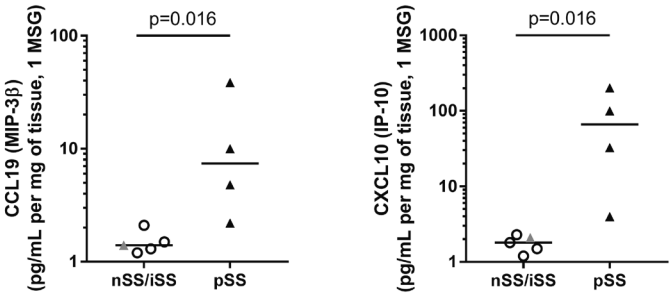
In the discovery cohort, 101 cytokines were measured by Luminex in weight-matched nSS vs pSS patients (both n=8)

Supplementary figures



Supplementary Figure 1. Significantly elevated cytokines in the salivary gland secretome of pSS versus nSS patients in the validation cohort.

The secretome levels of IL-2, IL-23, IL-31, sIL-2R, GM-CSF, and TIM-1/KIM-1 measured by Luminex in the validation cohort are shown. Cytokine concentrations below the detection limit were converted to the lowest point on the calibration curve (lower limit of quantification) multiplied by 0.5 (lower limit of detection).



Supplementary Figure 2. Elevated levels of CCL19 and CXCL10 in single salivary gland culture supernatants of pSS versus nSS patients.

Single minor salivary glands from biopsies from nSS, iSS and pSS patients were cultured for one hour at 37°C in RPMI with 10% fetal calf serum. Levels of CCL19 and CXCL10 were normalized per mg of biopsy tissue.

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CHAPTER 3

Epigenetically quantified immune cells in salivary glands of Sjögren's syndrome patients: a novel tool that detects robust correlations of T follicular helper cells with immunopathology

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Submitted for publication

Abstract

Introduction. To investigate whether epigenetic cell counting represents a novel method to quantify immune cells in salivary glands of patients with different forms of Sjögren's and sicca syndrome to capture immunopathology and potentially aid in diagnosis.

Methods. DNA from frozen salivary gland tissue sections of sicca patients was used for bisulphite conversion of demethylated DNA cytosine residues, followed by cell-specific quantitative PCR to calculate cell percentages in relation to total tissue cell numbers as quantified by housekeeping gene demethylation. Percentages of epigenetically-quantified cells were correlated to RNA expression of matched salivary gland tissue and histological and clinical parameters.

Results. Percentages of epigenetically quantified CD3, CD4, CD8, T follicular helper (Tfh)-cells, FoxP3+ regulatory T-cells and B-cells were significantly increased in the salivary glands of patients with Sjögren's syndrome. Unsupervised clustering using these percentages identified patient subsets with increased LFS and local B-cell hyperactivity and classifies patients different from conventional classification criteria. In particular, Tfh-cells were shown to strongly correlate with expression of CXCL13, lymphocytic focus scores, local B-cell hyperactivity and anti-SSA positivity.

Conclusion. Epigenetic cell counting is a promising novel tool to objectively and easily quantify immune cells in the labial salivary gland of sicca patients with relatively low amount of tissue needed. In view of the potential of this technique to include a huge number of (cell-specific) biomarkers, this opens up new standardized ways for salivary gland analysis with high relevance for patient classification, understanding of immunopathology and monitoring drug responses in clinical trials.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic, systemic autoimmune disorder characterized by dryness and lymphocytic infiltration of the exocrine glands.(1) PSS occurs in the absence of other autoimmune diseases and secondary SS (sSS) presents along with other autoimmune diseases.(1) Salivary gland biopsy is widely used for the diagnosis of pSS. The lymphocytic focus score (LFS), as enumerated in H&E-stained salivary gland tissue sections, forms part of routine clinical diagnostics and has an important position in the classification criteria for SS.(2-4) However, this method of analyzing salivary gland inflammation has several drawbacks, including lack of standardization of acquisition and processing of the salivary gland tissue, subjective histological interpretation and poor correlation of the scored abnormalities with dryness assessments. The latter is not necessarily a consequence of lack of standardization, but the limited information that is collected from the current histology simply does not allow for more in depth analyses of different cell subsets. In this respect the immunological processes that lead to immunopathological events such as lymphoma development lack detailed information on the inflammatory cell types. In addition, molecular markers that might be associated with aberrances in function of tissue cells like epithelial cells or fibroblasts are currently lacking. More in depth analysis might help to reveal the relationship between inflammation and damage of tissue cells.

Optimization of existing histology protocols to determine salivary gland inflammation will help to improve diagnosis of SS.(5) In parallel, exploration of complementary methodologies for analysis of local immunopathology could enable diagnostics, prognostics and patient-tailored treatment. Novel techniques that allow for better precision by high-throughput cell quantification in salivary gland tissues would be very helpful. We here explored whether epigenetic cell counting (ECC) can serve as a complementary tool to objectively classify patients and to improve characterization of immune cell infiltrate to better understand immunopathology in Sjögren's syndrome and other patients with sicca symptoms. ECC might also have the potential to stratify patients and to provide biomarkers to monitor response to therapy.

Epigenetic mechanisms such as DNA methylation are important modulators of gene expression. Epigenetic cell counting is a novel form of immune cell quantification based on quantitative real-time PCR that is based on certain genomic loci that remain specifically unmethylated in selected cell types, whereas they are methylated in all other cell types. This biological phenomenon can be turned into the primary sequence by treating DNA with bisulfite, leading to conversion of demethylated cytosines of the cellular DNA and allowing specific qPCR. The methylation status of the chromatin structure of either actively expressed or silenced genes is the basis of the epigenetic-based cell identification and quantification technology. Discovery of cell type-specific removal/absence of methyl groups

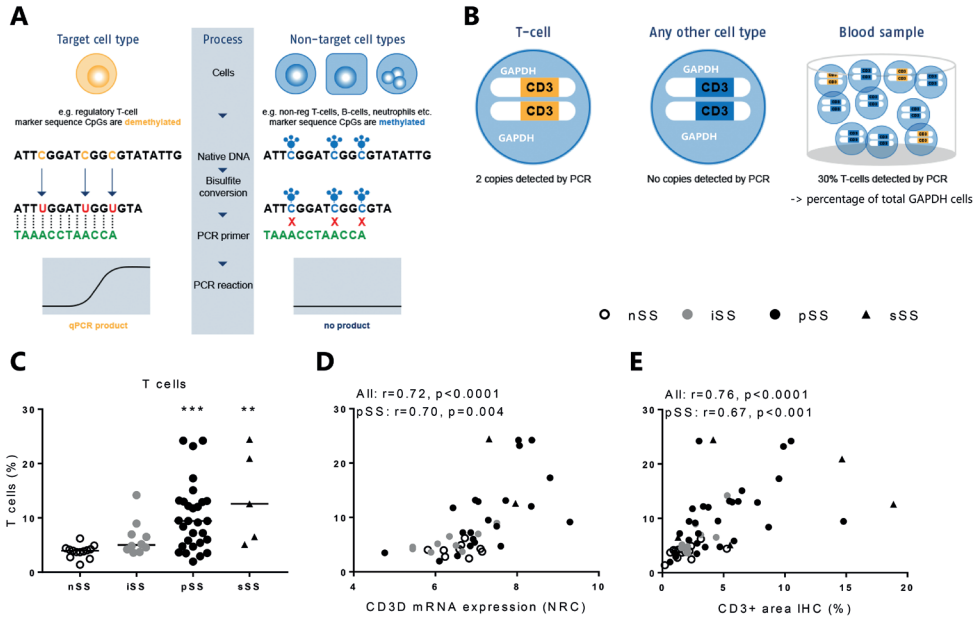

Figure 1. Epigenetic cell counting detects increased CD3 lymphocytes in salivary gland biopsies strongly correlating with CD3D gene expression and CD3 protein expression using immunohistochemistry.

(**A**)Epigenetic cell-counting is based on cell type-specific, epigenetic biomarkers. These genomic biomarker regions are marked by the absence of CpG methylation (demethylated) in the respective cell types of interest, while all other cell types show complete methylation. Only demethylated biomarker regions will react with bisulfite, a chemical that converts demethylated cytosines into a uracil nucleotide allowing a PCR reaction. Real time PCR is then employed to quantify the number of demethylated biomarker regions, and thus the precise number of the cell type of interest, in a wide range of sample matrices including whole blood, PBMCs, tissue or in isolated genomic DNA (**B**) In T-cells, the CD3 gene region is epigenetically active (yellow), whereas in all other cell types, this region is epigenetically inactive (blue). In each T cell, two epigenetically active (yellow) alleles are present and will be amplified by PCR. Non-T-cells are not detected. The number of epigenetically active CD3 gene copies directly translates into the number of T-cells in the sample. The parallel measurement of epigenetic reference systems, e.g. the housekeeping gene GAPDH, allows for total cell number determination.
(**C**) ECC detects increased CD3 percentages in salivary gland of iSS, pSS and sSS patients as compared to nSS patients. (**D**) Epigenetically quantified CD3 T-cells strongly correlate with CD3D gene expression levels and (**E**) CD3 expression as digitally quantified following immunohistochemistry. Individual patients and medians are shown. Kruskal-Wallis test with Dunn's multiple-comparison test was used. ** and *** indicate statistical significant differences vs nSS patients of $p<0.01$ and $p<0.001$. Spearman correlations coefficients (r) are given for all sicca patients and for pSS patients.

(demethylation) permits precise and robust quantification of immune cells from only small amounts of human blood or tissue samples (**Figure 1A**, **B**). These epigenetic biomarkers located on genomic DNA are also stably associated with a cell type of interest. This may prove superior to RNA expression which can be subject to enzymatic degradation and is often poorly associated with protein expression and cell function. This is exemplified by the Treg-specific demethylated region, which to date provides the most accurate identification and quantification of Tregs.(6). Another advantage of ECC is that it overcomes the requirement

of immune cells from blood or tissue samples to be fresh as ECC uses DNA, which can be stored long term. This is currently a major obstacle of studies on inflammatory cells and immunopathology.

In the current study we epigenetically quantified total CD3 T-cells, total B-cells and subsets of T-cells, including FoxP3 Tregs, IL-17-producing Th-cells, T follicular helper (Tfh) cells, CD4 and CD8-cells. Considering the important role of Tfh-cells in formation of ectopic lymphoid structures (ELS) (7-9), the associations of these cells with clinical and immunological parameters were studied in more detail. In addition, the relationship of Tfh-cells with clinical scores, B-cell hyperactivity, anti-SSA positivity, CD21+ follicular dendritic cells and ELS were studied. Finally, associations of Tfh-cells with RNA expression levels were investigated, including CXCL13, which is the major chemokine involved in directing Tfh-cells into the ELS(8) and is suggested to be crucial for B cell hyperactivity(8,10).

Our data demonstrate that epigenetic quantification of immune cells in salivary glands of Sjögren's syndrome patients is a novel tool to objectively detect significant correlations of inflammatory cell subsets with immunopathology and disease parameters.

Methods

Patients

pSS (n=29) and sSS patients (n=5) were diagnosed by a rheumatologist and fulfilled the AECG classification criteria.(2) The sSS patients were diagnosed as having Sjögren's syndrome in addition to another rheumatic autoimmune disease. Non-Sjögren's sicca (nSS) patients (n=13) were defined as patients with sicca complaints, without a connective tissue disease including pSS, with an LFS of zero, and without anti-Ro/SSA or anti-La/SSB autoantibodies. Patients with incomplete SS (iSS) (n=10) were defined as patients with sicca complaints, without a connective tissue disease, not fulfilling the classification criteria for pSS, but that do show signs of limited lymphocytic infiltration and/or the presence of anti-Ro/SSA or anti-La/SSB autoantibodies (clinical data are described in **Table 1**). Salivary gland tissue was surplus tissue that was provided pseudonymized, for which ethical approval was obtained according to the guidelines of the hospital's ethical committee.

	nSS n=13	iSS n=10	pSS n=29	sSS n=5
Gender (female %)	77	100	90	100
Age (yr, mean±SD)	47±15	49±15	53±13	53±15
Anti-Ro/SSA+ (%)	0	60	69	60
Anti-La/SSB+ (%)	0	0	30	0
LFS (foci/4mm^2)	0	0.4±0.4	2.3±1.4	3.5±2.1
IgG+ and IgM+ plasma cells in LSG biopsy (%)	24±14	26±14	51±16	42±21
Serum IgG (g/L)	11.0±3.1	10.8±2.4	16.3±8.2	14.4±3.1
ESR (mm/h)	11±9	17±14	17±15	19±14
Schirmer (mm/5min)	5±7	7±6	6±7	6±2
Immunosuppressive medication (n)	5	3	13	4
Hydroxychloroquine (n)	3	1	6	0
Corticosteroids (n)	0	1	3	1
Other (n)	2	1	4	3

Table 1. Patients' characteristics.

nSS: non-Sjögren's sicca; iSS: incomplete Sjögren's syndrome; pSS: primary Sjögren's syndrome; sSS: secondary Sjögren's syndrome; LFS: lymphocytic focus score; LSG: labial salivary gland; ESR: erythrocyte sedimentation rate.

Epigenetic cell counting

Epigenetic-based quantification of inflammatory cells was carried out by Epiontis GmbH (Berlin, Germany), as previously reported(10). Briefly, 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)(11). The latter was used to allow for determination of total cell numbers (**Figure 1B**). PCR systems including primers, probes and templates for all assays are taken from (Baron et al. 2018 Science Translational Medicine, accepted for publication). 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 one hundred. (Baron et al. 2018 Science Translational Medicine, accepted for publication).

Statistical analyses

Statistical analyses were performed in Graphpad Prism 6 and SPSS Version 21. Differences between the groups were assessed by Kruskal-Wallis test with Dunn's multiple comparisons post hoc test. Spearman's rank correlation coefficient was used for correlation analyses. Heatmap visualization and unsupervised hierarchical clustering based on Euclidian distances were performed in MeV (Multiple Experiment Viewer).

More information on methods used is described in the **Supplementary methods**.

Results

Epigenetic cell counting in salivary gland allows for robust detection of inflammatory cells
To assess whether epigenetic cell counting can be used as a tool to identify and quantify inflammatory cells in labial salivary gland biopsy tissue, we measured 7 different cell types in all 57 sicca patients (see patient details in **Table 1,** experimental procedure is described in **Supplementary Figure 1).**

Epigenetically quantified CD3 T-cells (**Figure 1C**) were significantly increased in pSS and sSS patients as compared to nSS patients. As a representative marker we next assessed whether epigenetically quantified CD3 T-cells could be technically validated by CD3 RNA assessment and quantification of CD3 T-cells at the protein level using IHC. Indeed strong correlations were observed between epigenetically quantified CD3 T-cells and *CD3D* gene expression (**Figure 1D**) as well as digitally quantified CD3 T-cells following IHC (**Figure 1E**).

ECC-based clustering identifies sicca patients with B cell hyperactivity and clusters different than diagnosis and classification criteria
In addition to CD3 T-cells, B-cells, CD4, and CD8-cells were all significantly increased in pSS and sSS patients as compared to nSS patients (all at least $p<0.05$, **Figure 2**). In iSS patients only CD8 cells were modestly yet significantly increased (data not shown). In addition, studying functionally related T cell subsets, we found both Tfh-cells and FoxP3-expressing Tregs strongly increased in the salivary glands of pSS and sSS patients (**Figure 3**, **Supplementary Figure 2**). In contrast, Th17-cells were present in substantial numbers, but no significant differences in the salivary gland of pSS, iSS and sSS patients as compared to nSS patients were observed (**Supplementary Figure 2**).

Next we assessed whether ECC-based clustering could identify patient subgroups. Unsupervised hierarchical clustering of cell counts identified 3 patient groups (**Figure 2A**). Interestingly, group 1 consisted of all nSS patients, but also 7 pSS, 5 iSS and 1 sSS patient. This group on average was characterized by low LFS scores (**Figure 2B**) and low B-cell activity as indicated by low percentages of salivary gland IgM and IgG-expressing plasma cells as quantified by IHC for diagnostic purposes and low levels of salivary gland IgM and IgG isotype gene expression (**Figure 2C** and **D**). Group 3 identified pSS and sSS patients with high LFS scores (**Figure 2B**) and high percentages of IgM and IgG expressing plasma cells in the LSG, corroborated by high levels of IgM, IgG1, IgG2 and IgG3 gene expression (**Figure 2D**).

Group 2 is an intermediate group based on the LFS and percentages of IgG+ and IgM+ plasma cells (mean±SD: LFS 2.4±2.0 vs. 0.6±0.9; IgG+ and IgM+ plasma cells 43±19% vs. 31±15% in group 2 and 1, respectively). Increased serum IgG levels were observed in group 2 (18.2±8.9 in

cluster 2 versus 11.2±2.8 g/L in cluster 1, p=0.005), whereas the increase in cluster 3 was not statistically significant (16.7±8.0, p=0.428).

In view of the association between type I IFN activity and disease activity as well as B cell hyperactivity in pSS(11), the expression of interferon inducible genes (IFIGs) was assessed in the salivary gland biopsies, as previously described in pSS.(11) The expression of most of these genes was elevated in a proportion of the pSS patients, especially those in cluster 3 (**Supplementary Figure 3**). In line with this, expression of IFIGs significantly correlated with B cell and Tfh-cell frequencies as quantified by ECC (**Supplementary Figure 3**).

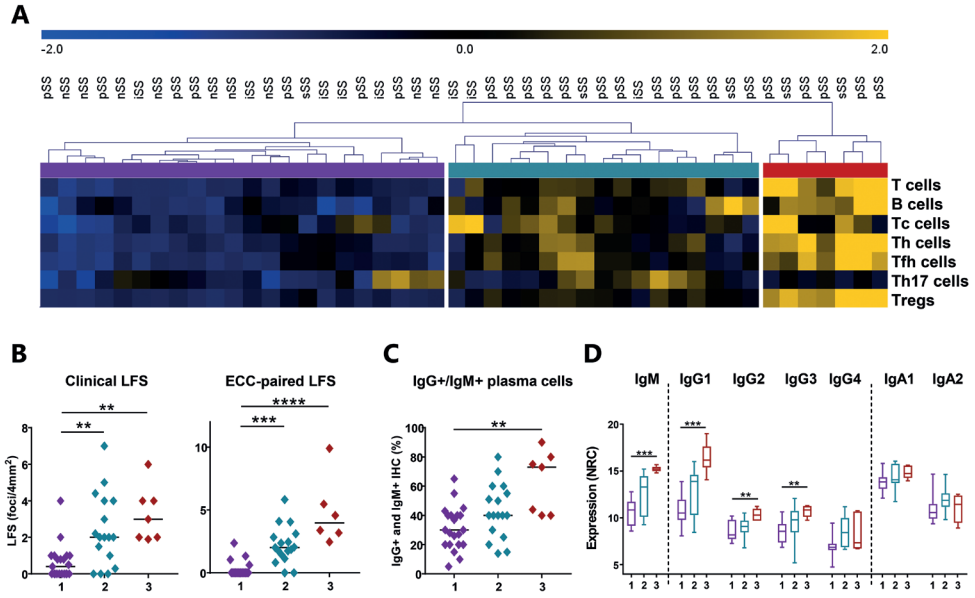

Figure 2. Epigenetic cell counting clusters patients with severe lymphocytic infiltration and B cell hyperactivity.

(**A**) Using unsupervised hierarchical clustering, three clusters of sicca patients are identified based on ECC counts. (blue to yellow represents Z scores from -2 to +2 standard deviations) (**B**) Lymphocytic focus scores as diagnostically assessed (Clinical) and of ECC-matched tissue are shown for all three clusters, with cluster 3 identifying patients with the highest LFS scores. (**C**) ECC-based clustering identifies patients with high B cell hyperactivity as indicated by percentages of IgG+ and IgM+ plasma cells as assessed by routine clinical practice by the pathologist and (**D**) by mRNA expression of the heavy chain constant region from IgG and IgA isotypes and IgM. Boxplot whiskers indicate the 5-95% interval. Medians are shown. Kruskal-Wallis test with Dunn's multiple-comparison test was used. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 respectively.

Increased epigenetically quantified T follicular helper cells strongly correlate to CXCL13 expression and B cell hyperactivity

Since Tfh-cells play a crucial role in lymphoid neogenesis and have potent B cell stimulatory capacities we assessed the frequencies of Tfh-cells as quantified by ECC in association with clinical parameters of lymphocytic infiltration and B cell hyperactivity. Numbers of Tfh-cells were elevated in pSS and sSS patients and correlated with both clinical LFS and even more strongly with LFS of ECC-paired tissue (**Figure 3A-C**). Tfh-cells express chemokine receptor CXCR5 by which CXCL13 directs these cells to B cell rich areas in ELS. Indeed, the number of Tfh-cells correlates with mRNA expression of CXCL13 (**Figure 3D**) and CXCR5 (r=0.60, p=0.009 in pSS patients). In addition, Tfh-cell numbers correlated with ICOS and MAF mRNA expression (r=0.76, p<0.001 and r=0.73, p<0.001 in pSS, respectively), characteristic genes for Tfh cells, but did not significantly correlate with gene expression characteristic for other T-cell subsets (CXCR3, RORC, GATA3 and FOXP3, data not shown).

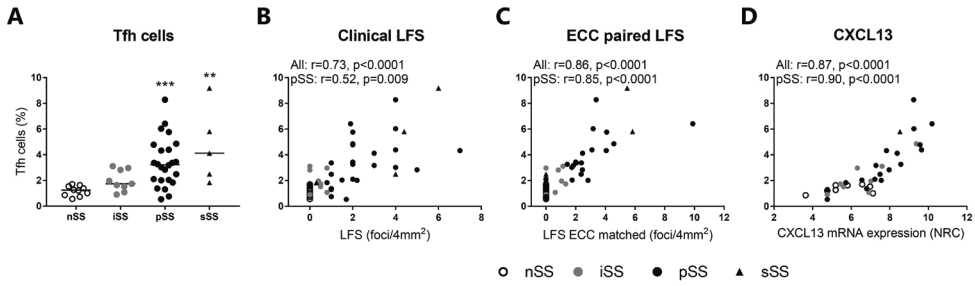

Figure 3. Tfh cell frequencies in salivary gland biopsies as assessed by epigenetic cell counting are increased in Sjögren's syndrome patients and correlate with LFS and expression of CXCL13.

(**A**) Tfh frequencies as assessed by ECC are elevated in pSS and sSS as compared to nSS patients. (**B**) Tfh cell frequencies correlate with clinical LFS scores and (**C**) strongly correlate with LFS scores of tissue sections that were paired to tissue sections that were used for ECC. (**D**) Tfh cells also robustly correlated with CXCL13 RNA expression (DNA/RNA isolated of the same tissue sections). Medians are shown. Kruskal-Wallis test with Dunn's multiple-comparison test was used. ** and *** indicate p<0.01 and p<0.001, respectively. Spearman correlations coefficients (r) are given for all sicca patients and for pSS patients.

Importantly, Tfh-cell frequencies correlated with clinical measures of B-cell hyperactivity including percentages of IgM and IgG-expressing plasma cells in the salivary glands, serum IgG levels, the presence of anti-Ro/SSA autoantibodies and the frequency of B-cells as assessed by ECC (**Figure 4A-D**). To assess ectopic lymphoid structures in the salivary gland biopsies, co-stainings of CD3 or CD20 with CD21 (indicating follicular dendritic cells) were performed. CD21-containing fDC networks were increased in pSS and sSS vs nSS patients (0.3±0.6 vs 0.0±0.0, p=0.016) but Tfh-cell frequencies only modestly correlated with the CD21 expressing cells in sicca patients (**Figure 4E**).

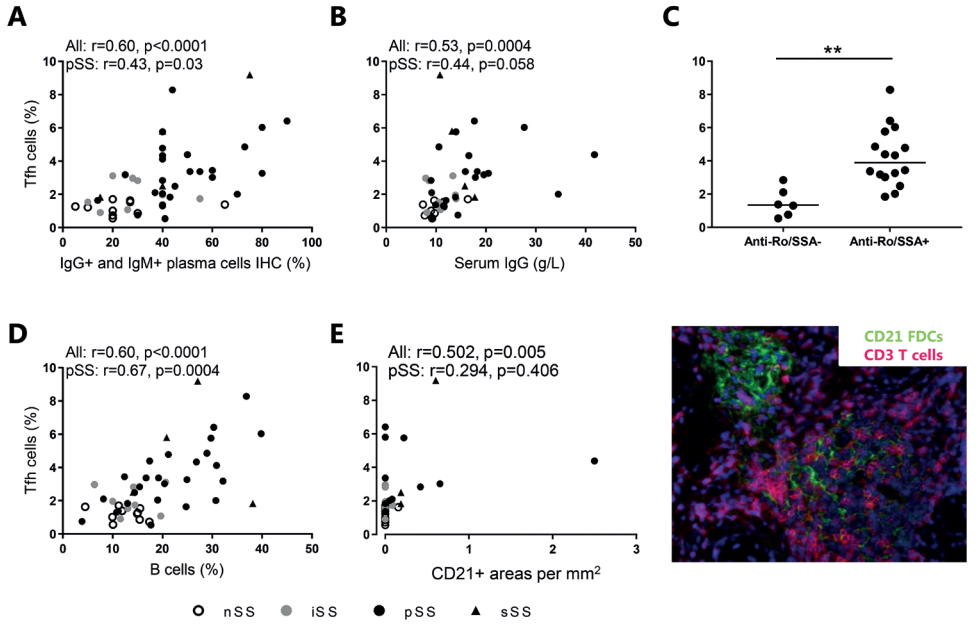

Figure 4. Tfh cell frequencies in salivary gland biopsies as assessed by epigenetic cell counting correlate with local and systemic measures of B cell hyperactivity in Sjögren's syndrome patients.

Tfh cell frequencies correlate with (**A**) the percentage of IgG and IgM expressing plasma cells, (**B**) serum IgG levels, and (**C**) presence of autoantibodies anti-Ro/SSA (median is shown, Mann-Whitney U was used). (**D**) In addition Tfh cell frequencies correlate with B cell frequencies as assessed by ECC and (**E**) are modestly associated with the presence of CD21+ FDC containing lymphoid structures in sicca patients. *, ** and *** indicate p<0.05, p<0.01 and p<0.001, respectively. Spearman correlations coefficients (r) are given for all sicca patients and for pSS patients.

Discussion

We here demonstrate that ECC is a valuable technique to measure inflammatory cell subsets in salivary glands, allowing to cluster patients that are characterized by high LFS and B-cell hyperactivity. Specifically, we show that epigenetically quantified Tfh-cells are strongly associated with markers of lymphoid neogenesis (incl. CXCL13, CXCR5, ICOS) and clinical parameters associated with disease severity (LFS, anti-SSA positivity and B cell hyperactivity).

Elevated frequencies of all studied inflammatory cells, except for IL-17-producing cells, are identified in pSS and sSS patients versus non-autoimmune sicca patients using ECC. Representative markers results were validated by RNA expression (for CD3 and Tfh-cells) and immunohistochemistry (for CD3), supporting the robustness of the ECC method. Clustering based on ECC results identified a cluster of pSS and sSS patients with the most severely affected salivary glands in terms of lymphocytic infiltration and B cell hyperactivity. Whether such differences are clinically meaningful and will help in decision making needs

to be demonstrated in larger cohorts that could typically be made available within large consortia that are currently being established (eg HarmonicSS). Interestingly, the current data demonstrated ECC-based clustering that classified patient distinct from classification criteria and hence this tool may complement existing tools. For future research, it is important to correlate ECC results with disease activity scores (ESSDAI) and patient reported scores (ESSPRI), which unfortunately were not available in the period in which the biopsies used for this study were collected. However, the results from this study indicate that ECC could be a valuable tool to improve tissue assessment for diagnostic and research purposes in sicca patients.

Although current histology has obvious advantages such as information on tissue architecture, currently, assessment of salivary gland biopsies is not performed according to standardized procedures and reliability is dependent on experienced technicians and pathologists(5). In addition, quantification of different cell subsets by IHC staining is laborious and time consuming and for such reasons often limited to a small tissue surface area. Using ECC, frequencies of immune cells are objectively quantified, which may improve reliability of tissue assessment, homogeneity of study populations and comparability between studies. Using ECC, many cell subsets can be quantified at the same time from one tissue sample. In addition, ECC is DNA-based, which is more stable than RNA, and may be applied to for example formalin-fixed paraffin-embedded tissue. As a future perspective, ECC potentially requires less tissue to perform reliable analyses as compared to currently used procedures, since it allows for analysis of cells in a large number of tissue layers, if necessary the analysis of all cells in the tissue sample. This potential is corroborated by the fact that epigenetically quantified immune cells (Tfh-cells) strongly correlated with LFS of paired tissue samples, indicating ECC to accurately capture lymphocytic foci. Interestingly, the size and number (n=1-2) of the available tissue sections was much smaller than what was used to assess the LFS for clinical practice (n=5-7). In this respect patients could benefit from this as much smaller number of tissue samples might be needed potentially allowing additional analyses and possibly reducing invasiveness of surgical procedures.

Identifying immune cell subsets using a combination of (surface) markers can be both a challenge due to technical limitations and time consuming, especially in tissues using immunohistochemistry. As we confirm in this study(12) ECC robustly identifies several immune cell subsets with specific functional properties. As an example we quantified FoxP3+ regulatory T-cells, whose exact markers for more than a decade have remained elusive. Stable expression of FOXP3 in Tregs was found to be largely controlled by a highly conserved CpG-enriched element in the FOXP3 gene, the Treg-specific demethylated region (TSDR)(13). Since activated CD4 T-cells can express both CD25 and FOXP3(14), and TSDR demethylation is not shared by activated T-cells, the Treg-specific epigenetic status provides the most accurate identification and quantification of Tregs(6). Hence, other unique epigenetic markers may

be used to provide detailed information on functional properties of inflammatory cells in the inflamed exocrine glands. In addition to the demethylation markers we have used in the current study, future studies could focus on a wide array of markers to identify specific pathologic events such as fibrosis and angiogenesis. Such markers might also be applied to identify therapeutic targets and to develop markers for stratification, prognosis and treatment response in sicca patients.

Although all activated T-cells can stimulate B-cell activity, Tfh cells in lymphoid organs and inflamed tissue are considered one of the most potent T-cell subsets inducing B-cell hyperactivity. In addition, Tfh-cells can play an important role in formation and maintenance of germinal centers. In the present study, epigenetically quantified Tfh-cell frequencies strongly correlated with important measures of immunopathology including lymphocytic infiltration, numbers of B-cells, elevated serum and salivary gland immunoglobulin levels and autoimmunity. Finally, numbers of Tfh-cells also strongly correlated with CXCL13 expression, a chemokine that tightly regulates movement of CXCR5+ Tfh-cells and B-cells. Elevated CXCL13 levels are expressed by a large proportion of patients with pSS in either serum or saliva (15), correlate with B-cell hyperactivity and disease activity and are elevated in patients with lymphoma(16,17). In addition, CXCL13 in serum and salivary gland has been shown to correlate with disease progression in several SS murine models. Furthermore, CXCL13 is elevated before disease becomes evident in a murine SS-like disease model, and neutralizing CXCL13 diminishes the disease (15). Thus, epigenetically quantified Tfh cells may capture CXCL13-driven inflammation and may be valuable as a biomarker for initial diagnosis, in the assessment of disease progression and severity.

CXCL13 can be produced by follicular stromal cells that mediate migration of CXCR5+ cells into lymphoid follicles. Moreover, overexpression of CXCL13 causes formation of ectopic lymphoid tissue(18). In our study CXCL13 and Tfh-cells only modestly correlated with presence of CD21+ ELS. This may be partly due to the fact that quantification of CD21+ FDCs is difficult, but on the other hand increased CXCL13 and Tfh-cells were also observed in patients that completely lacked CD21+ ELS. In these patients increased cellular infiltrates and B-cell hyperactivity was also observed. These observations could be explained by production of CXCL13 by other cells such as monocytes/macrophages, antigen-experienced T-cells, and Tfh-cells (12,15,19,20). This could subsequently facilitate Tfh-driven B-cell activation in the absence of ELS. Although other T cell-driven processes could play a role the present data suggest that CXCL13/Tfh-driven immune responses could contribute to B-cell hyperactivity and potentially lymphoma in the absence of ELS/germinal centers (21). This could also help to explain some of the inconsistencies in the literature on the association of ELS in salivary glands as an important prognostic factor for developing lymphoma in pSS patients(22-25).

In conclusion, epigenetic cell counting is a robust method to quantify immune cell subsets in salivary gland biopsies from sicca patients, and this novel tool hence could be valuable in studying immunopathology and could aid in diagnostics, prognostics and monitoring therapy response in clinical trials in the future.

Acknowledgements

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Supplementary material

Supplementary methods

RNA analysis

RNA sequencing was performed on an Illumina NextSeq500 sequencer providing approx. 20 million 75 bp single-ended trimmed reads for each sample. The sample qualities were assessed by FastQC and the reads were mapped to the human genome (GRCh38) using STAR aligner.(26) Since the samples were sequenced in two batches, the batch effect was corrected using RUVSeq package in R.(27) The normalized expression values (vsd normalized read counts) for the genes were calculated using the DESeq2 package in R.(28) RNAseq data were used to assess the correlations of mRNA expression with ECC counts.

DNA and RNA isolation

DNA and RNA were isolated from labial salivary gland tissue samples to perform epigenetic cell counting and RNA sequencing (the latter was performed from 8 nSS, 10 iSS, 21 pSS patients and 1 sSS patient). Cryo-preserved salivary gland tissue was stored in Tissue-Tek at -80°C. Sixty sections of 20μm were cut from each biopsy and lysed in RLT-plus (Qiagen) supplemented with beta-mercaptoethanol. Lysates were stored at -80°C until DNA/RNA purification. DNA and RNA were isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) on the QIAcube (Qiagen). DNA and RNA concentration and quality was measured using NanoDrop (Thermo Fisher Scientific). RNA quality was further assessed using Agilent 2100 Bioanalyzer. DNA and RNA solutions for each subject were stored at −20°C and -80°C, respectively, until analyses took place. RNA sequencing methods are described in the supplementary file.

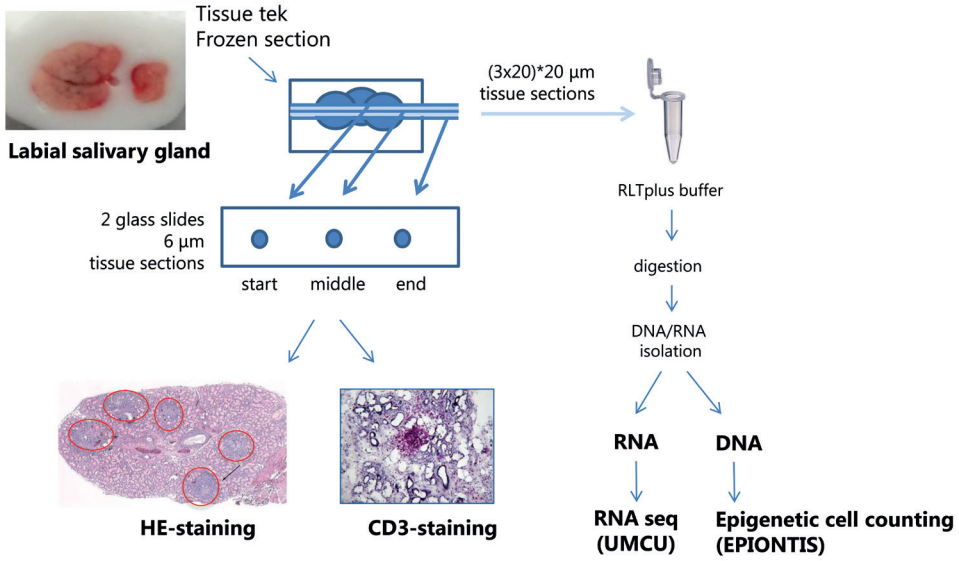
Histology

To validate the epigenetically quantified CD3 cells these were correlated to CD3 T-cells quantified by immunohistochemistry (IHC). For quantification of CD3 cell numbers on protein level mouse anti-human CD3 (clone SK7, BD Biosciences) was used. The number of CD3-expressing T-cells was digitally quantified using PhotoShop and Image J software and was calculated as the percentage area of positive cells. In addition to the lymphocytic focus scores (LFS) that were determined by the pathologist using FFPE-based tissue sections (clinical LFS), the LFS was scored based on H&E-staining of the tissue sections that were adjacent to the sections used for DNA and RNA analyses (ECC-paired LFS, see suppl figure 1 for experimental design). LFS was scored by two observers, blinded to the patient's diagnosis, and was expressed as LFS per 4 mm2 as was assessed with Image J.

For immunofluorescence staining, after deparaffinization and epitope retrieval formalin-fixed paraffin embedded tissue sections were stained for CD21 using mouse monoclonal anti-

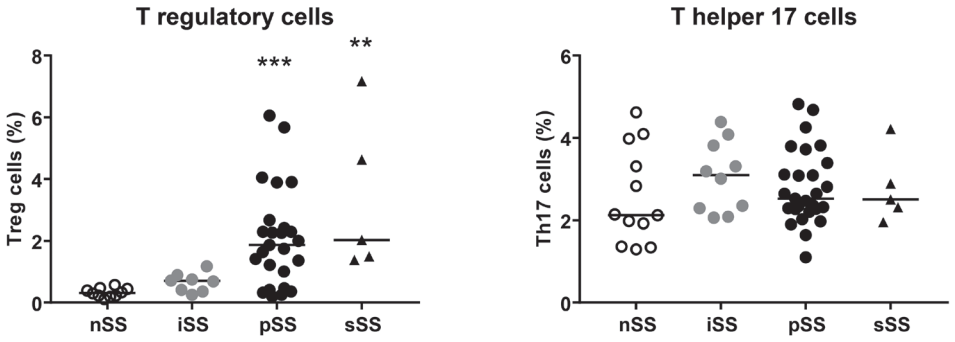
human CD21 antibody (clone 2G9, Novocastra/Leica Biosystems) in combination with either rabbit monoclonal CD3 or CD20 (Cell Marque). This was followed by fluorochrome-labeled secondary antibodies (Goat anti-Mouse IgG (H+L) Alexa Fluor 488 and Goat anti-Rabbit IgG (H+L) Alexa Fluor 555 Highly Cross-Adsorbed Secondary Antibodies, used for diagnostic purposes). Nuclei of the cells were visualized using counterstaining with DAPI. CD21 areas were considered if more than 10 CD21+ follicular DCs were co-localized. CD21 cells did not co-express B cell marker CD20 (data not shown).

Supplementary figures



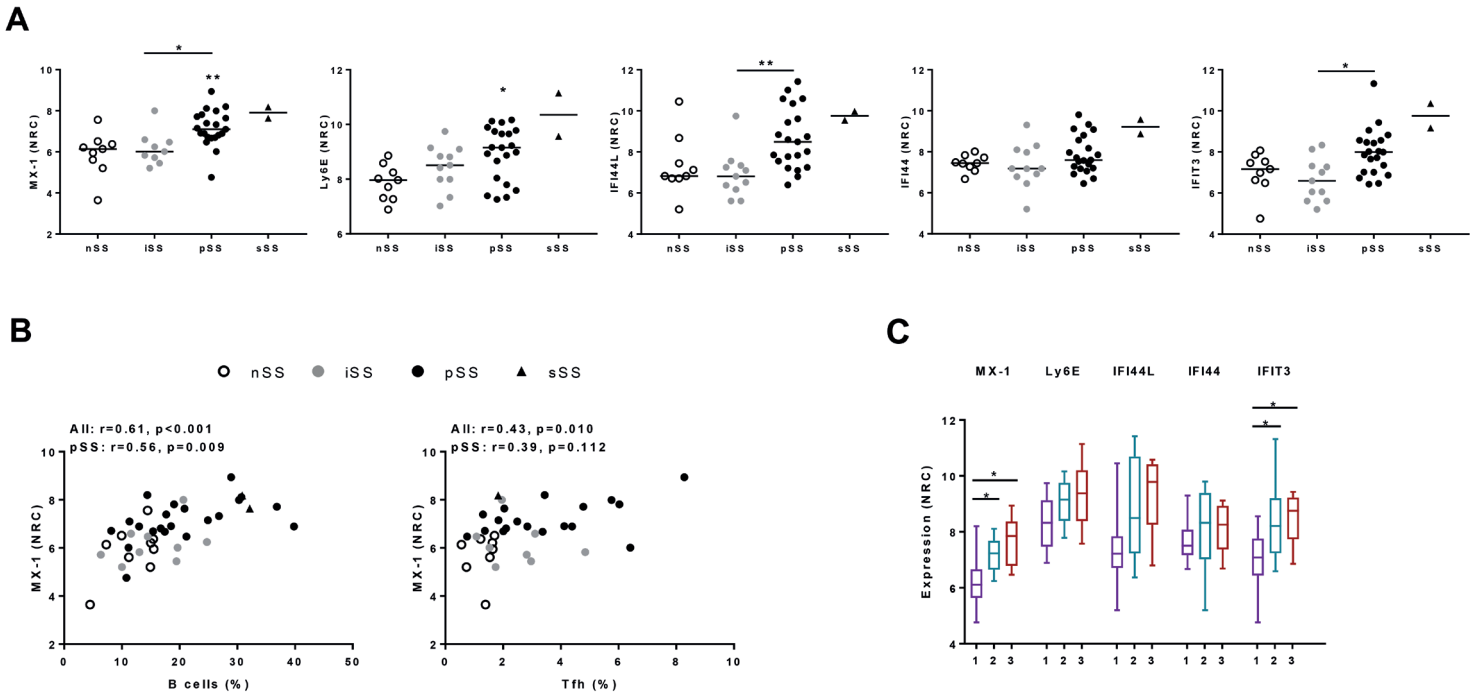
Supplementary Figure 1. Experimental procedure of collection of frozen tissue sections for IHC, RNA for RNA sequencing and DNA for epigenetic cell counting from labial salivary gland biopsies.

Frozen labial salivary gland tissue was cut into sections of 6µm for IHC at the start, middle and end of the biopsy, using a Cryotome. In between, 3 times 20 sections of 20µm were collected and lysed in RLT buffer with beta-mercaptoethanol. Subsequently, RNA and DNA were extracted from the lysates and stored for later usage for RNA sequencing and epigenetic cell counting, respectively.



Supplementary Figure 2. Regulatory T-cells, but not T helper 17 cells as quantified by epigenetic cell counting, are increased in salivary gland biopsies from Sjögren's syndrome patients.

Next to Tfh cell and Th17 T cell subsets frequencies of Th, regulatory cells and T helper 17 cells as assessed by ECC are shown in the studied groups of patients. Medians are shown. Kruskal-Wallis test with Dunn's multiple-comparison test was used. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively.



Supplementary Figure 3. Expression of Interferon-induced genes is increased in the salivary glands patients with pSS and correlates with the number of local B-cells and Tfh cells.

Messenger RNA expression of the five prototypical interferon-induced genes (IFIG) in Sjögren's syndrome was assessed using RNA sequencing. Differences in expression between the subgroups were assessed. pSS patients have higher expression of the majority of the IFIG as compared to nSS/iSS patients (**A**). Local expression of the quintessential IFIG MX-1 is correlated with the number of B-cells as well as T-follicular helper cells (Tfh) in the salivary glands as measured by epigenetic cell counting (**B**). Expression of MX-1 and IFIT3 is increased in patients from clusters 2 and 3 as compared to cluster 1 (**C**). Boxplot whiskers indicate the 5-95% interval. Kruskal-Wallis test with Dunn's multiple-comparison test was used. *, ** and *** indicate p<0.05, p<0.01 and p<0.001, respectively. Spearman correlations coefficients (r) are given for all sicca patients and for pSS patients.

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CHAPTER 4

High soluble IL-7 receptor expression in Sjögren's syndrome identifies patients with increased immunopathology and dryness

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Increased expression of interleukin (IL)-7 and its receptor is suggested to play a critical role in immunopathology of primary Sjögren's syndrome (pSS).(1-3) Data from humans and mice demonstrate that IL-7 drives a range of processes involved in pSS immunopathology, including epithelial cell apoptosis, lymphocyte infiltration, and reduction of salivary output(3). The IL-7/IL-7R axis is involved in formation of (ectopic) lymphoid structures in salivary glands,(3,4) which is a predictor for lymphoma development in pSS.(5, 6) IL-7 activity is potentiated by the soluble form of its receptor (sIL-7R), which is produced in inflamed tissues by activated stromal cells.(7) As sIL-7R is a possible biomarker for IL-7-driven immune activation and lymphoid neogenesis, we studied the expression of sIL-7R in pSS in relation to markers of inflammation and saliva production.

95 pSS patients were diagnosed according to the 2002 criteria (**Table 1**).(8) sIL-7R was measured in serum of 68 pSS patients using ELISA as previously described (9) and compared to 51 healthy individuals (HC). Labial salivary gland (SG) biopsy tissues were taken from 27 patients and, after thorough rinsing, were incubated in 200uL of sterile saline for 1 hour at room temperature. In these tissue supernatants, sIL-7R was measured using multi-cytokine analysis and compared to 24 sicca patients not meeting criteria for pSS or any other connective tissue disease and defined as non-Sjögren's sicca (nSS). Weights of biopsy tissues analyzed did not differ between the groups. sIL-7R^{high} patients were defined as those pSS patients with sIL-7R concentrations in serum or SG tissue supernatant above the mean plus twice the standard deviation of the concentrations measured in the respective control group. We used Mann Whitney U-test to compare groups, Spearman's rank correlation coefficient to assess correlations, and Fisher's exact test to compare proportions. Values of $p<0.05$ were considered statistically significant, p-values were not corrected for multiple hypothesis testing.

Serum levels of sIL-7R were significantly higher in pSS patients compared to healthy controls (**Figure 1A**). In the pSS patients, serum sIL-7R concentrations correlated with serum IgG (ρ=0.30, p=0.015), lymphocytic focus score (LFS) (ρ=0.36, p=0.008), stimulated whole saliva (SWS) (ρ=-0.48, p=0.011), and unstimulated whole saliva (UWS) (ρ=-0.54, p=0.004). A group of pSS patients with sIL-7R levels above those of HC could be discerned and was defined as sIL-7R^{high} (n=23/34%). These patients showed increased serum IgG and LFS compared to the other pSS patients (sIL-7R^{low}) (**Figure 1B**). Furthermore, these sIL-7R^{high} patients had significantly decreased salivary output, as measured by SWS (median [range]: 0.2 [0.0-0.9] vs. 1.2 [0.0-4.6] mL/min, p=0.015) and UWS (**Figure 1C**).

In addition, we observed increased sIL-7R production in SG supernatants from pSS patients compared to nSS (**Figure 1D**). In the pSS patients, supernatant sIL-7R concentrations correlated with serum IgG (ρ=0.52, p=0.007). Similar to the serum data, a sIL-7R^{high} group could be discerned (n=13/46%), with higher levels of serum IgG (**Figure 1E**), increased ESSDAI

scores (median [range]: 3.0 [2.0-11] versus 1.0 [0.0-7.0], p=0.017), and increased prevalence of anti-Ro/SSA and anti-La/SSB autoantibodies (**Figure 1F**) compared to the sIL-7R^{low} group.

Thus, sIL-7R is increased in serum and SG supernatant of pSS patients, in which high sIL-7R expression identifies patients with increased markers of inflammation and decreased salivary output. The sIL-7R increase in pSS SG supernatants indicates that the increased systemic sIL-7R levels are at least partially mediated by local production. High local production of sIL-7R was related to increased B-cell activity and autoimmunity, in line with the described role of IL-7 in lymphoid neogenesis.(10)

Our data indicate that sIL-7R may be a biomarker for patients with more severe disease, which is in line with data from systemic lupus erythematosus where increased serum sIL-7R is a marker for increased disease activity and lupus nephritis (9). As the IL-7/IL-7R axis is critical for lymphoid neogenesis,(10) increased sIL-7R may also contribute to lymphoma development. However, larger patient cohorts are necessary to validate the potential of sIL-7R as a disease marker and study its possible contribution to lymphoid neogenesis. Dissecting the functional role of sIL-7R in immunopathology may have important implications with regards to therapeutic targeting of the IL-7/IL-7R axis in pSS.

	Serum	SG supernatant	
	pSS	nSS	pSS
N (M/F)	68 (2/66)	24 (3/21)	27 (4/23)
Age (yr.)	56 [19 – 76]	44 [24 – 71]	53 [23 – 75]
ESR (mm/hour)	26 [2.0-109]	7.0 [2.0 – 23]	16 [3.0 – 63]
sIgG (g/L)	15 [2.9 – 45]	11 [6.5 – 15]	14 [8.5 – 30]
LFS (foci/4mm^2)	2.0 [0.0 – 4.0]	0.0 [0.0 – 0.8]	2.0 [0.4- 6.0]
C3 (g/L)	1.0 [0.7 – 1.4]	1.1 [0.6 – 1.7]	1.1 [0.5 – 1.6]
C4 (g/L)	0.2 [0.1 – 0.3]	0.3 [0.1 – 0.4]	0.3 [0.1 – 0.6]
ESSDAI	1.0 [0.0 – 12]	-	2.5 [0.0 – 11]
ANA pos (%)	52 (78%)	12 (50%)	20 (74%)
Anti-Ro/SSA pos (%)	47 (78%)	0 (0%)	19 (70%)
Anti-La/SSB pos (%)	26 (43%)	0 (0%)	7 (26%)

Table 1. Patients' characteristics.

SG: Salivary gland; ESR: erythrocyte sedimentation rate; sIgG: serum IgG; LFS: lymphocytic focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ANA: anti-nuclear antibodies; SSA: serum anti-SSA/Ro; SSB: serum anti-SSB/La. Values are median [range] unless specified otherwise.

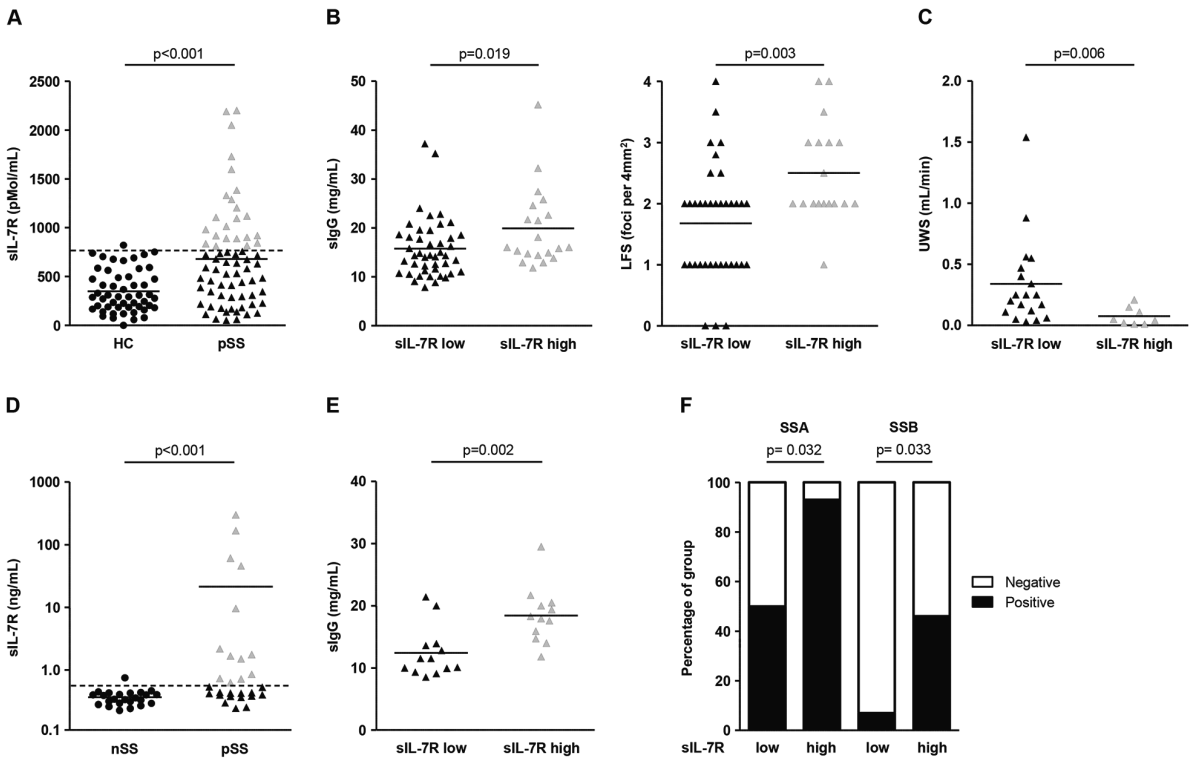

Figure 1. High sIL-7R expression identifies pSS patients with increased serum IgG, LFS and dryness.

pSS patients have significantly higher levels of serum sIL-7R than healthy controls (HC). A group of patients with high sIL-7R expression levels was identified (sIL-7R high; grey) (**A**). Serum sIL-7R high patients have higher serum IgG (sIgG) levels and lymphocytic focus scores (LFS) than the other patients (sIL-7R low; black) (**B**). Salivary output was measured in 27 of the pSS patients. sIL-7R high patients have less unstimulated whole saliva (UWS) production compared to sIL-7R low patients (**C**). sIL-7R was measured in salivary gland tissue supernatant of a separate cohort. In these supernatants, sIL-7R expression is higher in pSS compared to nSS patients and a sIL-7R high group can be distinguished (**D**). sIgG levels (**E**) and serum anti-Ro/SSA and anti-La/SSB prevalence is significantly increased in patients with high tissue sIL-7R concentrations (**F**). Age of the healthy controls used for serum measurement was 52 [19 – 75] (median [range]) with a male/female ratio of 2/49. Means are shown.

Acknowledgements

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CHAPTER 5

Increased expression of Fas on group 2 and 3 innate lymphoid cells is associated with an interferon signature in systemic lupus erythematosus and Sjögren's syndrome

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Abstract

Introduction. The role of innate lymphoid cells (ILCs) in the pathophysiology of rheumatic diseases is emerging. Evidence from animal studies implicate type I interferon (IFN), produced by plasmacytoid dendritic cells (pDCs), to be involved in regulating the survival of group 2 and group 3 ILCs (ILC2s and ILC3s) via the upregulation of Fas (CD95) expression. For the first time, we explored the frequency and phenotype of circulating ILCs in systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS) in relationship to the IFN signature.

Methods. Frequencies and phenotypes of ILC subsets and pDC were assessed by flow cytometry in peripheral blood of patients with SLE (n=20), pSS (n=20) and healthy controls (n=17). Patients were stratified by the presence or absence of an IFN signature as assessed by RT-qPCR on circulating mononuclear cells.

Results. ILC1 frequencies were increased in peripheral blood of patients with SLE and in pSS patients with high disease activity compared to healthy controls. Overall, the frequencies of ILC2s or ILC3s did not differ between patients with SLE, pSS and healthy controls. However, patients with a high type I IFN signature expressed elevated levels of Fas on ILC2s and ILC3s, which coincided with decreased frequencies of these cells in blood.

Conclusion. The presence of a type I IFN signature is related to Fas expression and frequencies of circulating ILC2s and ILC3s in patients with SLE and pSS, potentially altering the homeostatic balance of ILCs.

Introduction

Innate lymphoid cells (ILCs) are immune cells of lymphoid morphology that play an important role in epithelial homeostasis. ILCs lack the expression of T cell receptors and other "lineage" markers characteristic for other hematopoietic cells, but do express CD45, and CD117 (c-Kit) and/or CD294 (CRTH2) in addition to high expression of IL-7 receptor (CD127).(1) Besides their location at barrier sites, ILCs can be found in low numbers in blood. In response to environmental triggers ILCs rapidly release cytokines. The expression of transcription factors and the production of cytokines distinguishes three subsets of ILCs, that mirror the well-known subsets of helper T cells (Th). ILC1s express T-bet and produce interferon (IFN) γ and tumor necrosis factor (TNF) α, ILC2s express GATA-3 and produce interleukin (IL) 4, 5 and 13 and ILC3s express RORγt and produce IL-17 and 22, analogous to Th1, Th2 and Th17 cells, respectively. Due to their high expression of the IL-7 receptor, survival of ILCs, in particular ILC2s and ILC3s, is critically dependent on IL-7.(1,2)

Recent studies have linked alterations in the frequency and function of ILCs to the pathophysiology of rheumatic diseases, in particular rheumatoid arthritis (RA) and spondyloarthropathies.(2) SLE and pSS are systemic autoimmune diseases with involvement of epithelial tissue including the salivary and lacrimal glands, skin and oral mucosa. Central to the pathogenesis of SLE and pSS is the increased activity of T and B cells leading to production of pathogenic autoantibodies. Increased activity of the IL-7 axis drives T and B cell hyperactivity in SLE and pSS and is related to major pathologic features such as lupus nephritis and dryness.(3,4)

In SLE and pSS, increased levels of type I IFNs (eg. IFNα) are associated with disease activity, and these IFNs regulate multiple key immunological processes, including B cell hyperactivity. Mechanistically, immune complexes of RNA/DNA binding autoantibodies have been shown to activate plasmacytoid dendritic cells (pDC) to produce type I IFN (IFNα) in SLE and pSS, resulting in an overexpression of type I IFN-inducible genes, known as the IFN signature.(5)

Evidence from animal studies that model HIV and asthma indicate a close relationship between IFNα-producing pDCs and the fate of group 2 and group 3 ILCs. pDC-derived IFNα induces the expression of Fas (First apoptosis signal receptor, CD95) and other proapoptotic molecules on ILC2s and ILC3s rendering them susceptible to apoptosis.(6-8) In patients with HIV, ILC2s and ILC3s are decreased in frequency and characterized by a high expression of Fas.(9) However, no previous studies have investigated ILCs in patients with SLE and pSS. Here we explored the frequency and phenotype of circulating ILCs in SLE and pSS in relation to pDC activation and the subsequent IFN signature.

Methods

Patients

Peripheral blood mononuclear cells (PBMCs) from patients and healthy controls were isolated by ficoll density gradient centrifugation and cryopreserved in liquid nitrogen until further use. A total of 20 patients with SLE, 20 patients with pSS and 17 age- and sex-matched healthy controls were included in this study. SLE patients met the American College of Rheumatology (ACR) classification criteria and patients with pSS met the American-European Consensus Group criteria (AECG). None of the included patients had clinical evidence of an ongoing infection. This study was approved by the ethical committee of the UMC Utrecht, in accordance with the Helsinki declaration. All patients and healthy controls signed informed consent prior to the donation of blood. Patient characteristics can be found in **Table 1**.

	HC (n=17)	**SLE (n=20)**	**pSS (n=20)**
Age	50 (40 - 56)	43 (27 - 55)	58 (46 - 67)
Female	100%	100%	100%
Current drug use			
Hydroxychloroquine		65%	10%
Prednisone		45%	10%
Azathioprine		25%	5%
Mycophenolate mofetil		15%	0%
Serology			
Antinuclear antibodies		100%	85%
anti-dsDNA antibodies (ELiA, IU/mL)		7 (2 - 14)	-
C3 (g/L)		0.94 (0.86 - 1.04)	1.09 (0.95 - 1.16)
C4 (g/L)		0.18 (0.14 - 0.21)	0.20 (0.16 - 0.27)
Anti-Ro/SSA		45%	80%
Anti-La/SSB		20%	50%
ESR (mm/hr)		7 (3 - 11)	16 (11 - 22)
Serum IgG (g/L)		-	14 (12 - 17)
Clinical manifestations			
ESSDAI		-	5 (2 - 7)
ESSPRI		-	6 (5 - 7)
Lymphocytic focus score (foci/4mm^2)		-	2 (1.3 - 2.4)
Schirmer (mm/5min)		-	10 (5 - 20)
SLEDAI		2 (0 - 4)	-
History of Lupus Nephritis		50%	-

Table 1. Clinical characteristics.

Percentages of total or medians with interquartile range. Abbreviations: ESR: erythrocyte sedimentation rate. ESSDAI: EULAR Sjögren's syndrome disease activity index. ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index. SLEDAI: SLE disease activity index.

Flow cytometry

ILCs were identified as previously described by our group.(10) A list of used antibodies can be found in **Supplementary table 1**. 5-10x10e6 PBMC were stained and subsequently acquired on an LSR Fortessa (BD). Dead cells were excluded using the fixable viability dye eF506 (eBioscience). From the lymphocyte gate, ILCs were identified as CD45+Lin-CD127+. The expression of CD117 (c-Kit) and CD294 (CRTH2) were used to identify ILC1s (CD117-CD294-), ILC2s (CD294+) and ILC3s (CD117+CD294-) subsets.

A second flow cytometry panel was used to identify the frequency of pDCs among CD45+ cells (defined as CD123+BDCA2+BDCA4+ cells) and the expression of Siglec-1 (Sialic acid-binding immunoglobulin-type lectin-1, CD169, an IFN inducible protein(11)) on CD14+ monocytes.

qPCR

RNA was extracted from PBMCs using the Allprep universal kit (Qiagen) according to the manufacturer's instructions. Complementary DNA was synthesized from 200ng RNA using iScript (BioRad). RT-qPCR was performed on a Quantstudio 12k Flex (Thermofisher). The expression of IFI44, IFI44L, MX1, Ly6E and IFIT3 normalized to the housekeeping gene GUSB were used to calculate an IFN scores. A list of primer sequences can be found in **Supplementary table 2**.

Statistics

The type I IFN scores were calculated as previously described.(12,13) An optimal cut-off to discern IFN-high and IFN-low patients subgroups was identified at the maximum J-statistic of the Youden's index of the receiver operating characteristics curve as described.(13) Mann Whitney U tests and Spearman rank correlations were used to test statistical significance. All tests were conducted two sided at an alpha level of 0.05.

Results

ILC1s are increased in SLE and are associated with disease activity in pSS

We readily identified circulating ILCs among PBMCs by their high expression of CD127 and the absence of lineage markers (**Figure 1A**). The total frequencies of ILCs did not differ among SLE and pSS patients as compared with HCs (**Figure 1B**). Using the expression of CD117 and CD294 we discerned three populations of ILCs: CD117-CD294- ILC1s, CD294+ ILC2s and CD117+CD294- ILC3s (**Figure 1C**). There were no changes in the proportions of ILC subsets among total ILCs among patients with SLE, pSS and HCs (**Figure 1D**). With the exception of ILC1s being increased in patients with SLE as compared with HCs (**Figure 1E**), no differences were found in the frequencies of ILCs subsets among patients with SLE, pSS and HCs (**Supplementary figure 1**). Within the pSS group, higher ILC1 frequencies were

associated with higher disease activity as measured by ESSDAI (r=0.68, p=0.001, **Figure 1F**) as well as serum IgG levels (r=0.53, p=0.015, data not shown) and with presence of anti-La/SSB autoantibodies (p=0.01, data not shown). No correlations with SLE clinical features were found.

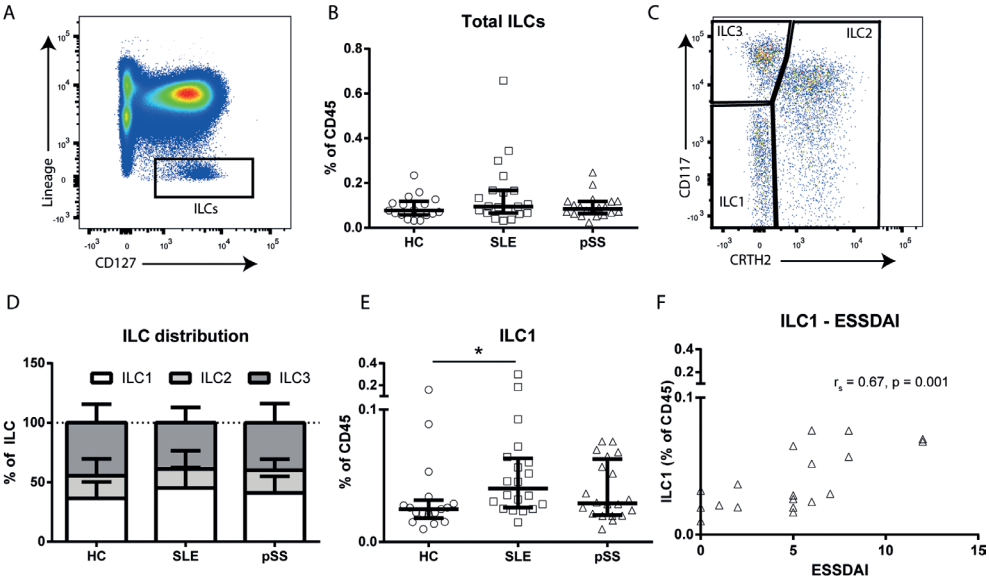

Figure 1. Increased circulating ILC1 frequency in patients with SLE and pSS with high disease activity.

(**A**) Identification of ILCs in the peripheral blood of SLE and pSS patients based on lack of expression of lineage markers and expression of IL-7Rα (CD127). (**B**) Frequency of total ILCs in patients with SLE and pSS does not differ from HCs. (**C**) Identification of the three ILC subsets on the basis of the expression of CD117 and CD294. (**D**) Distribution of ILC subsets does not significantly differ between SLE, pSS and HC. (**E**) ILC1s are increased in patients with SLE. (**E**) Increased frequencies of ILC1s correlate with higher disease activity in patients with pSS.

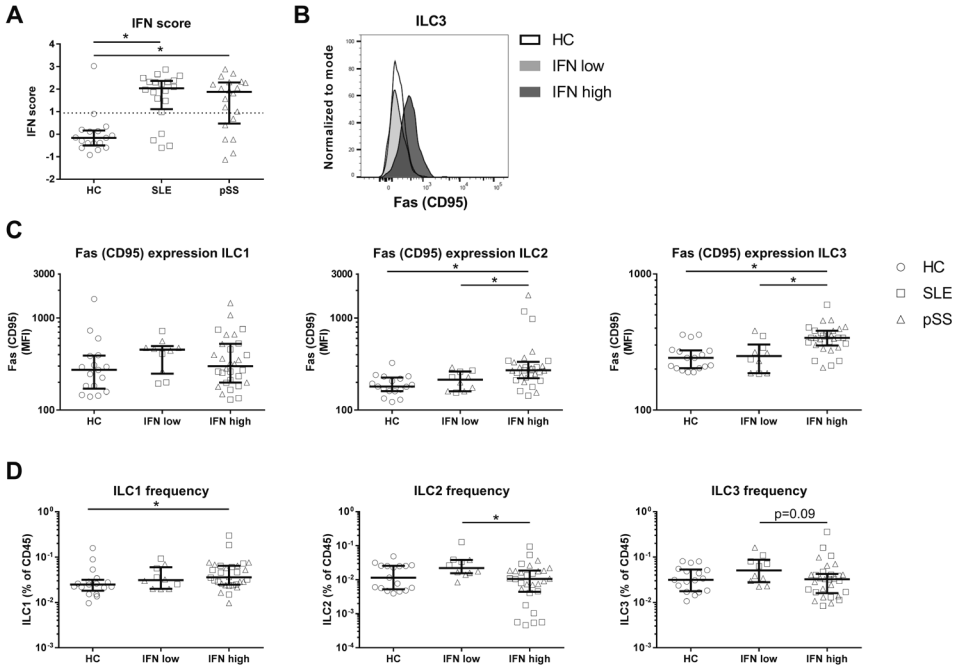

Figure 2. Fas expression is elevated on ILC2s and ILC3s from patients with a high type I IFN signature in association with decreased frequencies of these cells.

(**A**) Elevated IFN scores are present in a subgroup of patients with SLE and pSS. (**B**) Representative FACS plot. (**C**) Increased expression of Fas (CD95) on ILC2s and ILC3s in patients with a high type I IFN signature. (**D**) Decreased frequencies of ILC2s and ILC3s in patients with a high type I IFN signature.

The presence of a type I IFN signature is associated with elevated Fas expression on ILC2 and ILC3 subsets and decreased cell frequency

Elevated IFN scores were observed in patients with SLE and pSS (p<0.001, **Figure 2A**), which strongly correlated to monocyte Siglec-1 (CD169) expression (r=0.82, p<0.001, **Supplementary figure 2**), confirming the robust identification of the IFN signature. Setting a threshold using the HCs, 16/20 (80%) and 14/20 (70%) patients with SLE and pSS respectively were characterized by a high type I IFN signature (IFN-high, **Figure 2A**). In patients with a high IFN signature, Fas expression was upregulated on both ILC2 and ILC3 subsets (**Figure 2B, C**). In contrast, Fas expression on ILC1s was not related to the IFN signature (**Figure 2C**). The increased expression of Fas on the ILC2 and ILC3 subsets in IFN-high patients was associated with a decrease in ILC2s (p=0.01, **Figure 2D**) and a similar trend in ILC3s (p=0.09, **Figure 2D**) as compared with IFN-low patients. ILC1 frequencies did not differ between IFN-high and IFN-low patients (**Figure 2D**). Circulating pDCs were decreased in SLE and pSS and correlated with Fas expression on ILC2 and ILC3 subsets (r=-0.39, p=0.01, and r=-0.38, p=0.02 for ILC2 and ILC3 respectively, **Supplementary figure 3**).

Discussion

Here we evaluated for the first time the frequency and phenotype of circulating ILC subsets in patients with SLE and pSS. ILC1s were found elevated in blood of patients with SLE and were associated with disease activity in pSS. When comparing SLE and pSS, no differences were observed in the frequencies of ILC subsets. However, high expression of Fas (CD95) on circulating ILC2 and ILC3 subsets was associated with an increased IFN signature, possibly rendering these cells more susceptible to apoptosis. These observations corroborate previous studies in mice that reported a link between type I IFN, pDC activation and apoptosis of circulating ILC2s and ILC3s(6-8). We here report unprecedented data on a potential similar regulation in the blood of patients with systemic autoimmune diseases.

ILCs were only recently identified. In the pre-ILC era, a subset of ILC1-like natural killer (NK) cells were reported to be increased in patients with SLE compared to HC.(1) Here we report that circulating ILC1s, classified according to current phenotypical definitions, are increased in patients with SLE and in patients with pSS with higher disease activity.

In mice models of rheumatoid arthritis (RA), the ILC2 subset is crucial for the resolution of autoimmune inflammation by producing IL-9 which promotes regulatory T cell function and restores immune homeostasis. In humans, circulating ILC2 are reduced in active RA and normalize to normal levels in remission.(14) Whether such differences represent systemic cell death or (re-)circulation events remains to be established. In our cohort, the frequency of circulating ILC2s is reduced in patients with a high IFN signature, which is associated with disease activity in these patients. Hence, possibly the decrease in ILC2s in IFN-high SLE and pSS patients may contribute to ongoing inflammation via similar mechanisms as in RA.

In systemic sclerosis, another disease characterized by a type I IFN signature, ILC2s are elevated in the skin.(15) Migration of ILCs into target tissues might therefore represent another explanation for the reduced levels of circulating ILC2s and ILC3s in IFN-high patients. No studies so far have assessed ILCs in affected tissues of patients with SLE. In pSS, IL-22 producing NKp44^{+} cells, which might represent ILC3s, are found in the salivary glands of patients with pSS and correlate with the amount of lymphocytic infiltration.(16)

Survival of ILCs, including lymphoid tissue inducer cells (LTi) is dependent on IL-7, which is elevated in salivary glands of pSS patients(17). The IL-7/IL-7R axis plays an important role in formation of ectopic lymphoid structures in non-lymphoid tissues, including the salivary gland, and this has been shown to require presence of LTi cells.(18) Altogether, this suggests a role for ILCs in development of lymphocytic infiltrates, including germinal center-like structures, in the glands of pSS patients.

Perturbations in the immune system are often shared between different autoimmune diseases, with the IFN signature as a clear example. It is becoming increasingly clear, that both from an immunological as well as a clinical perspective, stratification of patients with SLE and pSS on a molecular level, as by the IFN signature, aids in the identification of subgroups with different immunological alterations and even response to therapy.(12,13,19) Here we report that alterations in the frequency and phenotype of circulating ILCs is related to the IFN signature in SLE and pSS. On the basis of our observations, we propose that future studies on ILCs in patients with autoimmune diseases, whether in blood or tissue, should take into account the presence or absence of the (local) IFN signature, since this may significantly impact their function and role in autoimmune diseases.

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Supplementary material

Supplementary tables

ILC panel

Laser	Marker	Label	Company	Clone
Blue1	CD3	FITC	BD	SK7
	CD19	FITC	BD	HIB19
	CD14	FITC	BD	M5E2
	CD16	FITC	BD	3G8
	CD94	FITC	eBioscience	DX22
	CD11c	FITC	Sony Biotechnology	Bu15
	CD141	FITC	Miltenyi	14H12
	CD34	FITC	Sony Biotechnology	561
	BDCA2	FITC	Miltenyi	AC144
	CD123	FITC	eBioscience	6H6
	FCeR1	FITC	eBioscience	AER-37
Red1	CD127 (IL7Ra)	AF647	Sony Biotechnology	HCD127
Red1/1	CD45	AF700	Sony Biotechnology	F10-89-4
YeGr2	CD294 (CRTH2)	PE-CF594	BD Horizon	BM16
Violet1	CD95	ef450	eBioscience	DX2
Violet3	CD117 (cKit)	BV605	Biolegend	104D2
Violet2	Fixable viability dye	ef506	eBioscience	

pDC panel

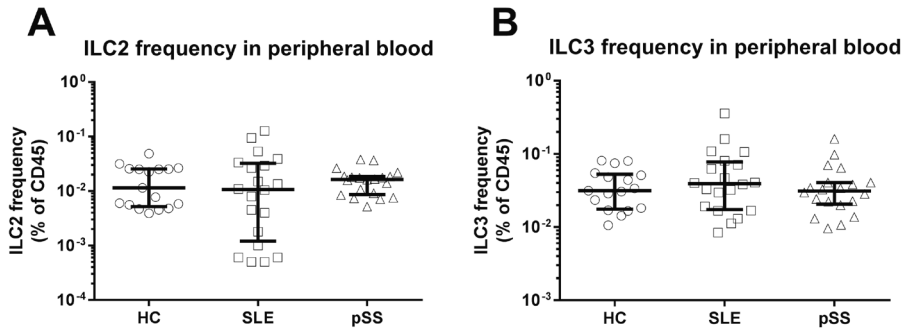
Laser	Marker	Label	Company	Clone
red2	CD14	APC-ef780	eBioscience	61D3
violet1	CD16	PB	BD	3G8
blue3	BDCA2 (CD303)	PerCP-Cy5.5	Sony Biotechnology	201A
red1	BDCA4 (CD304)	APC	Miltenyi	AD5-17F6
red1/1	CD45	AF700	Sony Biotechnology	F10-89-4
blue1	CD123	FITC	eBioscience	6H6
yegr1	CD169	PE	eBioscience	7-239

Supplementary table 1. Flow cytometry antibodies

Gene symbol	Forward primer	Reverse primer
Ly6E	ATCTGTACTGCCTGAAGCCG	GTCACGAGATTCCCAATGCC
IFIT3	ACTGTTTCAACGGGTGTTGG	CCTTGTAGCAGCACCCAATC
IFI44L	CCACCGTCAGTATTTGGAATGT	ATTTCTGTGCTCTCTGGCTT
GUSB	CACCAGGGACCATCCAATACC	GCAGTCCAGCGTAGTTGAAAAA
MX1	GCATCCCACCCTCTATTACTG	CGCACCTTCTCCTCATACTG
IFI44	TTTGCTCTTTCTGACATCTCGGT	TCCTCCCTTAGATTCCCTATTTGC

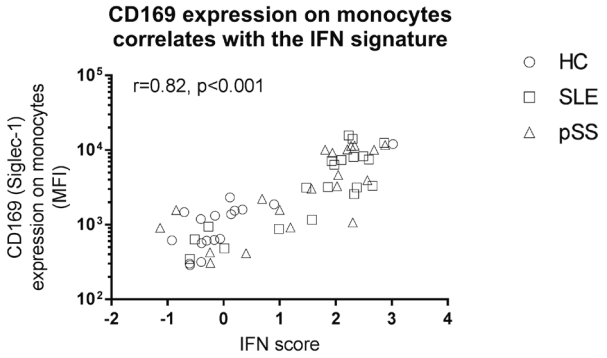
Supplementary table 2. Primers used for assessment of the IFN signature

Supplementary figures



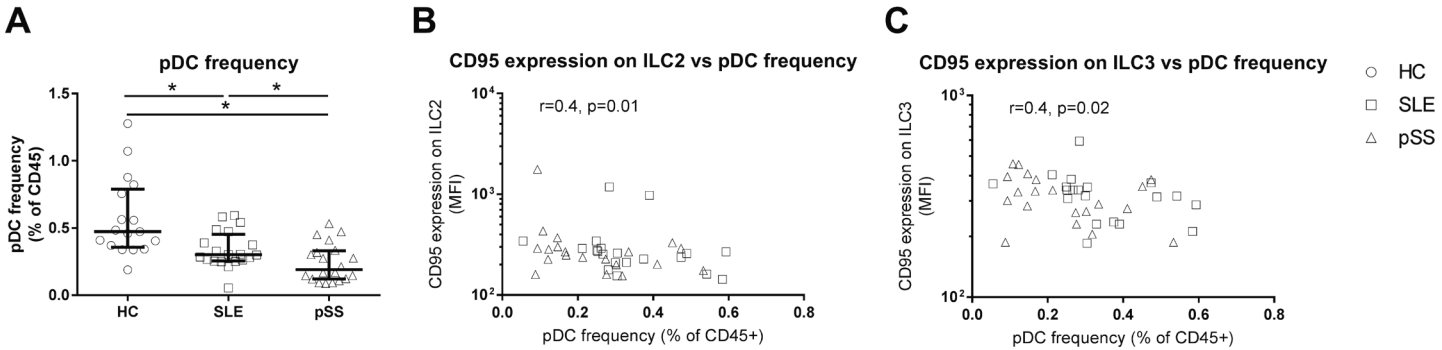
Supplementary figure 1. Peripheral blood frequencies of ILC2s and ILC3s do not differ between HC, SLE and pSS.

Frequencies of (**A**) ILC2 and (**B**) ILC3 in peripheral blood of HC and patients with SLE and pSS.



Supplementary figure 2. CD169 expression by monocytes correlates with the IFN signature in SLE and pSS patients.

Correlation between CD169 (Siglec-1) expression by monocytes and the IFN signature in HC, SLE and pSS.



Supplementary figure 3. Fas expression by ILC2s and ILC3s correlates with pDC frequency in SLE and pSS patients.

(**A**) Plasmacytoid DC frequencies in peripheral blood of HC, SLE and pSS. Correlation between pDC frequency and Fas expression by (**B**) ILC2 and (**C**) ILC3 in SLE and pSS patients.

CHAPTER 6

Increased CCL25 and T Helper Cells Expressing CCR9 in the Salivary Glands of Patients With Primary Sjögren's Syndrome: Potential New Axis in Lymphoid Neogenesis

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Abstract

Introduction. T follicular helper (Tfh)-cells play a critical role in germinal center formation and B-cell activation, both hallmarks of primary Sjögren's syndrome (pSS). CCR9-expressing Th-cells have "Tfh-like" characteristics and are increased at mucosa-associated sites in several inflammatory conditions. Because of their unique characteristics and limited evaluation we investigated the local and systemic CCL25/CCR9-axis in pSS.

Methods. CCL25 protein and mRNA levels and CCR9+ Th-cells were assessed in labial salivary glands (LSG) of pSS and non-Sjögren's sicca (nSS) patients and their correlation with inflammatory and clinical parameters was evaluated. Circulating CCR9+ and CXCR5+ Th-cells were compared based on phenotypic and functional properties.

Results. CCL25 protein and mRNA levels were elevated in LSG from pSS versus nSS patients and associated with B-cell hyperactivity, autoimmunity and levels of IL-21 and soluble IL-7Rα. The frequency of CCR9-expressing cells was increased in the LSG of pSS patients. Circulating CCR9+ Th-cells expressing PD-1 and ICOS were elevated in pSS patients. CCR9+ Th-cells displayed higher expression of IL-7Rα and secreted higher levels of IFN-γ, IL-17, IL-4 and IL-21 as compared to CXCR5+ Th-cells, *ex vivo* and upon triggering with antigen or IL-7. Both CCR9+ and CXCR5+ Th-cells induced IgG production by B-cells more potently than CCR9-CXCR5- Th-cells.

Conclusion. Enhanced CCL25 expression in pSS LSG can facilitate attraction of CCR9+ Th-cells, secreting high levels of pro-inflammatory cytokines when triggered with antigen or IL-7. Associations with B-cell hyperactivity, autoimmunity and markers of lymphoid neogenesis indicate the CCL25/CCR9-axis plays a significant role in pSS immunopathology, representing a novel therapeutic target.

Introduction

Primary Sjögren's syndrome (pSS) is characterized by inflammation of the exocrine glands associated with dryness of eyes and mouth. A hallmark feature of pSS is B-cell hyperactivity, reflected by autoantibody production, elevated serum IgG levels and increased risk of lymphoma development (in ~10% of patients).(1,2) Both the presence of germinal centers (GCs) and a high number of lymphocytic infiltrates in the salivary glands are associated with lymphoma development.(3,4)

T follicular helper (Tfh)-cells are potent B-cell stimulating cells and reside in GCs in lymph nodes. They are characterized by expression of Bcl-6, CXCR5, ICOS, PD-1 and cytokines such as IL-21, IL-4 and CXCL13 which attracts CXCR5+ Tfh-cells and CXCR5+ B-cells.(5,6) Tfh-cells are elevated in peripheral blood of pSS patients and the frequency of Tfh-cells correlate with autoantibodies, disease severity and aberrant memory B-cell and plasma cell subsets. Expression of IL-21, IL-4 and CXCL13 as well as Tfh numbers are increased in the salivary glands of pSS patients.(7-10)

Recently, a novel CD4+ T helper (Th)-cell subset was described that shares characteristics with Tfh-cells including IL-21 production, ICOS and Bcl-6 expression and expresses the chemokine receptor CCR9 but not CXCR5. These cells are therefore called "Tfh-like" cells. (11) Both in mice and humans, CCR9+ Th-cells are present in secondary lymphoid organs. (12,13) In humans, these CCR9+ Th-cells produce high levels of IFN-γ in addition to IL-17, IL-10 and IL-4 and induce robust B-cell responses.(12,14,15) They specifically migrate to mucosal sites in response to the chemokine CCL25 (thymus expressed chemokine, TECK). (16,17) CCR9+ Th-cells are important for maintenance of mucosal immune homeostasis but also may have a function in mucosal inflammation, potentially contributing to inflammatory bowel disease (IBD) and primary sclerosing cholangitis (PSC).(18-20) 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.(21,22) Inhibition of CCR9 decreased 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 therapy.(23-25)

Recently, in non-obese diabetic (NOD) mice, CCR9+ T-cells were also shown to mediate immunopathology in mucosa-associated tissues in accessory organs of the digestive tract, including the pancreas and salivary glands. The NOD mice spontaneously developed pSS-like symptoms and had infiltration of IL-21 expressing CCR9+ Th-cells in the salivary glands.(11)

CCL25 mRNA is not detectable in healthy human salivary gland tissue, but is up-regulated during oral inflammation.(26) Reduced methylation of the CCL25 gene is found in gingival

tissue of periodontitis patients.(27) In mice, CCL25 gene expression is upregulated in the oral mucosa upon antigenic triggering and during wound healing.(28,29)

Apart from demonstrating a pivotal role for CCR9+ Th-cells in experimental Sjögren-like disease, McGuire et al. found that CCR9+ Th-cells are enriched in the circulation of pSS patients, indicating that these cells might play a role in the disease.(11) We confirmed these findings and further characterized the role of CCR9+ Th-cells in pSS. We investigated the presence of CCR9+ Th-cells and its specific ligand CCL25 in the labial salivary gland (LSG) of pSS patients. In addition, phenotypic and functional properties of circulating CCR9+ Th-cells were studied in comparison to CXCR5+ Th-cells.

Materials and methods

Patients and controls

All pSS patients were classified according to the American-European Consensus Group (AECG) criteria.(30) Sicca patients not fulfilling the AECG criteria were defined as nSS patients. Autoantibody status and labial salivary gland biopsy were assessed for all nSS and pSS patients. All healthy volunteers, pSS and nSS patients were from the University Medical Center Utrecht, demographic and clinical data are shown in **Table 1**. The UMC Utrecht ethical committee approved the study and all participants gave written informed consent.

CCL25 and CCR9 expression analysis

CCL25 and *CCR9* mRNA expression levels in the LSG were assessed by qRT-PCR. *18S* was used as a housekeeping gene, expression was calculated using the $2^{-\Delta\Delta CT}$ method normalized against the median of the control group. To detect CCL25 secretion by LSG tissues, these were thoroughly rinsed directly after the biopsy procedure and incubated in 200µL of saline for 1h at room temperature. The tissue supernatant was frozen. CCL25 and IFN-γ, IL-17, IL-4, IL-21, IL-7, soluble IL-7Rα and CXCL13 were measured using Luminex multi-analyte analysis as previously described.(31)

Immunohistochemistry was performed to detect CCR9 expression in LSG. Frozen tissue sections were incubated with anti-CCR9 mAb or mouse IgG2a isotype control or by omitting the first antibody. Antigen–antibody complexes were visualized using the BrightVision poly-HRP detection system.

For immunofluorescence staining to assess co-localization, anti-CCR9, anti-CD3, anti-CD4 and anti-ICOS were used with secondary AF488 and AF555-labelled antibodies (gene expression and IHC details are given in **Supplementary File 1**).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Paque. Monocytes, CD19+ B-cells and CD4+ T-cells were isolated from PBMCs by autoMACS. To compare functional characteristics of CCR9+ Th-cells with CXCR5+ Th-cells, these subsets were sorted using BD FACSAria III after incubation of 5-12.10^7 PBMCs from healthy donors and patients based on CD3, CD4, CXCR5 and CCR9 expression, after which they were cultured.

Flow cytometry

To characterize CCR9+ Th-cells, 1.10^6 PBMCs from HCs and patients were stained for CD3, CD4, CD45RO, CXCR5, CCR9, CD127 (IL-7Rα), ICOS and PD-1. For intracellular cytokine staining 1.10^6 CD4+ T-cells/mL were stimulated with PMA and ionomycin in the presence of brefeldin A for 4h at 37°C and cells were fixed and permeabilized. Cells were stained for CD3, CD4, CD45RO, CD27, CXCR5, CCR9, CD127, IFN-γ, IL-17A, IL-4, IL-21 and IL-10 (flow cytometry details are given in **Supplementary File 1**).

Cell culture

For analysis of cytokine secretion, 2.10^4 CCR9+ or CXCR5+ Th-cells were cultured with 5.10^3 monocytes 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. For analysis of B-cell stimulation 2.10^4 CCR9+, CXCR5+ or CCR9-CXCR5- Th-cells were cultured with 2.10^5 B-cells and 5.10^3 monocytes for 12 days with 1 ng/mL IL-7 and 0.01 ng/mL SEB superantigen. IgG production was measured by ELISA (Human IgG quantification kit, Bethyl laboratories Montgomery, TX, USA) according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed in Prism 6 software and SPSS. Student's t-test, paired parametric t-test, Mann-Whitney U test and Wilcoxon non-parametrical paired test were used where appropriate. For correlations with disease parameters, Pearson's correlation and Spearman's rho were used where appropriate. Differences and correlations were considered statistically significant at $p<0.05$.

	Serum		Biopsy supernatants		Immuno-histochemistry		Gene expression		Flow cytometry	
	HC (n=5)	pSS (n=12)	nSS (n=34)	pSS (n=26)	nSS (n=9)	pSS (n=10)	nSS (n=9)	pSS (n=9)	HC (n=9)	pSS (n=12)
Female gender, n (%)	5 (100)	9 (75)	30 (88)	23 (88)	9 (100)	9 (90)	8 (89)	8 (89)	8 (89)	12 (100)
Age, years (mean ± SD)	58.2 ± 5.8	50.0 ± 11.1	50.3 ± 15.6	52.6 ± 13.8	45.8 ± 15.5	48.2 ± 12.8	53.3 ± 7.5	43.7 ± 19.7	43.6 ± 14.1	54.1 ± 14.5
Anti-Ro/SSA positive, n (%)	-	8 (67)	10 (29)	18 (69)	1 (11)	9 (90)	2 (22)	9 (100)	-	11 (92)
Anti-La/SSB positive, n (%)	-	3 (25)	0 (0)	5 (19)	1 (11)	5 (50)	0 (0)	3 (33)	-	7 (58)
ANA positive, n (%)	-	10 (83)	10 (29)	19 (73)	2 (22)	7 (70)	1 (11)	7 (78)	-	10 (83)
RF positive, n (%)	-	4 (33)	1 (3)	10 (38)	1 (11)	3 (30)	1 (11)	3 (33)	-	3 (25)
Lymphocytic focus score (foci/4mm2)	-	1.5 (1.0-2.4)	0.0 (0.0-0.1)	1.7 (1.0- 2.7)	0 (0-0)	4.5 (3.0-6.0)	0 (0-0)	3.0 (1.5-5.0)	-	2.0 (1.6-2.8)
IgA positive plasma cells (%)	-	50 (46-60)	75 (70-80)	59 (47-65)	85 (51-89)	40 (15-50)	>70	43 (16-50)	-	40 (15-59)
Schirmer (mm/5min)	-	8.5 (1.0-27.5)	5.3 (2.3-12.1)	7.0 (3.0-10.0)	0.0 (0.0-14.0)	3.5 (0-13.8)	1.5 (1.0- 5.0)	4.0 (0.8-17.0)	-	4.5 (0.3-12.5)
Serum IgG (g/L)	-	15.3 (10.3-20.4)	10.6 (8.1-12.8)	14.0 (10.8-19.7)	11.5 (9.8-12.8)	15.1 (12.7-24.6)	11.4 (11.0-12.4)	17.4 (10.4-28.5)	-	16.5 (11.8-18.1)
ESR (mm/h)	-	16 (7-25)	7 (6-14)	16 (7-28)	22 (13-28)	27 (10-56)	6 (3-21)	14 (10-52)	-	27 (12-33)
ESSDAI score (0-123)	-	2.5 (2.0-6.5)	-	3.0 (0.5-5.5)	-	-	-	-	-	6.0 (5.0-9.0)
ESSPRI score (0-10)	-	5.7 (2-7.7)	-	5.3 (2.3-6.0)	-	-	-	-	-	5.0 (5.0-7.0)
Immunosuppressants	-	3	3	3	5	4	1	2	-	1
Hydroxychloroquine		1	2	0	0	0	0	0		1
NSAIDs daily		0	0	0	5	2	1	1		1
Other		2	1	3	0	2	0	1		0

Table 1. Patients' characteristics.

Medians with interquartile range (IQR) are shown unless specified otherwise. HC: healthy controls, pSS: primary Sjögren's syndrome, nSS: non-Sjögren's sicca; SD: standard deviation; SSA: Sjögren's syndrome A antigen; SSB: Sjögren's syndrome B antigen; ANA: antinuclear antibodies; RF: rheumatoid factor; IgA: immunoglobulin A; IgG: immunoglobulin G; ESR: erythrocyte sedimentation rate; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; NSAIDs: non-steroidal anti-inflammatory drugs.

Results

Increased CCL25 levels in salivary glands from pSS patients are associated with B-cell hyperactivity and factors associated with lymphoid neogenesis

Levels of serum CCL25 were not significantly different in pSS patients (n=12) as compared to healthy controls (HC, n=5) (median 2748 pg/mL [IQR 2056-3748] versus 1992 pg/mL [IQR 1904-2449], p=0.064) and no significant correlations with clinical parameters were found.

In the LSG, CCL25 mRNA expression was significantly increased in pSS patients as compared to nSS patients (**Figure 1A**). In most individuals, CCR9 mRNA expression in this tissue was not detectable or very low. CCL25 protein levels were significantly increased in LSG biopsy supernatants from pSS patients as compared to nSS patients and levels were found to be elevated in anti-Ro/SSA-positive patients (**Figure 1B-C**). In all sicca patients, the level of CCL25 in LSG supernatants correlated with lymphocytic focus scores (LFS). In pSS patients, CCL25 protein levels significantly correlated with increased percentage of IgM and IgG expressing plasma cells and increased serum IgG levels (**Figure 1D**). No significant correlations with Schirmer's tests, EULAR Sjögren's syndrome disease activity index (ESSDAI) or EULAR Sjögren's syndrome patient reported index (ESSPRI) were found (data not shown).

In view of the potential role of CCL25 to attract CCR9+ Th-cells, we assessed whether CCL25 protein in the tissue supernatants was associated with cytokines present in the tissue supernatants that are typically associated with Tfh-cells and lymphoid neogenesis, including IL-21, CXCL13, IL-7 and sIL-7Rα.(6,32) In addition, Th1, Th2 and Th17 major defining cytokines IFN-γ, IL-4 and IL-17 were assessed. IFN-γ, IL-4, IL-17 and IL-7 were detectable in a minority of samples, which hampered the assessment of correlations (data not shown). Levels of IL-21 and sIL-7Rα, however, were significantly increased in pSS patients and robustly correlated with CCL25 levels. CXCL13 was significantly increased in pSS patients as compared to nSS controls, but did not correlate with CCL25 levels (**Figure 1E**).

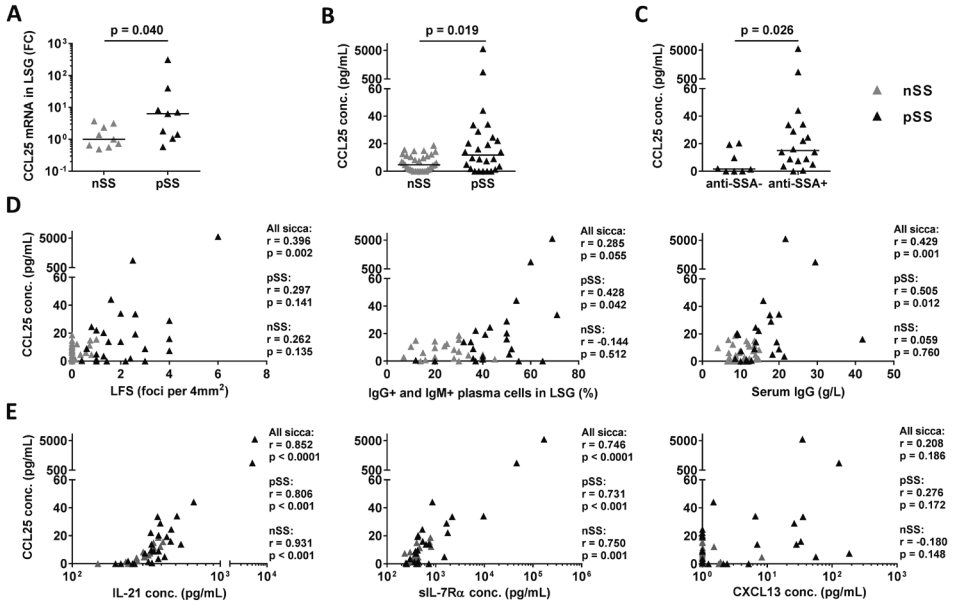

Figure 1. Elevated expression of CCL25 in labial salivary glands of pSS patients is associated with autoimmunity and B-cell hyperactivity.

(**A**) CCL25 mRNA expression is increased in labial salivary gland (LSG) tissue from pSS patients (n=9) compared to nSS (n=9) patients. 18S was used as a housekeeping gene, relative expression was calculated normalized against the median of the control group (nSS). FC: fold change. (**B**) CCL25 protein levels were elevated in LSG biopsy supernatants from pSS patients (n=26) compared to nSS (n=34) patients. (**C**) CCL25 levels are higher in pSS patients with anti-Ro/SSA autoantibodies. (**D**) CCL25 levels in LSG biopsy supernatants from all sicca patients (nSS + pSS) correlates with LFS. In pSS patients CCL25 levels correlate with percentage of IgM and IgG-expressing plasma cells and serum IgG levels. (**E**) In LSG biopsy supernatants CCL25 correlated significantly with IL-21 and a marker of the IL-7/IL-7Rα pathway, soluble IL-7Rα (sIL-7Rα), but not with CXCL13. P values (p) and Spearman correlation coefficients (r) are indicated.

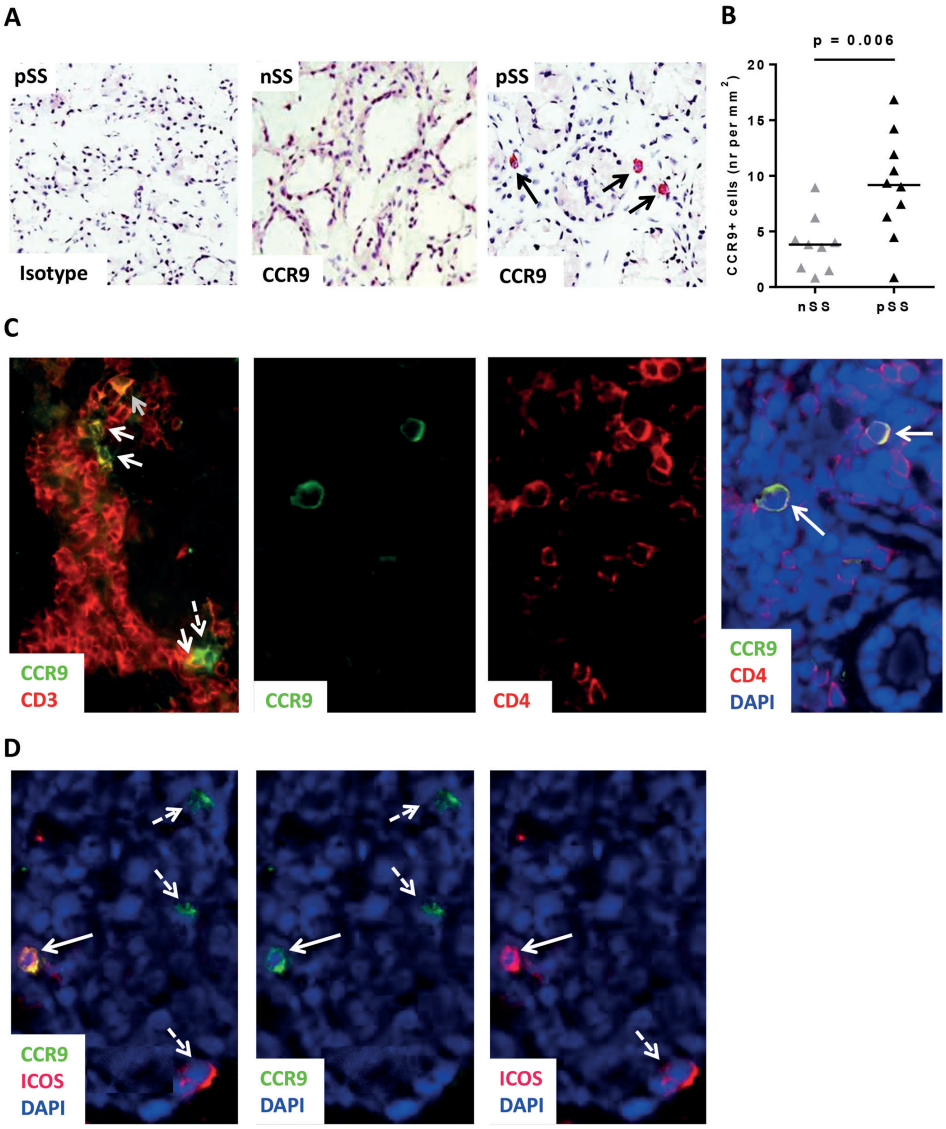

Figure 2. CCR9 expressing T-cells are increased in the salivary glands of pSS patients.

(**A**) Representative isotype control and immunohistochemical CCR9 stainings (red) of LSG tissue from an nSS patient and a pSS patient, respectively. Black arrows indicate CCR9+ cells. (**B**) CCR9 expressing cells are significantly increased in pSS (n=10) versus nSS patients (n=9). (**C**) Representative immunofluorescence images demonstrating that in a CD3 (red) T-cell rich lymphoid aggregate the majority of CCR9+ cells co-express CD3 and CD4 respectively, (yellow/orange). White arrows indicate co-localization, dashed white arrow indicates CD3-/CCR9+ cells (green). (**D**) Representative immunofluorescence staining of the LSG of a pSS patient demonstrating that CCR9+ICOS+ cells are present (CCR9: green, ICOS: red, DAPI: blue). White arrows indicate co-localization, dashed white arrows indicate single expression of CCR9 or ICOS.

Numbers of CCR9-expressing cells are enhanced in the salivary glands of pSS patients

We confirmed the finding published by McGuire et al. (11) that the CCR9+ CD4+ T-cell frequency is increased in the peripheral blood of pSS patients as compared to HC (3.0±1.3 vs 2.4±0.9%, p=0.041) and extended these findings by evaluating the numbers of CCR9+ cells in the LSG by IHC. CCR9+ cells were increased in the LSG of pSS patients as compared to nSS patients (**Figure 2A-B**) and their presence largely co-localized with CD3 and CD4 (**Figure 2C**). In addition 41.4±15.7% of CCR9+ cells expressed ICOS in the salivary gland of pSS patients (n=4 pSS) confirming the presence of functional molecules associated with B-cell activation (**Figure 2D).** CCR9+ cells were mostly found near epithelial cells, in and around lymphocytic infiltrates. No significant correlations of CCR9+ cell numbers with LFS, percentage of IgG+/ IgM+ plasma cells or serum IgG were found (data not shown).

Circulating CCR9+ Th-cells express high levels of IL-7Rα as compared to CXCR5+ Th-cells and elevated proportions of circulating CCR9+ Th-cells co-express ICOS and PD-1 in pSS

Since the IL-7/IL-7Rα pathway plays an important role in pSS pathogenesis (8,33-37) and (ectopic) GC formation, including in the LSG, expression of IL-7Rα was studied on circulating CCR9+ and CXCR5+ Th-cells from HC and pSS patients.(32,38,39) CCR9+ Th-cells showed higher median fluorescent intensity (MFI) level of IL-7Rα expression as compared to CXCR5+ Th-cells in both HC and pSS (**Figure 3A-C**).

As CCR9+ Th-cells share many features with Tfh-cells, it was of particular interest to compare them to CXCR5+ Th-cells. Expression of ICOS and PD-1, activation markers characteristically expressed on Tfh-cells, was assessed.(6) Since the majority of PD-1 and ICOS in both subsets was expressed on the CD45RO+ effector memory and central memory cells, CD45RO+CCR9+ and CD45RO+CXCR5+ Th-cells from HCs and pSS patients were compared.(40,41) Primary SS patients displayed significantly increased numbers of ICOS and PD-1 co-expressing cells in the CCR9+ Th subset compared to CCR9+ Th-cells from HC, which was not observed for CXCR5+ Th-cells. Within HC or pSS patients no statistically significant difference in ICOS+PD-1+ percentages was found between CCR9+ and CXCR5+ Th-cells (**Figure 3D-E**).

Enhanced proportions of circulating CCR9+ Th-cells produce IL-21 and IL-10 and have a Th1/Th17.1 phenotype as compared to CXCR5+ Th-cells

Intracellular cytokine staining was performed to characterize Th-cell subset polarization in total *ex vivo* peripheral blood CCR9+ and CXCR5+ Th-cells from pSS patients versus HCs (representative FACS images in **Figure 4A**). In both pSS patients and HC, high percentages of CCR9+ Th-cells produced IFN-γ (Th1 cells, not expressing IL-4 or IL-17) as compared to CXCR5+ Th-cells, (54.9±21.1% vs 12.8±4.5%, respectively, p<0.001). In addition, IFN-γ+ cells co-expressing IL-17, a typical cytokine profile for pro-inflammatory Th17.1 cells (42), were elevated in the CCR9+ Th-cell subpopulation (0.8±0.6% versus 0.1±0.1%, p<0.001).

Furthermore, cells that produce IL-21 (3.8±1.6% versus 2.3±1.3%, p=0.003) and IL-10 (2.2±1.4% vs 1.1±1.1%, p=0.002) were increased in the CCR9+ Th population as compared to CXCR5+ Th-cells. Percentages of Th17 (IL-17 producing, not IFN-γ or IL-4) and Th2 (IL-4 producing, not IFN-γ or IL-17) cells did not significantly differ (1.1±0.6 versus 0.8±0.6% and 1.3±0.7 versus 1.0±1.0%). Between pSS patients and HC no significant differences in circulating Th subset polarization were found in either CCR9+ Th-cells or CXCR5+ Th-cells (**Figure 4B**).

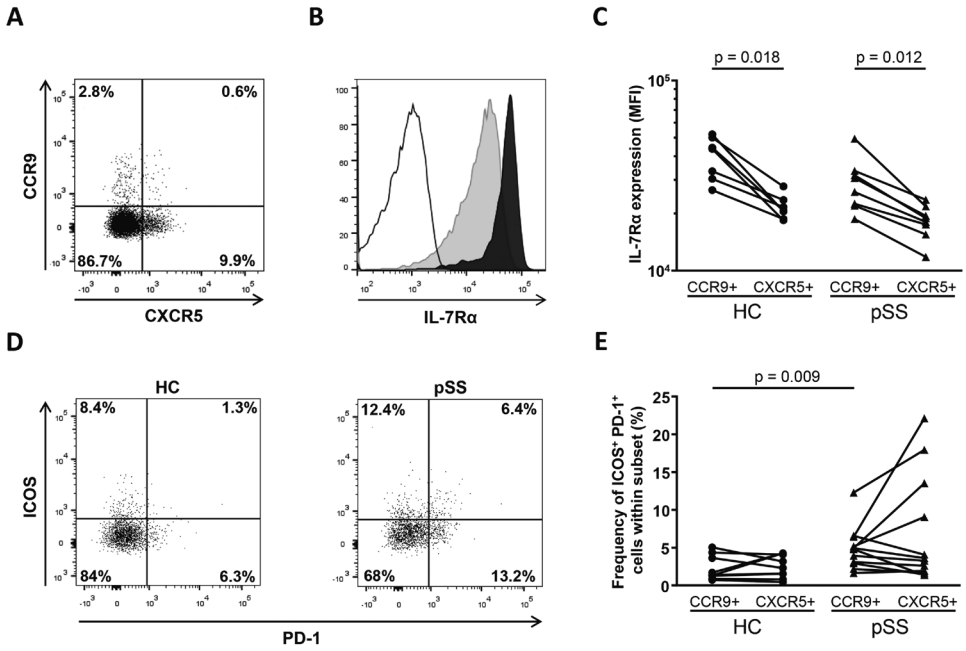

Figure 3. CCR9+ Th-cells are characterized by high levels of IL-7Rα and increased co-expression of ICOS and PD-1 in pSS patients.

(**A**) Representative flow cytometry dot plot identifying distinct CCR9 and CXCR5 expressing CD4+ T-cells. (**B**) Representative histogram of IL-7Rα expression by CCR9+ (black) versus CXCR5+ Th-cells (grey) versus unstained control (black line). (**C**) Increased expression of IL-7Rα (MFI) by CCR9+ Th-cells as compared to CXCR5+ Th-cells from both HC (n=7) and pSS patients (n=8). (**D**) Representative figure of ICOS and PD-1 expression by CCR9+ Th-cells from a healthy individual and from a pSS patient. (**E**) Percentages of ICOS and PD-1 co-expressing CCR9+ Th-cells are significantly increased in pSS patients (n=12) as compared to HC (n=9) CCR9+ and CXCR5+ Th-cells.

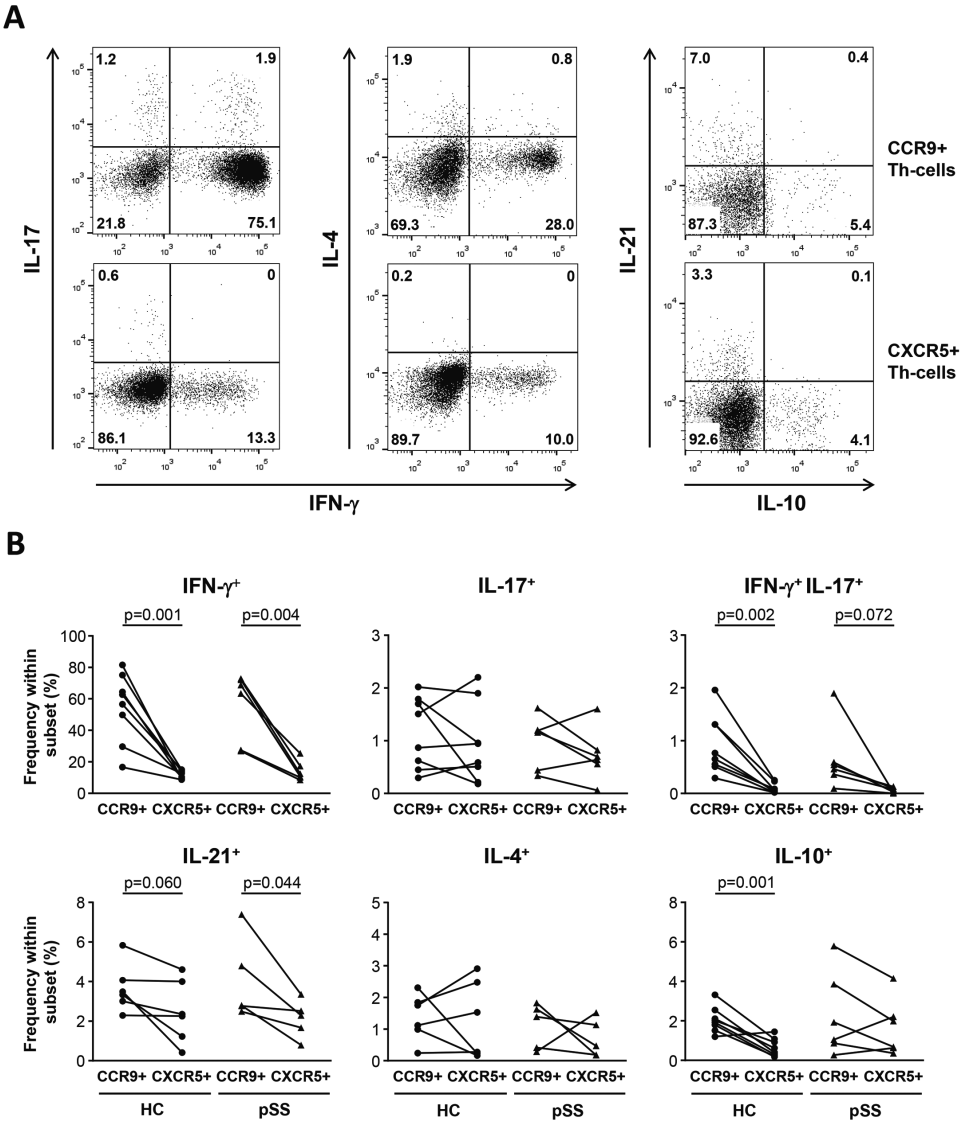

Figure 4. Enhanced proportions of ex vivo peripheral blood CCR9+ Th-cells produce IL-21 and IL-10 and have a more robust Th1 (IFN-γ+) and Th17.1 (IFN-γ+IL-17+) phenotype compared to CXCR5+ Th-cells.

(**A**) Representative flow cytometry dot plots of IL-17, IFN-γ, IL-21, IL-4 and IL-10 expression by CCR9+ Th-cells as compared to CXCR5+ Th-cells. (**B**) Percentages of CCR9+ Th-cells secreting IFN-γ, both IFN-γ and IL-17, IL-21, and IL-10 were significantly higher than those of CXCR5+ Th-cells. IL-4 and IL-17 single positive cells were not significantly different in HC or pSS patients (dots HC n=8, triangles pSS, n=6, for IL-21 staining HC n=6, pSS n=5). Percentages of cytokine secreting CCR9+ or CXCR5+ Th-cells did not differ between HC and pSS patients.

Naïve, effector, effector memory and central memory Th-cells were expected to produce different levels of cytokines. Therefore frequencies of these subsets within the CCR9+ and CXCR5+ Th-cell populations were assessed, showing that the CCR9+ Th subset contains a higher frequency of effector and effector memory cells and the CXCR5+ Th subset contains a higher frequency of central memory cells (**Supplementary Figure 1**). These subsets were distinguished based on CD45RO and CD27 expression and subsequently cytokine production was assessed. In these subsets, similar differences in cytokine production between CCR9+ and CXCR5+ Th-cells as for total CD4+ T-cells were observed. Frequencies of IFN-γ+ cells were increased in all CCR9+ subsets, frequencies of IFN-γ+IL-17+ and IL-10+ cells were increased in the naïve, effector memory and central memory CCR9+ subsets and frequency of IL-21+ cells In the naïve and central memory CCR9+ subset (**Supplementary Figure 2**).

Both memory CCR9+ and CXCR5+ Th-cells showed higer IL-7Rα expression and percentages of ICOS+PD-1+ cells than CCR9-CXCR5- naïve Th-cells (**Supplementary Figure 3**). Also, CCR9+ and CXCR5+ Th-cells that consisted primarily of central memory cells (**Supplementary Figure 1**) showed higher percentages of cytokine secreting cells (IL-21, IFN-γ, IL-17, IL-4 and IL-10) as compared to CCR9-CXCR5- Th-cells consisting mainly of naïve Th-cells (**Supplementary Figure 4**).

Antigen and IL-7-triggered CCR9+ Th-cells robustly produce IFN-γ, IL-4, IL-17 and IL-21 while antigen drives only CXCR5+ Th-cells to produce CXCL13

The function of CCR9+ Th-cells from HC and pSS patients co-cultured with monocytes were studied in view of their crucial role in immunopathology.(43) No significant differences in *in vitro* cytokine production were found between cultures with cells from HC and pSS patients, hence *in vitro* cytokine production data from HC and pSS were pooled to compare function of CCR9+ and CXCR5+ Th-cells. Upon restimulation, CCR9+ Th-cells showed elevated IFN-γ, IL-4 and IL-17 production as compared to CXCR5+ Th-cells (**Figure 5A**). IL-21 and CXCL13 production under this condition were very low or undetectable (data not shown) and IL-10 production was not different between CCR9+ and CXCR5+ Th-cells (**Figure 5A**). To investigate TCR-triggered T-cell activation, which should occur in the target organs when Th-cells enter the tissue, CCR9+ and CXCR5+ Th-cells were cultured in the presence of SEB antigen. Interestingly, CXCR5+ Th-cells stimulated with superantigen produced significantly higher levels of CXCL13 than CCR9+ Th-cells (p=0.043). However, in response to SEB, CCR9+ Th-cells produced higher levels of IFN-γ and IL-4 (both p=0.043). In addition, CCR9+ Th-cells produced higher levels of IL-17, IL-21 and IL-10 as compared to CXCR5+ Th-cells, although this did not reach statistical significance (**Figure 5B**).

IL-7R-mediated stimulation was also investigated because of its role in pSS immunopathology (8,33-37), the strong correlation of sIL-7Rα with CCL25 and the high IL-7Rα expression on CCR9+ Th-cells. Stimulation with IL-7 induced CCR9+ Th-cells to produce significantly higher

amounts of IFN-γ, IL-4 and IL-21 as compared to CXCR5+ Th-cells (p=0.018, p=0.018, p=0.028, respectively). In addition, a trend towards increased production of IL-17 was found (p=0.091). CXCL13 production was not significantly induced and IL-10 production did not significantly differ between CCR9+ Th and CXCR5+ Th cultures upon IL-7 stimulation (**Figure 5A-B**).

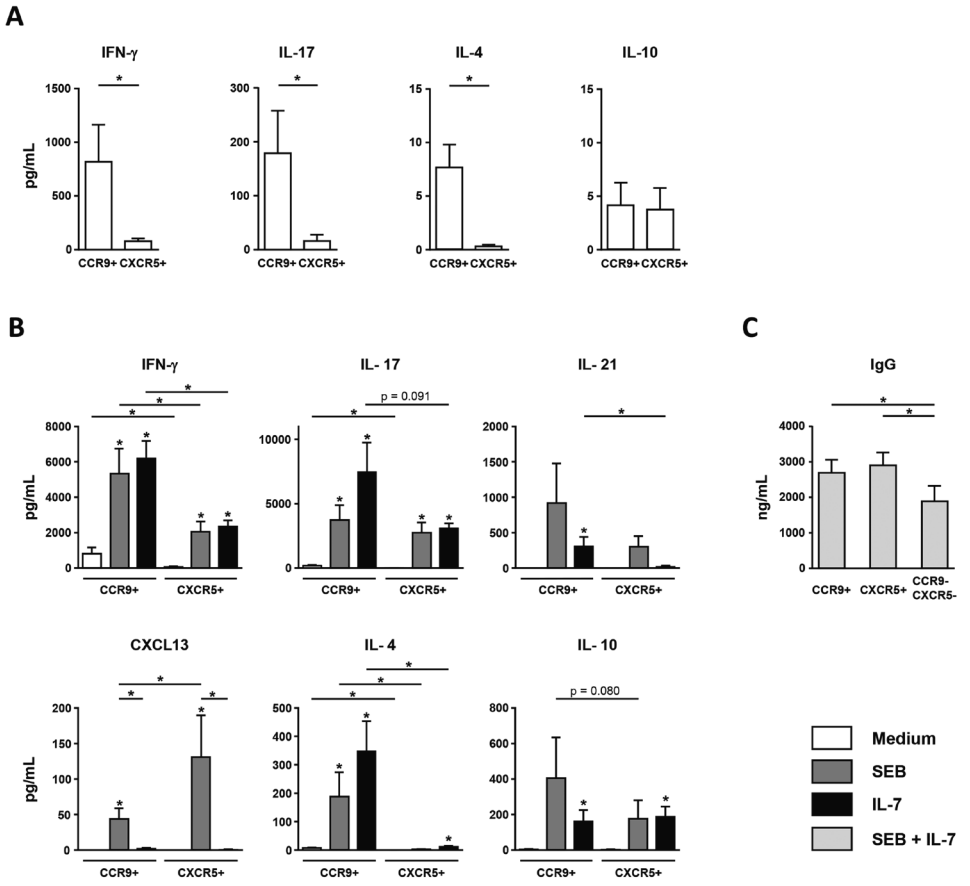

Figure 5. CCR9+ Th-cells robustly produce IFN-γ, IL-17, IL-4 and IL-21 upon antigen and IL-7 challenge while antigen-triggered CXCR5+ Th-cells more potently produce CXCL13. Both CCR9+ and CXCR5+ Th-cells have increased capacity to stimulate IgG production.

(**A**) CCR9+ and CXCR5+ Th-cells (2.104, n=7, HC n=4, pSS n=3) were co-cultured with monocytes (5.103) for 3 days and restimulated with PMA and ionomycin. Culture of CCR9+ Th-cells without stimuli showed increased IFN-γ, IL-17 and IL-4 production as compared to CXCR5+ Th-cells. (**B**) CXCR5+ Th-cells stimulated with superantigen (Staphylococcal Enterotoxin B (SEB), n=5; HC n=3, pSS n=2) produced significantly more CXCL13 than CCR9+ Th-cells. However, in response to SEB CCR9+ Th-cells produced higher levels of IFN-γ and IL-4. Stimulation with IL-7 (n=7; HC n=4, pSS n=3) induced CCR9+ Th-cells to produce significantly more IFN-γ, IL-21 and IL-4 as compared to CXCR5+ Th-cells. (**C**) CCR9+, CXCR5+ and CCR9-CXCR5- Th-cells (2.104, HC n=6) were co-cultured with B cells (2.105) and monocytes (5.103) for 12 days in the presence of IL-7 and SEB. IgG levels were measured in the supernatants. In the cultures with CCR9+ and CXCR5+ Th-cells IgG production was significantly higher as compared to cultures with CCR9-CXCR5- Th-cells.

CCR9+ and CXCR5+ Th-cells more potently induce IgG production by B cells than CCR9-CXCR5- Th-cells

Since both CCR9+ and CXCR5+ Th-cells have been described to potently induce antibody responses by B-cells, co-culture of these cells with B-cells was performed.(6,14) Stimulation with IL-7 and SEB was performed to activate the Th-cells. Both CCR9+ and CXCR5+ Th-cells induced IgG production, which was significantly increased as compared to CCR9-CXCR5- Th-cells (p=0.025 and p=0.013, respectively) (**Figure 5C**).

Discussion

For the first time, the present study demonstrates increased levels of CCL25 and elevated numbers of CCR9 expressing cells in the salivary glands of pSS patients. Elevated CCL25 levels correlate with lymphocytic focus scores, B-cell hyperactivity, autoimmunity (presence of anti-Ro/SSA autoantibodies) and mediators potentially involved in lymphoid neogenesis (IL-21 and sIL-7Rα). Circulating CCR9+ Th-cells from pSS patients displayed increased ICOS and PD-1 expression. In addition, we demonstrate that CCR9+ Th-cells have increased IL-7Rα expression compared to CXCR5+ Th-cells and that increased proportions of CCR9+ Th-cells, *ex vivo* and in response to antigen and IL-7, produce higher levels of IFN-γ, IL-4, IL-17 and IL-21 than CXCR5+ Th-cells. The latter subset in turn produces more CXCL13. Both CCR9+ and CXCR5+ Th-cells more potently stimulated IgG production by B-cells than CCR9-CXCR5- Th-cells. Altogether, these findings suggest a role for the CCL25/CCR9-axis in pSS pathogenesis, in a manner that is distinct from CXCR5+ Th-cells.

Both CCR9-expressing Th-cells from HCs and pSS patients (data not shown) migrate in response to CCL25.(16,26,44) CCL25 is found in the intestinal mucosa of both HC and patients with IBD and is primarily produced by epithelial cells.(21,26) However, CCL25 expression has not been detected in healthy LSG.(26) Here, we show increased mRNA and protein expression of CCL25 in the salivary glands of pSS patients as compared to nSS controls and consistent with CCL25 production by epithelial cells, we mainly observed CCR9+ cells in the vicinity of epithelial cells. Although the trigger for CCL25 induction in the salivary gland of pSS patients is unknown, studies in cell lines and mice demonstrated that viral and bacterial infections and tissue damage can up-regulate CCL25 expression in mucosal tissues.(28,29,45-48)

We and others have shown that incubation of CCR9+ Th-cells with CCL25 dramatically reduces CCR9 cell surface expression ((44,49), and data not shown). This might explain why we found a low frequency of CCR9+ Th-cells at the site of inflammation without significant correlations with LFS. The lack of detection of CCR9 mRNA expression may be due to chemokine receptor expression characteristics. Since chemokine receptor genes are known to be low copy number genes and the chemokine receptor proteins are recycled on the cell

membrane, mRNA expression of chemokine receptors is often low and often not directly correlated with the protein present.(50) In support of a role for the CCL25/CCR9-axis in pSS, we demonstrated significant correlations of CCL25 in LSG supernatants with LFS, B-cell hyperactivity and autoimmunity in pSS patients. In line with this, increased expression of PD-1 and ICOS on CCR9+ Th-cells in pSS was observed, indicating activation and potentially superior interaction with B-cells. Finally, in this study we demonstrate the strong capability of CCR9+ Th-cells to produce high levels of IFN-γ, IL-4, IL-10, IL-17 and IL-21 and to stimulate increased IgG production by B-cells. Together this suggests that these cells play a role in activation of B-cells in pSS. Expression of ICOS and PD-1 and production of cytokines IL-4 and IL-21 by Tfh-cells have been shown to play important roles in germinal center processes inducing long-term, high affinity B-cell responses.(5,6) The Tfh-like role for CCR9+ Th-cells is supported by the finding that they are potent inducers of antibody production.(14) A role for the CCL25/CCR9 Th cell-axis in autoimmunity and immunopathology in pSS is further supported by the increased frequencies of CCR9+ Th-cells in blood of pSS patients and the capacity of CCR9+ Th-cells to induce experimental sialadenitis, glandular pathology and autoimmunity.(11) Hence, despite their low numbers, increased numbers of CCR9+ T-cells in the salivary gland are shown to play a pivotal role in autoimmunity by production of high levels of cytokines and capacity to stimulate B-cells.(14)

Corresponding with previous studies that focused on CCR9+ Th-cells in HC, in pSS patients we now show that *ex vivo* CCR9+ Th-cells produce high levels of Th1 and Th17 cytokines, IFN-γ and IL-17 as compared to CXCR5+ Th-cells.(14,15) In addition, we demonstrate in pSS patients that CCR9+ Th-cells producing IL-10 are enriched.(14) Here we for the first time demonstrate that human CCR9+ Th-cells encompass enriched proportions of IL-21 producing cells, as well as the pro-inflammatory Th17.1 subset, which produces both IFN-γ and IL-17.

In vitro CCR9+ Th-cells were superior IFN-γ, IL-4, IL-17 and IL-21 producers as compared to CXCR5+ Th-cells, depending on the stimulus. Upon TCR triggering, elevated IFN-γ and IL-4 production by CCR9+ Th-cells was found, but CXCR5+ Th-cells were superior CXCL13 producers. Interestingly, IL-7 induced activation caused strong up-regulation of all cytokines measured, except for CXCL13, and CCR9+ Th-cells produced significantly elevated levels of IFN-γ, IL-4 and IL-21 compared to CXCR5+ Th-cells. In addition, we demonstrate a strong correlation of CCL25 and sIL-7Rα, which recently was shown to strongly enhance IL-7-induced immune activation *in vivo*.(51) Considering this and the pivotal role of the IL-7/IL-7Rα-pathway in lymphoid neogenesis in salivary glands, IL-7-driven activation of CCR9+ Th-cells may play a pivotal role in this process.(32)

Our results show that CCL25 does not correlate with CXCL13 in the LSG and that CCR9+ Th-cells do not produce high levels of CXCL13 as compared to CXCR5+ Th-cells. In addition, CCR9+ Th-cells are much more potent cytokine producers in response to IL-7 than CXCR5+

Th-cells. These findings in combination with the Tfh-like characteristics of CCR9+ Th-cells indicate a distinct role for the IL-7/IL-7R-associated CCL25/CCR9-axis versus the CXCL13/CXCR5-axis in B-cell activation and lymphocytic infiltration including germinal center-like structure formation in pSS patients.

Both in the *ex vivo* and *in vitro* experiments, we demonstrated equally potent cytokine production by CCR9+ Th-cells from pSS patients and HCs. In view of the increased expression of PD-1 and ICOS on CCR9+ Th-cells from pSS patients, this is still unexplained. Currently we are investigating co-expression of other chemokine receptors on CCR9+ Th-cells to support the hypothesis that subsets of CCR9+ Th-cell migrate in response to co-expressed chemokine receptors, hence explaining lack of increased circulating cytokine secreting cells.

In conclusion, elevated expression of CCL25 and increased numbers of CCR9+ cells in the LSG of pSS patients correlate with clinical features including lymphocytic infiltration in the LSG, B-cell hyperactivity and autoimmunity. Peripheral blood CCR9+ Th-cells from pSS patients displayed increased ICOS and PD-1 expression. When compared to CXCR5+ Th-cells, the CCR9+ Th-cells are IL-7Rα^{high} and produce elevated levels of IFN-γ, IL-4, IL-17 and IL-21, especially upon IL-7R-mediated stimulation. Considering the pivotal role of CCR9+ T-cells in an experimental Sjögren-like model, our findings suggest an important role for the CCL25/CCR9-axis in pSS immunopathology, representing a novel therapeutic target in this disease.

Acknowledgements

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Supplementary material

Supplementary File 1. Materials and methods.

Gene expression analysis

Frozen LSGs embedded in Tissue-Tek (Sakura) were cut into sections of 20μM and RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions and quantified using Qubit (Qiagen). *CCL25* and *CCR9* expression were assessed by qRT-PCR using 25ng RNA per reaction. *18S* was used as a housekeeping gene, expression was calculated using the $2^{-\Delta\Delta CT}$ method normalized against the median of the control group.

Immunohistochemistry and immunofluorescence

Frozen 6μm sections of LSG tissue were fixed with acetone, air-dried and washed with PBS. Sections were incubated with anti-CCR9 mAb (R&D Systems, monoclonal mouse IgG2a, clone 248621) in PBS with 1% BSA and 1% goat serum for 1h at room temperature after blocking with 10% goat serum for 30 minutes. Control stainings were performed using a mouse IgG2a isotype control or by omitting the first antibody. Thymus tissue was used as positive control for CCR9 staining. Antigen–antibody complexes were visualized using the BrightVision poly-HRP detection system (ImmunoLogic). Slides were briefly washed in a Tris-HCl pH 8.4 solution. The reactivity was then visualized with new fuchsin in solution to which levamisole was added. Subsequently, slides were washed in demineralized water, counterstained with haematoxylin solution according to Mayer and washed in running tap water. Finally, sections were air dried and mounted on coverslips.

For immunofluorescence stainings, sections were prepared in the same manner including blocking and fixing, and the same anti-CCR9 mAb was used. For co-localization experiments, anti-CD3 (polyclonal rabbit, Dako), anti-CD4 mAb (monoclonal rabbit, Cell Marque) and anti-ICOS (monoclonal rabbit, AbCam) were used. Secondary anti-mouse and anti-rabbit antibodies labelled with AF488 and AF555, respectively, were used (Life Technologies). Additionally, VectaShield containing DAPI was used for nuclear staining. Images were visualized using a Leica fluorescence microscope.

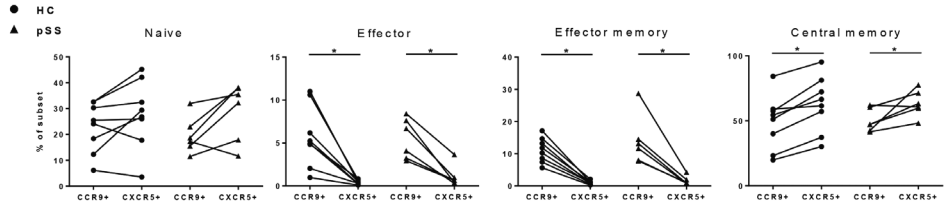
Flow cytometry

To analyze extracellular and intracellular markers, cells were characterized by flow cytometry using a LSRFortessa flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software version 10 (Tree Star Inc).

To characterize CCR9 Th-cells, 1.10^6 PBMCs from healthy donors and patients were stained with anti-CD3 AF700, anti-CD45RO FITC, anti-CXCR5 PerCP-Cy5.5, anti-CD127 (IL-7Rα) BV605 (BioLegend), anti-CCR9 PE (R&D, clone 248621), anti-CD4 APC-ef780, anti-ICOS APC (eBioscience) and anti-PD-1 BV711 (BD). Cells were stained for 30 minutes at 4°C in PBS containing 1% BSA and 0.1% sodium azide.

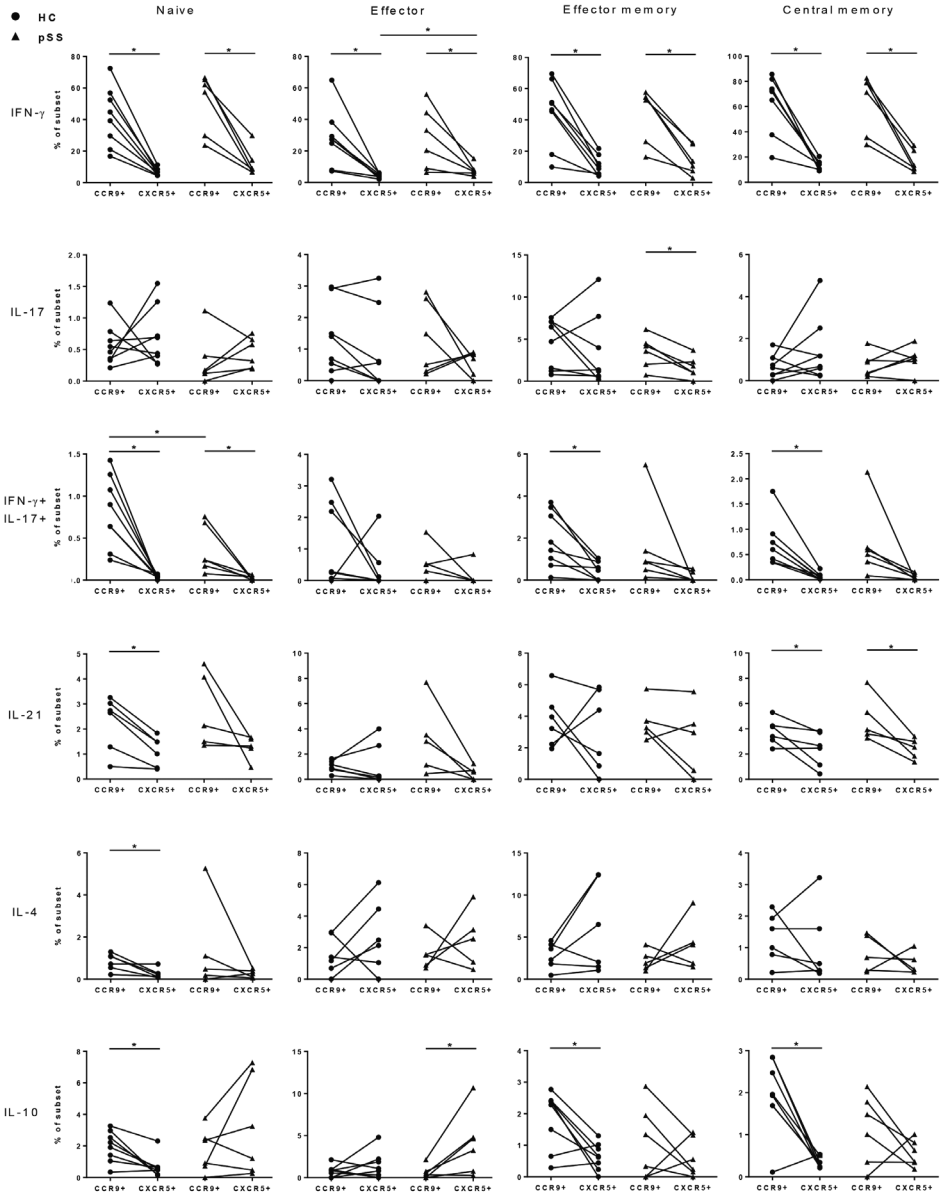
For intracellular cytokine staining 1.10^6 CD4 T-cells/mL were stimulated with PMA and ionomycin in the presence of brefeldin A for 4h at 37°C in 24-wells plates in RPMI 1640 Glutamax medium (Gibco BRL, Life Technologies) containing 1% penicillin and streptomycin and 10% fetal calf serum. After culture, extracellular staining was performed for 15 minutes at room temperature and after fixation and permeabilization intracellular staining was performed for 15 minutes at room temperature. The following antibodies were used, divided into two panels: anti-CD3 AF700, anti-CD4 BV785 , anti-CD45RO BV711, anti-CD127 BV605, anti-IL-21 APC (BioLegend), anti-CCR9 PE (R&D), anti-CXCR5 BV421, anti-IL-10 PE-CF594 (BD Horizon), anti-CD27 eFluor780, anti-IFN-γ PE-Cy7, anti-IL-17a FITC and anti-IL-4 APC (eBioscience).

Supplementary figures



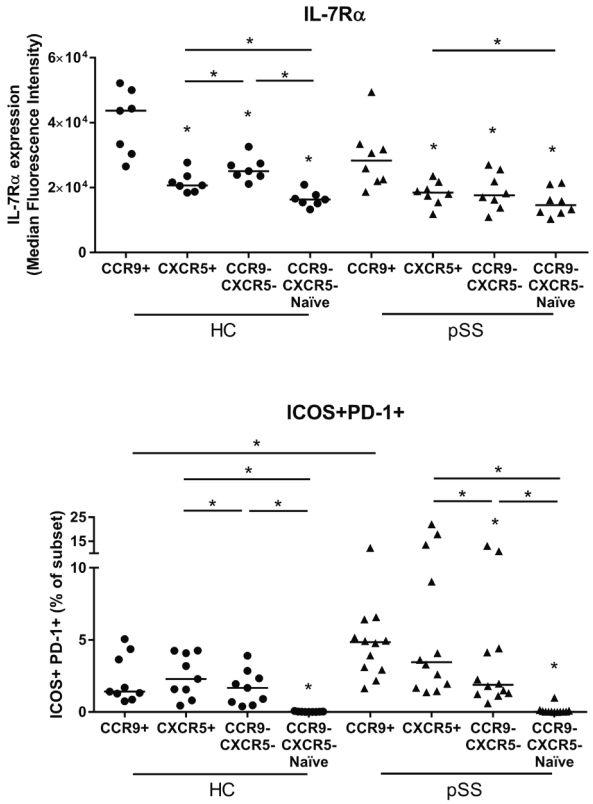
Supplementary Figure 1. Frequencies of naïve, effector, effector memory and central memory cells within the CCR9+ and CXCR5+ CD4+ Th subsets.

Naïve, effector, effector memory and central memory Th-cells within the CCR9+ and CXCR5+ Th subsets were distinguished based on CD27 and CD45RO expression (n=8 HC, n=6 pSS). Naïve: CD45RO-CD27+; effector: CD45RO-CD27-; effector memory: CD45RO+CD27-; central memory: CD45RO+CD27+.



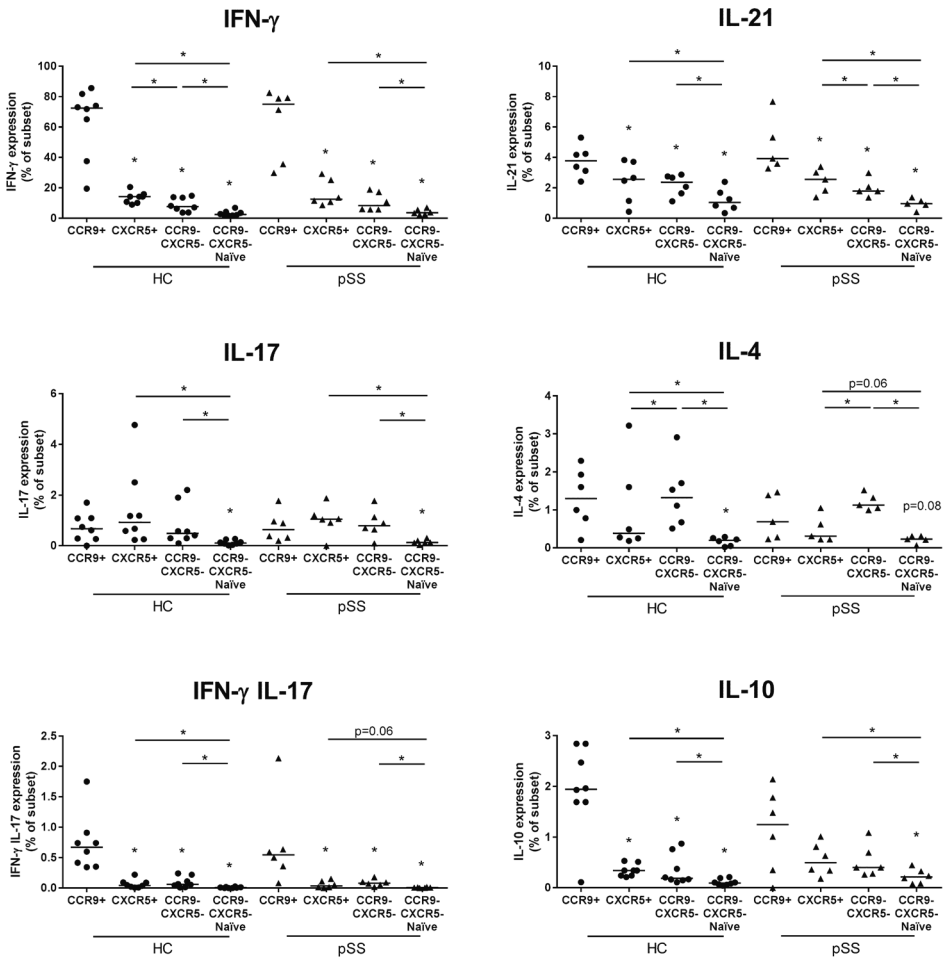
Supplementary Figure 2. Intracellular cytokine staining of naïve, effector, effector memory and central memory CCR9+ and CXCR5+ CD4+ Th-cells.

Intracellular cytokine staining by flow cytometry was performed to assess cytokine expression by naïve, effector, effector memory and central memory CCR9+ and CXCR5+ Th-cells from n=8 HC and n=6 pSS. Percentages of IFN-γ+, IL-17+, IFN-γ+IL-17+, IL-21+, IL-4+ and IL-10+ cells within these subsets are shown.



Supplementary Figure 3. Increased IL-7Rα expression and ICOS+PD-1+ proportions in CCR9+ and CXCR5+ Th-cells as compared to CCR9-CXCR5- Th-cells.

Ex vivo expression of IL-7Rα and ICOS and PD-1 was assessed by flow cytometry and are shown for CCR9+, CXCR5+ and CCR9-CXCR5- memory Th-cells and CCR9-CXCR5- naïve Th-cells in HC (n=7) and pSS patients (n=8).



Supplementary Figure 4. Increased percentages of IFN-γ IFN-γ/IL-17, IL-17, IL-21 and IL-10 secreting cells in CCR9+ and CXCR5+ Th-cells as compared to naïve CCR9-CXCR5- Th-cells.

Intracellular cytokine staining by flow cytometry was performed to assess ex vivo cytokine expression by CCR9+, CXCR5+ and CCR9-CXCR5- Th-cells from n=8 HC and n=6 pSS. Percentages of IFN-γ+, IL-17+, IFN-γ+IL-17+, IL-21+, IL-4+ and IL-10+ cells within central memory CCR9+, CXCR5+, CCR9- CXCR5- and naïve CCR9-CXCR5- subsets are shown.

CHAPTER 7

Dysregulated transcriptome of CCR9-expressing pathogenic T helper cells from primary Sjögren's syndrome patients identifies CCL5 as a novel effector molecule

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Manuscript in preparation

Abstract

Introduction. CCR9+ Tfh-like pathogenic Th-cells and their ligand CCL25 are elevated in patients with primary Sjögren's syndrome (pSS) and indicated to play an important 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 were FACS-sorted from blood of 7 healthy individuals and 7 pSS patients and RNA sequencing was performed. Computational analysis was used to identify differentially expressed genes (DEG), coherent gene expression networks and differentially regulated pathways. Target genes were replicated in additional cohorts by qPCR, flow cytometry and functional experiments.

Results. In the CCR9+ Th-cell subset 2777 DEGs were identified between healthy controls and pSS patients, and 1416 and 1077 in the CXCR5+ and CCR9-CXCR5- subsets, respectively. Using network analysis 15 modules or clusters of genes were constructed, consisting of genes showing coherent expression patterns. Modules of interest were selected based on eigengene expression and functional relevance. These modules contained pathways involved in e.g. migration and adhesion, cytokine and chemokine production and proliferation. Selected DEGs of interest within one specific network that were upregulated in CCR9+ Th cells were validated, including *HOPX, SOX4, ITGAE, ITGA1, NCR3, ABCB1, C3AR1, NT5E, CCR5* and *CCL5*. Ex vivo, CCR9+ Th cells produced significantly higher levels of CCL5 than CXCR5+ and CCR9-CXCR5- Th cells and this was strongly induced upon IL-7 and antigen triggering.

Conclusion. Transcriptomic analysis of CCR9+ Th-cells reveals multiple pathways involved in effector T-cell function, including transcription factors involved in Th1 function *HOPX* and *SOX4*, chemokine *CCL5*, chemokine receptor *CCR5*, adhesion molecules *ITGAE* and *ITGA1*, complement receptor *C3AR1,* cytotoxicity receptor *NCR3*, and multidrug resistance gene *ABCB1*. These key molecules indicate novel pathogenic properties of CCR9+ Th-cells.

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 and B cells.(1-3) 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.(4)

T follicular helper (Tfh) cells are potent B cell stimulating cells and reside in GCs in lymph nodes.(5) In addition, Tfh cells are elevated in salivary glands and peripheral blood of pSS patients correlating with autoantibodies, disease severity and aberrant memory B cell and plasma cell subsets.(6-8) Recently, a novel "Tfh-like" cell subset was described that shares characteristics with Tfh-cells including IL-21 production, ICOS and Bcl-6 expression but these cells express CCR9 instead of CXCR5.(9) CCR9+ Th-cells are present in secondary lymphoid organs of both mice and humans.(10,11) In humans, these CCR9+ Th-cells produce high levels of IFN-γ in addition to IL-17, IL-10 and IL-4 and strongly induce B-cell responses.(10,12,13) They specifically migrate to mucosal sites in response to the chemokine CCL25.(14,15) CCR9+ Th-cells are important for mucosal immune homeostasis but also may have a function in mucosal inflammation, potentially contributing to inflammatory bowel disease (IBD).(16,17) 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.(18,19) Inhibition of CCR9 decreased 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 therapy.(20-22)

CCL25 mRNA is not detectable in healthy human salivary gland tissue, but is up-regulated during oral inflammation.(23) In mice, CCL25 gene expression is upregulated in the oral mucosa upon antigenic triggering and during wound healing.(24,25) 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.(9) 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.(9,26) Interestingly, the CCR9+ Th cell subset shares characteristics with the newly described pathogenic T 'peripheral helper' (Tph) cells (ICOS+PD-1+CXCR5-) which drive B cells in rheumatoid arthritis patients.(27) Circulating CCR9+ Th cells from pSS patients express elevated percentages of ICOS and PD-1. 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.(28-35) To investigate which processes may drive CCR9+ Th cell pathogenicity, we performed transcriptomic profiling of circulating CCR9+ Th cells from healthy controls and pSS patients. Here we demonstrate the dysregulated transcriptome of CCR9-expressing pathogenic T helper cells from primary Sjögren's syndrome patients and identify CCL5 as a novel effector molecule of CCR9+ Th cells.

Methods

Patients and healthy controls

For cell sorting and subsequent RNA sequencing, n=7 healthy controls (HC) and n=7 pSS patients were included. For qPCR validation, flow cytometry and in vitro validation n=18 HC and n=9 pSS patients, n=23 HC and n=22 pSS patients, and n=4 HC and n=3 pSS patients were included, respectively. All pSS patients were diagnosed by a rheumatologist and met the American-European Consensus Group (AECG) criteria and the ACR-EULAR criteria.(36,37) All healthy volunteers and pSS patients were included in the University Medical Center (UMC) Utrecht, demographic and clinical data are shown in **Table 1**. The UMC Utrecht ethical committee approved the study and all participants gave written informed consent.

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

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.

Flow cytometry and cell sorting

Fresh peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. 70-100mL of blood was used, containing 50-120 mln PBMCs, which were incubated with fluorochrome-conjugated antibodies against CD3, CD4, CXCR5 and CCR9 (**Supplementary table 1**). CCR9+CXCR5-, CXCR5+CCR9- and CCR9-CXCR5- (further also called 'double negative' (DN)) Th cells were sorted by flow cytometry using BD FACSARIAIII into tubes with RPMI 1640 containing 10% FCS and penicillin/streptomycin (gating strategy, **Figure 1A**). Cells were lysed in RLT+ (Qiagen) with beta-mercaptoethanol.

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 Table 1**). ABCB1 was stained intracellularly using the Fixation-Permeabilization protocol from eBioscience.

RNA sequencing and computational analyses

RNA was extracted from the sorted Th cell subsets from HC and pSS using QIACube (Qiagen). Two CCR9+ Th cell samples from healthy controls had to be excluded for quality control reasons. RNAseq 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 and the sequencing reads were aligned to human genome (GRCh38 build 79) using STAR aligner.(38) 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.(39) For differential expression between Th subsets, paired analyses were performed (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. We accumulated all the differentially expressed genes (DEGs) from all comparisons for further analyses. 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 WGCNA (weighted gene correlation network analysis) modules were constructed of genes showing coherent eigengene expression. Using the DEG assigned to modules, 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, module membership, size of differential expression between Th subsets and between HC and pSS (see description in **Figure 3**).

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, *SOX4* Hs04987498_s1 and *HOPX* Hs04188695_m1, and *B2M* Hs00187842_m1 was used as housekeeping gene, ThermoFisher, LifeTechnologies) using RNA extracted from FACS sorted CCR9+, CXCR5+ and CCR9-CXCR5- Th cell subsets from HC and pSS 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 (Life Technologies). qPCR reactions were performed using Quantstudio. mRNA expression values were calculated according to the comparative threshold cycle, using *B2M* as endogenous control and calculating the fold change (FC) versus the mean of the CCR9-CXCR5- Th cell subset from the HC as a reference set at 1.

Culture

For analysis of cytokine secretion, 2.10^4 CCR9+, CXCR5+ or CCR9-CXCR5- Th-cells were cultured with 5.10^5 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 Supernatants were measured by Luminex technology as previously described.(40)

Statistical analysis

RNA sequencing data were analyzed as described above. Flow cytometry and cytokine data were analyzed using Graphpad Prism 6 and IBM SPSS Statistics 21. Student's t-test, paired parametric t-test, Mann-Whitney U test and Wilcoxon non-parametrical paired test were used where appropriate. 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- (further also called '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 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 indicate that 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

We used WGCNA to cluster DEGs into 15 different modules each containing a set of genes exhibiting coherent expression patterns. We label each module by a color name. We further selected modules with the consensus expression pattern (eigengene expression) of interest, for example, modules that contained genes which were 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**).

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, mainly comprised genes involved in metabolism, transcription and translation and interferon signaling (cyan, purple, and darkgreen, respectively) (**Figure 2D**).

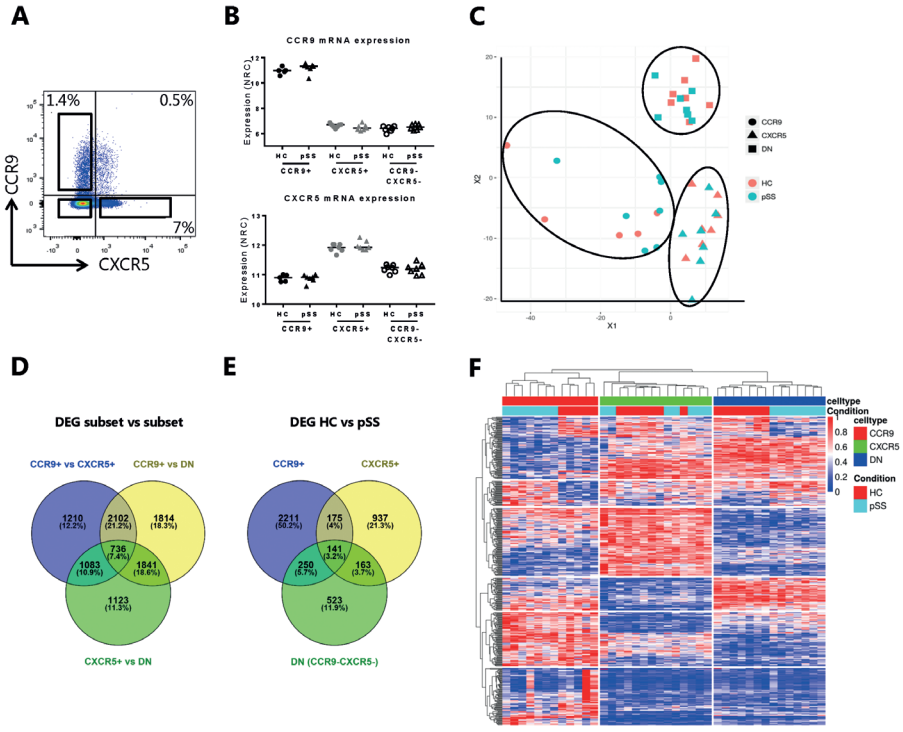

Figure 1. Transcriptomic profiling robustly separates CCR9+, CXCR5+ and CCR9-CXCR5- Th cell subsets and identifies differentially expressed genes in pSS patients.

RNA-sequencing was performed on FACS-sorted CCR9+, CXCR5+ and CCR9-CXCR5- (further also called '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). (**A**) Representative flow cytometry plot of CCR9 and CXCR5 expression on CD4+ Th cells used for FACS sorting. (**B**) Confirmation of elevated mRNA expression of CCR9 in the sorted CCR9+ Th cells and CXCR5 expression in CXCR5+ Th cells, respectively, supports the experimental procedure. NRC: normalized read counts, log2 normalized. (**C**) Multidimensional scaling plot (MDS) showing that the Th cell subsets are robustly distinguished by their transcriptomes. DEGs between the subsets are used for this analysis. (**D**) Venn diagram shows differentially expressed genes (DEGs) between the CCR9+ vs CXCR5+ vs DN Th cell subsets. (**E**) Venn diagram showing numbers of DEG between HC and pSS CCR9+, CXCR5+ and CCR9-CXCR5- Th cell subsets, based on nominal p-values. (**F**) Heatmap representation of hierarchical clustering analysis using the top 100 DEGs of all comparisons, showing distinct gene expression profiles between the subsets and between HC and pSS.

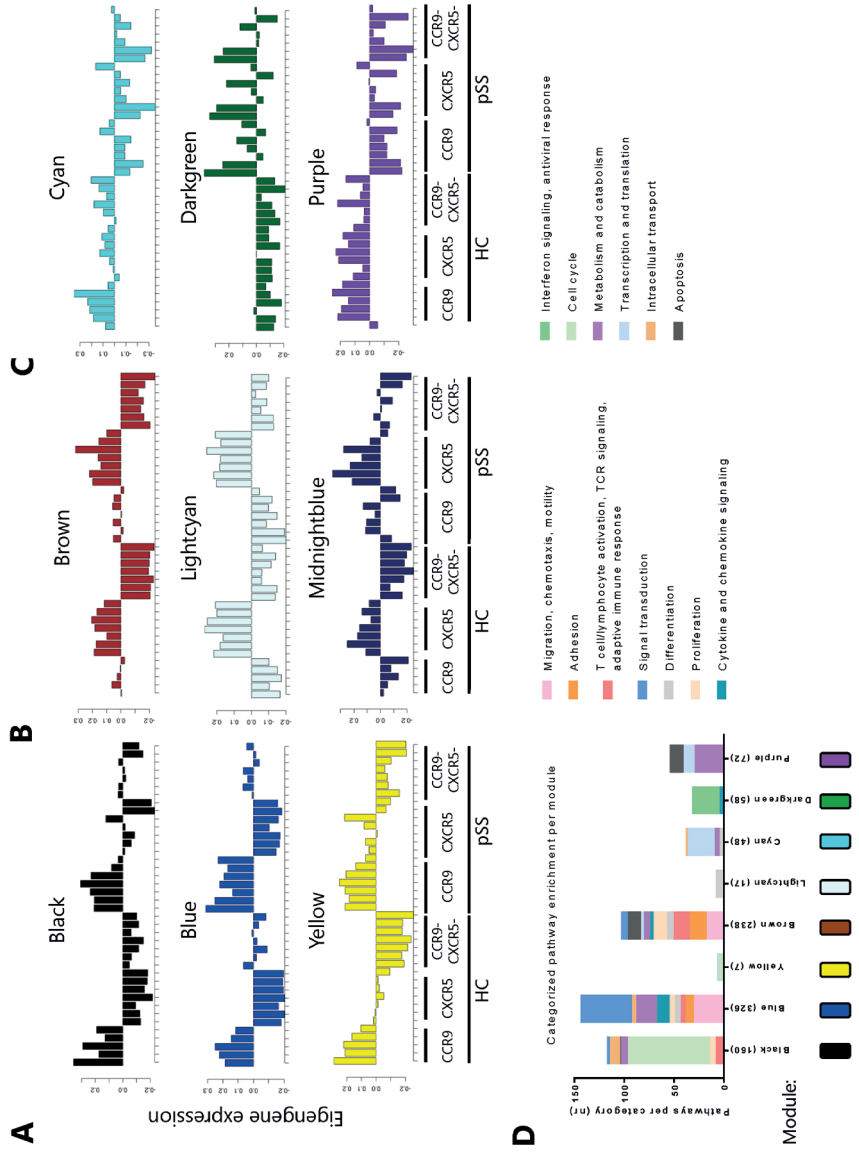

Figure 2. WGCNA analysis reveals networks of genes with coherent expression patterns involved in distinct Th cell functions.

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 (**C**) are selected. Each bar represents the eigengene expression of one sample of the indicated Th cell subset of a donor. Two CCR9+ Th cell samples from healthy controls had to be excluded for quality control reasons. (**D**) Pathway enrichment analysis reveals different pathways contained in the modules. Pathways are categorized, 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. In the midnightblue module no enrichment for pathways was found, therefore it is excluded from the graph. WGCNA: weighted correlation network analysis.

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 positive correlation of the expression pattern of a gene with the eigengene expression of the module, 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**.

From this selection, we next aimed to identify genes upregulated in CCR9+ Th cells present in coherent gene networks, which showed differential expression between HC and pSS and may play an important role in Th cell function. 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, showing the largest number of pathways crucial for effector Th cell function. 9 target genes out of 12 (**Figure 3A**) that were also differentially expressed in HC vs pSS and were shown to have known function based literature evidence, were subsequently selected. These included two transcription (co) factors *HOPX* and *SOX4*, and in addition *ITGAE, NT5E, C3AR1, CCL5, ITGA1* and *ABCB1*. Additionally from the 32 genes identified in the blue module that were not differentially expressed in HC vs pSS, *CCR5* and *NCR3* were selected given their effector function and potential role in pSS(41-43). 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, NT5E* (CD73), *NCR3* (NKp30), *CCR5* and, *ABCB1* (MDR-1, P-glycoprotein) (CCR9+ vs CXCR5+ and CCR9-CXCR5- all p<0.05, **Figure 4C** and **D**, representative flow cytometry images are shown in **Supplementary figure 2**). However, the differential expression of these genes between CCR9+ Th cells from HC versus pSS patients were not confirmed in the additional cohort on mRNA or protein level.

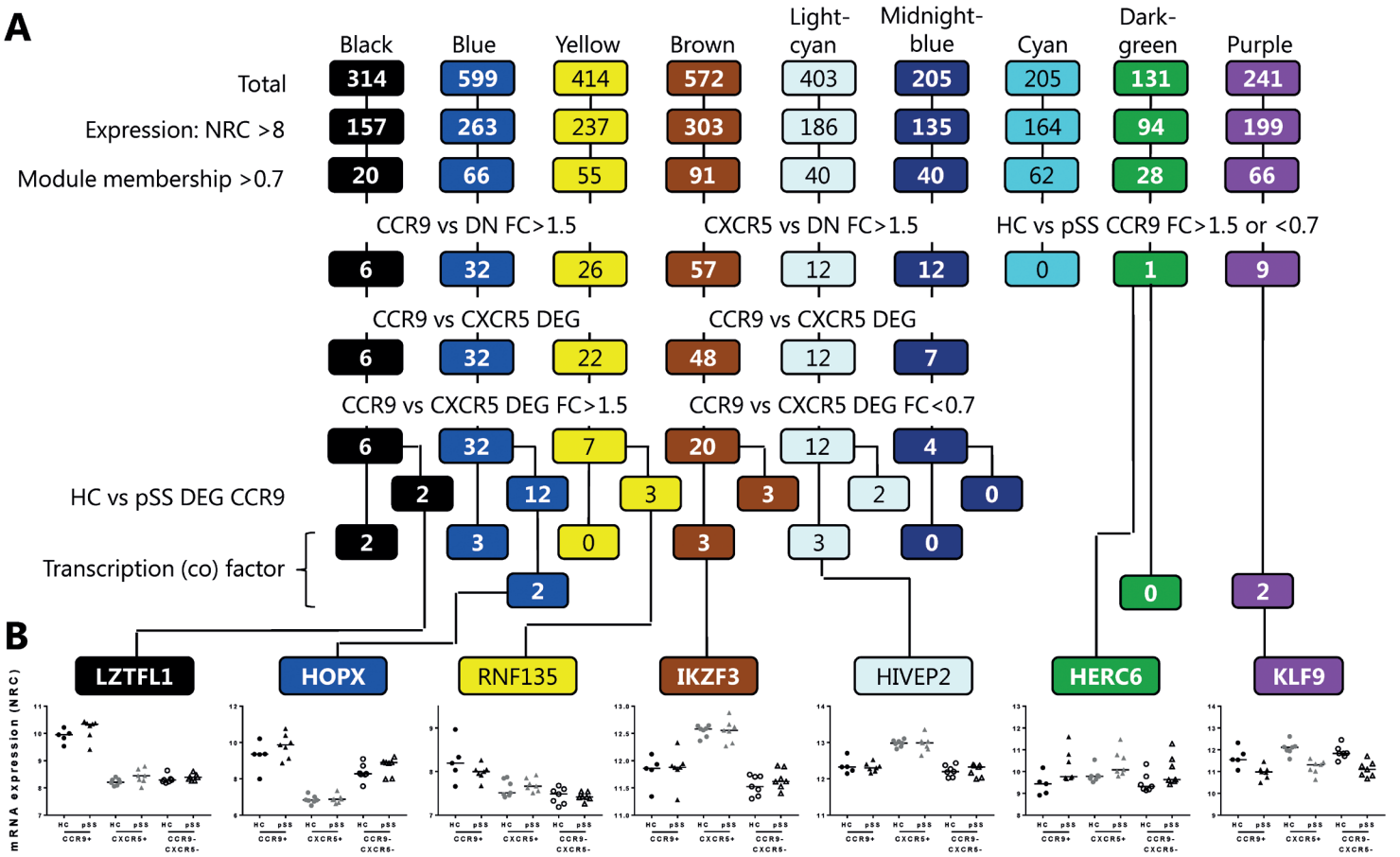

Figure 3. Selection of target genes for validation per module of interest.

(**A**) 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 are selected that are also supported by literature evidence. (**B**) Examples of gen Examples of genes selected based on the criteria and per module are shown.

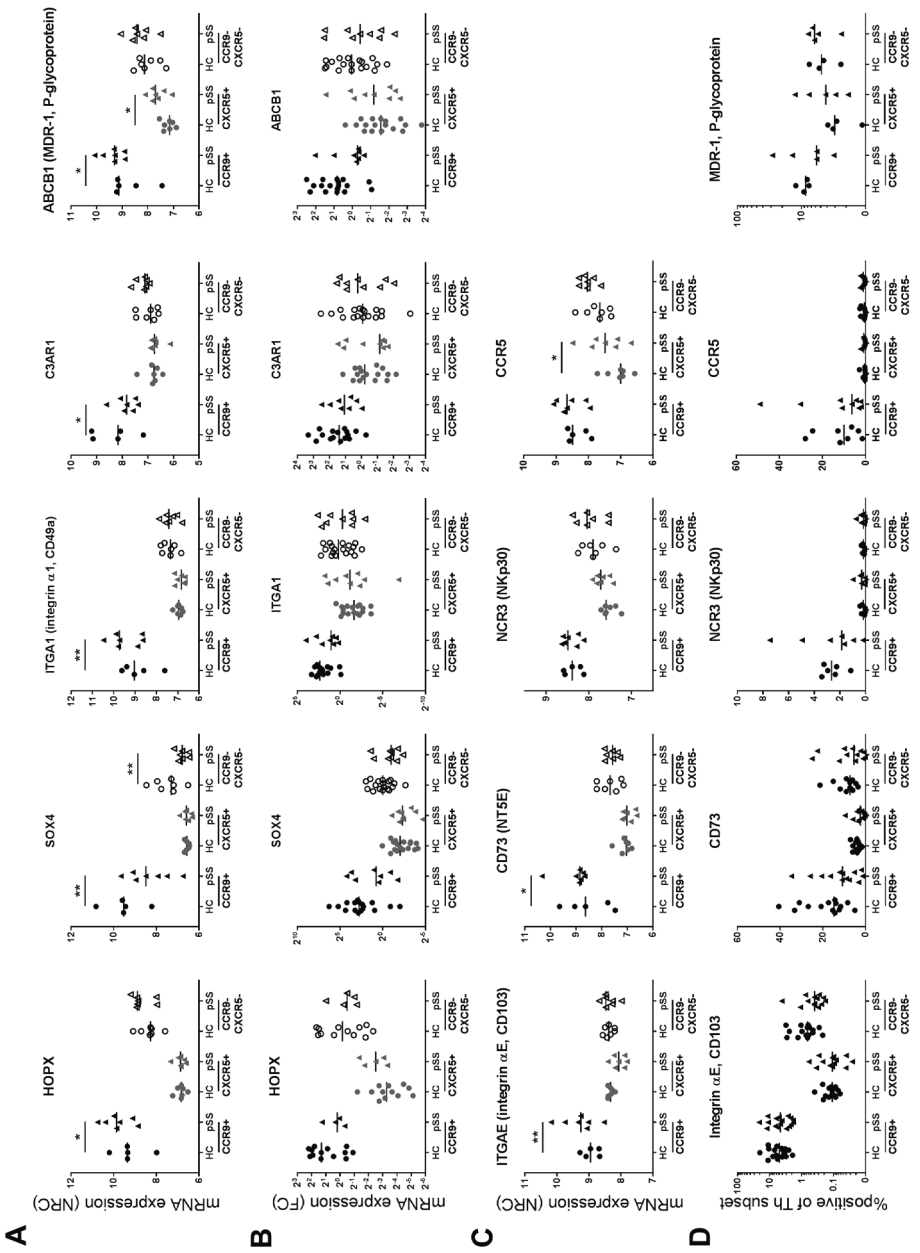

Figure 4. Selected target genes from the blue module are confirmed in additional cohorts.

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. RNAseq 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 cohort and elevated expression on CCR9+ Th cells vs CXCR5 and CCR9-CXCR5- Th cells was confirmed (all p<0.001), but there was no significant difference between HC and pSS. For genes encoding for proteins or which flow cytometry antibodies were available: ITGAE (CD103), NCR3 (NKp30), ABCB1, CCR5, CD73, protein expression could be assessed on fresh peripheral blood mononuclear cells. For these markers, elevated expression in the CCR9+ subset vs CXCR5+ and CCR9-CXCR5- was confirmed (all p<0.05). Differences between HC and pSS in the CCR9 subset according to the RNAseq data were not confirmed on protein level. NRC: normalized read counts; FC: fold change, DN HC mean is set at 1.

CCR9+ Th cells from healthy controls and pSS patients produce high levels of CCL5 and respond more potently to IL-7

CCL5 (RANTES) is elevated in CCR9+ Th transcriptomes compared to the other subsets and passed the selection criteria shown in **Figure 3**. Interestingly, the expression of *CCL5* is elevated in the CCR9+ Th cells from pSS patients compared to HC (**Figure 5A**). To functionally validate the production of this chemokine on protein level, CCR9, CXCR5 and CCR9-CXCR5- Th subsets were co-cultured with monocytes and CCL5 was measured in supernatants upon restimulation with PMA and ionomycin. Significantly elevated levels of CCL5 were measured in the CCR9+ Th cell cultures as compared to CXCR5+ and CCR9-CXCR5- Th cells (**Figure 5B**). Since IL-7 and antigen triggering may be key drivers of CCR9+ Th activation and thereby CCL5 secretion, cells were cultured with IL-7 or superantigen SEB. Interestingly, IL-7 strongly upregulated CCL5 production by co-cultured CCR9+ Th cells, significantly higher than CXCR5+ or CCR9-CXCR5- Th cells (**Figure 5C**). 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).

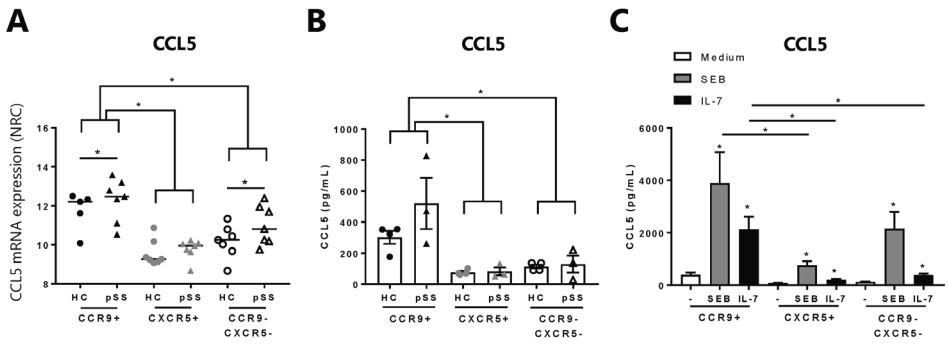

Figure 5. CCR9+ Th cells secrete increased levels of CCL5 which is enhanced by IL-7 and antigenic triggering.

(**A**) mRNA expression as assessed by RNA sequencing is elevated in CCR9+ Th cells as compared to CXCR5+ and CCR9-CXCR5- Th cells. (**B**) CCR9+, CXCR5+ and CCR9-CXCR5- Th cells from healthy controls (n=4) and pSS patients (n=3) were cultured with monocytes for 72 hours followed by restimulation by PMA and ionomycin, showing elevated production of CCL5 by CCR9+ Th cells. (**C**) The same experiment was performed with or without SEB (Staphylococcal Enterotoxin B) or IL-7 followed by restimulation by PMA and ionomycin. With and without stimulation, CCR9+ Th cells produce elevated levels of CCL5 protein as compared to the other Th cell subsets.

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 and 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. In addition, high production of CCL5 by CCR9+ Th cells was identified.

Our strategy to select target genes for validation, used strict data-driven criteria and was focused on upregulated genes in the CCR9+ Th cell subset in one specific (blue) module showing robust differences between the Th subsets. Using this strategy we identified a number of robustly differentially expressed genes that were validated in an additional cohort 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 are likely playing key roles in the function of CCR9+ Th cells, For example, *LZTFL1* that has coherence within the black module encodes for a factor that is upregulated by all-trans retinoic acid and by TCR signaling. Its overexpression has been shown to enhance NFAT mediated signaling, potentially contributing to the production of cytokines by CCR9+ Th cells.(44) 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 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 a 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.(45) 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* in developing Th1 cells induces more IFN-γ-producing cells.(46) Together, this suggests that *HOPX* and *SOX4* may drive the high IFNγ production by

CCR9+ Th cells, which we and others have previously shown(12,26). However, future research is needed to confirm the role of *HOPX* and *SOX4* in regulating Th1 activity in CCR9+ Th cells.

In addition to *HOPX*, *SOX4*, we found that *CCL5* and its receptor *CCR5* are upregulated in CCR9+ Th cells. Both CCL5 and CCR5 have been associated with a Th1 phenotype.(47,48) 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.(49) In human studies, CCR5 expression has been shown to increase in the conjunctival epithelium of patients with dry eye syndrome.(42,50) Interestingly, CCL5 and its receptor CCR5 were increased in inflamed gland in Sjögren-like disease and blockade of CCL5 can significantly reduce disease.(51) The ligands for CCR5, CCL3 and CCL5 are elevated in pSS salivary glands potentially facilitating migration of CCR5-expressing cells including CCR9+ Th cells.(43) In this paper we demonstrate strongly increased secretion of CCL5 by co-cultured CCR9+ Th cells as compared to CXCR5+ and CCR9-CXCR5- Th cells. Interestingly, IL-7 which is a key early mediator of salivary gland inflammation(28-31) and is a crucial factor in lymphoid structure organization(35,52), significantly increased CCL5 production by CCR9+ Th cells as compared to CXCR5+ and CCR9-CXCR5- Th cells. As IL-7 has previously been shown to increase responsiveness of auto-reactive T cells(53,54) this could implicate that IL-7-driven self-reactive T cell responses associated with CCL5 production play a 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.(55) Also, laminin a ligand for CD49a has been found to be upregulated in pSS salivary glands.(56). However, whereas we did find a subset of CCR9 Th cells with increased expression of CD103, we could 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.(41) 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.(57) CD73 is expressed by regulatory T cells but can be upregulated on all Th cells upon activation.(58) As a homeostatic process, regulatory molecules are upregulated upon activation of T cells, and this may be the case for CD73 on the pro-inflammatory 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.(59) 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).(60) Further research is needed to study the relevance of expression of ABCB1 on CCR9+ Th cells and in pSS.

In some pSS patients, hypocomplementaemia of in particular C3 and C4 is found, which is associated with lymphoma development.(61) 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.(62) 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.(63)

Although robust differences between Th subsets were detected and replicated, the differences in mRNA expression between HC and pSS were rather small, especially the CCR9+ Th cell subset-specific differences between HC and pSS, and could not be replicated. In this respect, DEGs from the modules showing the largest differences between HC and pSS i.e. cyan, darkgreen including IFN induced genes, which indeed have been shown to be upregulated in part of pSS patients(64), and purple, the three modules in which the differences between HC and pSS were present in all Th subsets, may have given a higher chance of being replicated. However, our main focus was to identify genes upregulated in CCR9+ Th cells present in coherent gene networks which showed differential expression between HC and pSS. As a

future perspective, bulk or single cell sorting of CCR9+ Th cells from salivary glands of pSS patients to perform RNA sequencing may reveal the local more robust activation profile of CCR9+ Th cells.

Finally, variation in gene expression data may partly be due to patient heterogeneity. Indeed differences were observed between patients used for discovery (RNAseq) and replication by qPCR. A larger proportion of patients of the RNAseq cohort was treated with immunosuppressants and there were differences in clinical parameters (LFS, in the proportion of patients with auto-antibodies, ESSDAI scores, serum IgG levels). However, none of these clinical parameters showed statistically significant correlations with the expression of the target genes.

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.

Acknowledgements

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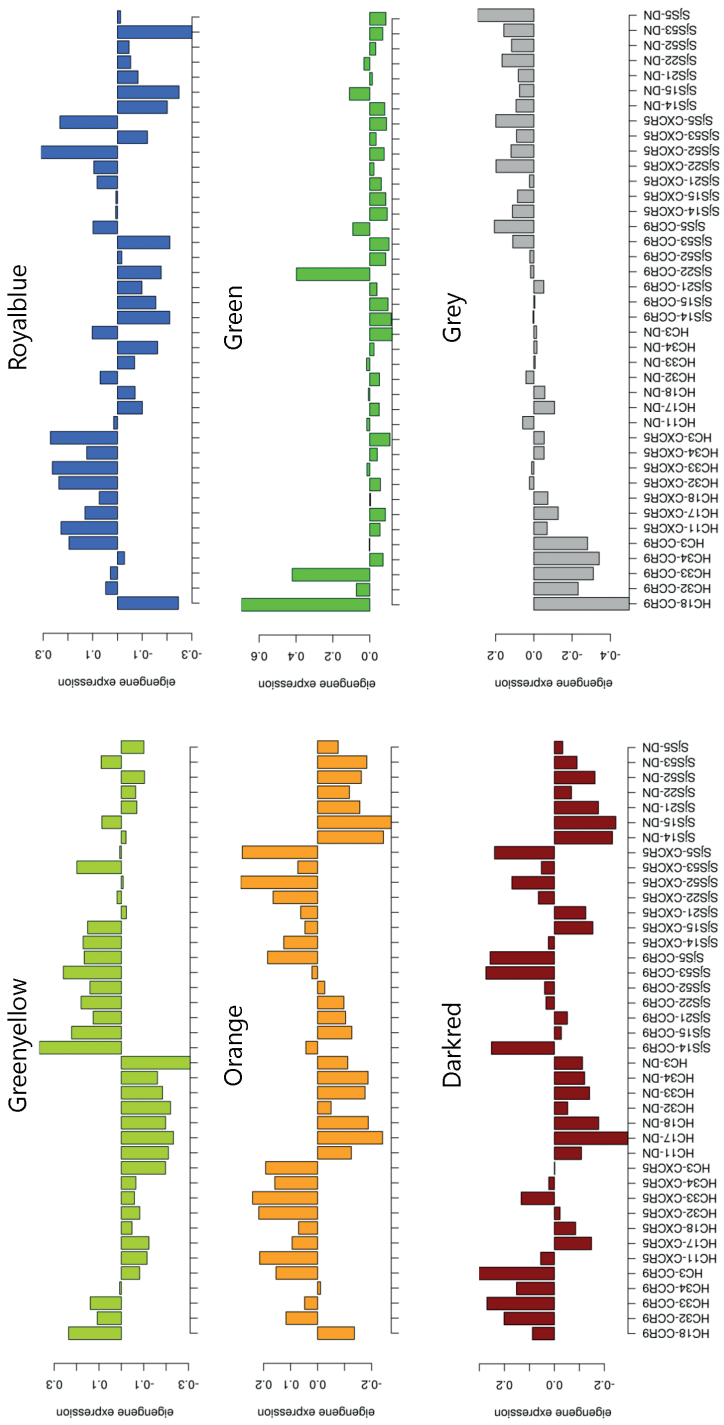
Supplementary material

Supplementary table

Marker	Fluorochrome	Company	Clone
CD3	AF700	Sony Biotechnology	UCHT1
CD4	PerCP	Biolegend	RPA-T4
	APC-ef780	eBioscience	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
CD56	FITC	Biolegend	HCD56
CD73	PE	BD Biosciences	AD2
CD45RO	BV711	Biolegend	UCHL1
CD27	APC-ef780	eBioscience	O323
CD103	FITC	Dako	Ber-ACT8
CD49a	APC	Sony Biotechnology	TS2/7
ABCB1	FITC	eBioscience	UIC2
CCR5	PE	eBioscience	eBioT21/8

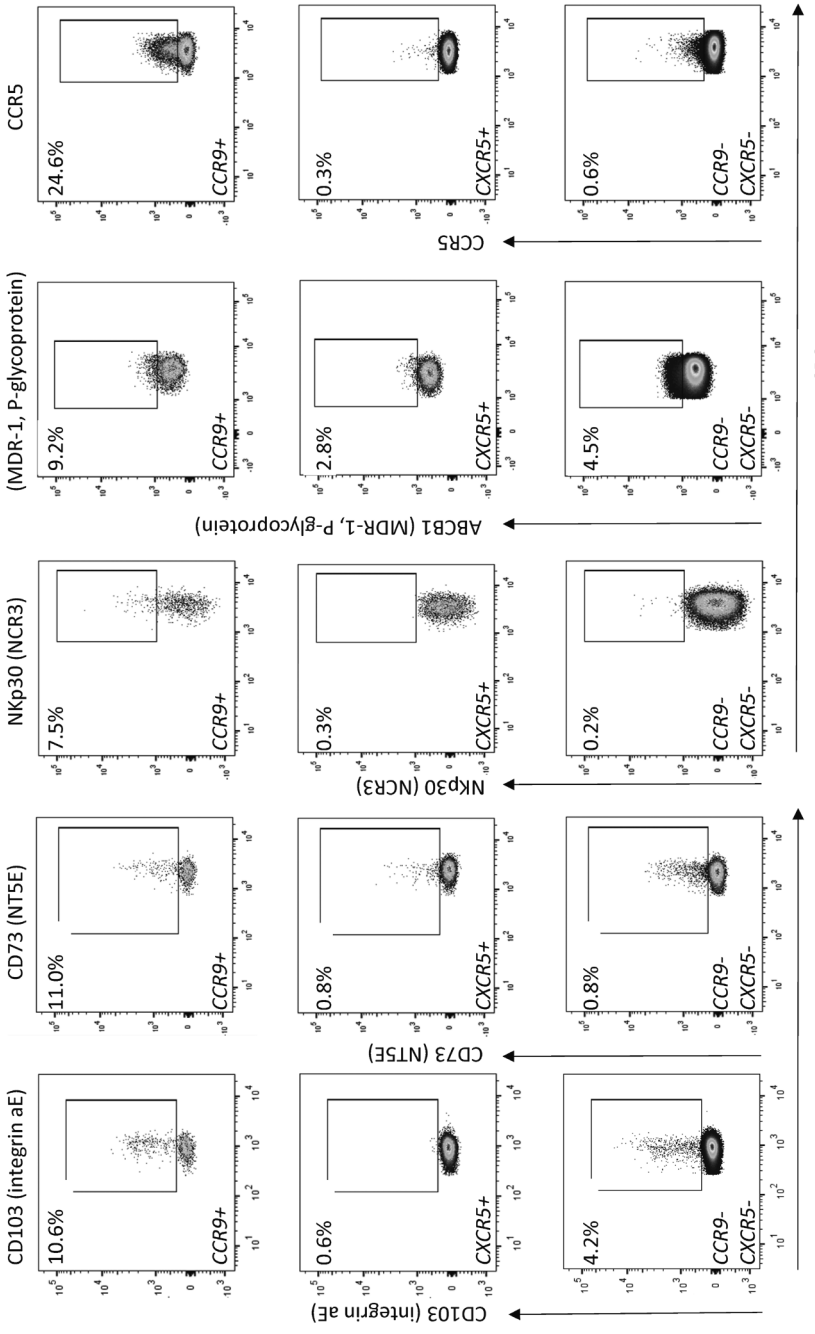
Supplementary table 1. Antibodies used for flow cytometry.

Supplementary figures



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 FACS 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.

CHAPTER 8

Decreased circulating CXCR3+CCR9+ Th cells are associated with elevated levels of their ligands CXCL10 and CCL25 in the salivary gland of patients with Sjögren's syndrome to facilitate their concerted migration

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Abstract

Introduction. CCR9+ Th cells can induce Sjögren-like symptoms in mice and both CCR9+ Th-cells and their ligand CCL25 are increased in the salivary glands of primary Sjögren's syndrome (pSS) patients. Increased circulating CCR9+ Th cells from pSS patients are hyperresponsive to IL-7, secrete high levels of IFN-γ, IL-21, IL-17 and IL-4 and potently stimulate B cells, all equally effective to healthy individuals. Our aim was to study co-expression of chemokine receptors on CCR9+ Th cells and whether in pSS this might differentially affect CCR9+ Th cell frequencies.

Methods. Frequencies of circulating CCR9+ and CCR9- Th-cells co-expressing CXCR3, CCR4, CCR6 and CCR10 were studied in pSS patients and healthy controls. CCL25, CXCL10, CCL17, CCL20 and CCL27 mRNA and protein expression of salivary gland tissue of pSS and non-Sjögren's sicca (nSS) patients was assessed. Chemotaxis assays were performed to study migration induced by CXCL10 and CCL25.

Results. Higher expression of CXCR3, CCR4 and CCR6 but not CCR10 was observed on CCR9+ Th cells as compared to cells lacking CCR9. Decreased frequencies of circulating memory CCR9+CXCR3+ Th-cells were found in pSS patients, which was most pronounced in the effector memory subset. Increased salivary gland CCL25 and CXCL10 expression significantly correlated and both ligands functioned synergistically based on *in vitro* induced chemotaxis.

Conclusion. Decreased memory CXCR3+CCR9+ Th-cells in blood of pSS patients may be due to a concerted action of overexpressed ligands at the site of inflammation in the salivary glands facilitating their preferential migration and positioning in the lymphocytic infiltrates.

Introduction

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disorder characterized by oral and ocular dryness and lymphocytic infiltration of the exocrine glands.(1) The largest part of the lymphocytic infiltrates consists of memory CD4+ T cells, which have been shown to play a major role in the immunopathology of Sjögren's syndrome.(2) Recruitment of leukocytes to tissues is dependent on chemokine-chemokine receptor interactions and adhesion molecules. Several chemokines and receptors have been shown to be upregulated in the salivary and lacrimal glands of pSS patients potentially leading to migration of T cells to the glands, including the interferon induced chemokines CXCL9, CXCL10, CXCL11 and their receptor CXCR3.(3, 4) Recently, CXCR5+ T follicular helper (Tfh) cells and their ligand CXCL13 and CCR9+ "Tfh-like" cells with their ligand CCL25 have been implicated in disease pathogenesis. Both levels of CXCL13 and CCL25 and numbers of CXCR5+ and CCR9+ Th cells are elevated in the salivary glands of pSS patients. CXCR5+ Tfh and CCR9+ Tfh-like cells produce pro-inflammatory cytokines and provide co-stimulatory signals that strongly stimulate B cell responses which play an important role in B cell hyperactivity pSS.(5-8) In addition, these Tfh and Tfh-like cells may be involved in the formation of germinal centre-like structures, which are present in the salivary glands of ~25% of pSS patients.(8) In one study, polarization of circulating Tfh cell subsets towards a Tfh1, Tfh2 or Tfh17 phenotype was not observed in pSS patients, while in another study an increase of Tfh17 was found.(9, 10) In the present study, we investigated co-expression of chemokine receptors on circulating CCR9+ Th cells, potentially mediating accumulation of effector Th cell subsets in the salivary glands of pSS patients. In addition we assessed local presence of chemokines in the salivary gland and tested whether increased chemokines may have a concerted action on Th cells.

Patients and methods

Patients

pSS patients, non-Sjögren's sicca (nSS) patients and healthy subjects were included from the outpatients clinic of the department of Rheumatology & Clinical Immunology of the University Medical Centre Utrecht and were randomly and cross-sectionally selected. All pSS patients were diagnosed by a rheumatologist and fulfilled the American-European Consensus Group (AECG) criteria(11). The nSS patients were defined as patients suffering from ocular and/or oral dryness without any other known cause and who were not diagnosed with an underlying rheumatic disease including Sjögren's syndrome, consequently, they could not be classified using the AECG-criteria. The study was approved by the hospital's medical ethics committee and all subjects signed informed consent. Patient characteristics are shown in **Table 1** for those that contributed to the gene expression, flow cytometry and chemotaxis studies and **Supplementary table 1** for those that contributed labial biopsies.

	Gene expression		Flow cytometry		Chemotaxis	
	nSS (n=9)	pSS (n=9)	HC (n=11)	pSS (n=17)	HC (n=6)	pSS (n=10)
Female gender, n (%)	8 (89)	8 (89)	11 (100)	17 (100)	6 (100)	10 (100)
Age, years (mean ± SD)	53.3 ± 7.5	43.7 ± 19.7	43.4 ± 12.3	54.6 ± 13.2	54.3 ± 6.8	58.6 ± 11.0
Anti-Ro/SSA positive, n (%)	2 (22)	9 (100)	-	16 (94)	-	8 (80)
Anti-La/SSB positive, n (%)	0 (0)	3 (33)	-	9 (53)	-	6 (60)
ANA positive, n (%)	1 (11)	7 (78)	-	13 (76)	-	8 (80)
Lymphocytic focus score (foci/4mm2)	0 (0-0)	3.0 (1.5-5.0)	-	2.0 (1.4-2.7)	-	2 (1.8-3)
IgA positive plasma cells (%)	>70	43 (16-50)	-	38 (25-51)	-	33 (19-46)
Schirmer (mm/5min)	1.5 (1.0-5.0)	4.0 (0.8-17.0)	-	3.5 (0.4-12.3)	-	4.5 (0.5-10)
Serum IgG (g/L)	11.4 (11.0-12.4)	17.4 (10.4-28.5)	-	17.2 (13.8-19.7)	-	13.6 (12.0-17.0)
ESR (mm/h)	6 (3-21)	14 (10-52)	-	22 (13-33)	-	13 (12-34)
ESSDAI score (0-123)	-	-	-	6 (5-8)	-	6 (2-8)
ESSPRI score (0-10)	-	-	-	6.5 (5-7)	-	5 (3-7)
Immunosuppressants	1	2	-	3	-	2
Hydroxychloroquine	0	0	-	3	-	1
Other	0	1	-	0	-	1

Table 1. Patients' characteristics.

Median (IQR, interquartile range) are shown unless specified otherwise. nSS: non-Sjögren's sicca; pSS: primary Sjögren's syndrome; HC: healthy controls; ESR: erythrocyte sedimentation rate; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index.

Chemokine and chemokine receptor assessment

Fresh PBMCs from healthy controls (HC) and pSS patients were assessed by flow cytometry, staining for CD3, CD4, CD45RO, CD27, CCR9, CXCR3, CCR4, CCR6 and CCR10 (**Supplementary Table 2**). Quantitative PCR was performed using the Applied Biosystem 7000 System (Thermo Fisher Scientific; Waltham, MA, USA), and TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific). As described (12) cDNA was analysed for the expression of CCL17 (Hs00171074_m1), CCL20 (Hs00171125_m1), CCL27 (Hs00171157_m1), CXCL10 (Hs00171042_m1), CCR4 (Hs00747615_s1), CCR6 (Hs00171121_m1), CCR10 (Hs00706455_s1) and CXCR3 (Hs00171041_m1). Expression was normalized to 18S and consequently was calculated as a fold change relative to the mean of the nSS group, using the delta-delta CT method. All primers were purchased from Thermo Fisher Scientific. Protein levels of these chemokines were measured by Luminex in labial salivary gland biopsy supernatants. Fresh labial salivary gland tissues were thoroughly rinsed and incubated with 200µL of saline (0.9% NaCl) in a 500µL vial (Sarstedt) for 1 hour at room temperature. The biopsy tissue was removed from the vial and the remaining tissue supernatants were rendered cell-free by centrifugation at 500g for 5 minutes and stored at -80°C. Chemokines were measured using Luminex multiplex technology as previously described.(13)

Chemotaxis

Transwell experiments were performed to assess chemotaxis induced by CCL25 and CXCL10. 1×10^5 PBMCs were transferred into the upper chamber of 5µm pore-size transwell plates (96 well ChemoTX®, NeuroProbe). Fresh medium alone, or containing CCL25 (0 or 100ng/mL, Peprotech) and/or CXCL10 (0, 10 or 100ng/mL, Peprotech) were added to the lower chamber. After 2 hours at 37°C, cells migrating to the lower chamber were quantified by flow cytometry with standardization of the volume and acquisition time per well.

Statistical analysis

Statistical analyses were performed in Prism 6 software and SPSS. Student's t-test, paired parametric t-test, Mann-Whitney U test and Wilcoxon non-parametrical paired test were used where appropriate. For correlations with disease parameters, Pearson's correlation and Spearman's rho were used where appropriate. Differences and correlations were considered statistically significant at $p<0.05$, $p=0.05$-0.10 was considered as a trend towards statistical significance and indicated in figures.

Results

Frequencies of circulating memory CXCR3+CCR9+ Th cells are decreased in pSS

Significantly increased frequencies of CXCR3+, CCR4+ and CCR6+ cells were found within the CCR9+ Th cell subset as compared to CCR9- Th cells in the circulation of healthy individuals (**Figure 1A, B**). Interestingly, in pSS patients a significant decrease in CXCR3+CCR9+ Th cells was found in the central memory (CD45RO+CD27+), effector (CD45RO-CD27-) and effector memory (CD45RO+CD27-) subsets, but not naïve Th cells (CD45RO-CD27+) (**Figure 1C**). In addition, decreased frequencies of CCR6-expressing CCR9+ effector memory Th cells. In contrast, increased frequencies of CCR4-expressing CCR9+ effector memory Th cells were observed in pSS patients (**Figure 1C**). The decreased frequencies of these subsets did not significantly correlate with clinical parameters (data not shown). Trends towards similar differences were observed in CCR9- Th cells, although less pronounced (**Supplementary Figure 1**). These results indicate a predominant decrease of CXCR3-expressing CCR9+ memory Th cells in the circulation of pSS patients.

CCL25 and CXCL10 are increased in the salivary glands of pSS patients and synergistically induce Th cell migration in vitro

Next we assessed whether mRNA and protein expression of representative chemokines that can attract CXCR3, CCR4, CCR6 and CCR10-expressing Th cells were expressed by salivary gland tissue, either expressed as mRNA in tissue lysates or in supernatant of tissue explants (14). We previously reported that mRNA expression of CCL25 was significantly increased in salivary glands of pSS patients as compared to nSS patients.(6) Increased CXCL10 mRNA expression as previously reported by others (3) was confirmed (**Figure 2A**). Also increased expression of the other CXCR3-ligands, CXCL9 and CXCL11 in pSS was in line with previous data (fold change (median, interquartile range): 10 (4-153) and 5 (2-42) vs nSS, p=0.012 and p=0.006, respectively).(3,15) In addition, there was a trend towards increased CCL20 expression (p=0.077), and a decrease of CCL27 expression (p=0.050). whereas CCL17 was not differentially expressed (**Figure 2A**). As we have previously reported, increased protein levels of CCL25 and CXCL10 in secretomes of pSS salivary gland tissue were observed.(3,6,14) We here report that upregulated CCL25 mRNA expression significantly correlated to CXCL10 (**Figure 2C**). In accordance with the mRNA data these chemokines also correlated on protein level (all sicca donors: r=0.47, p=0.002, pSS: r=0.42, p=0.04). Only very low levels of CCL17 and CCL20 (CCR4 and CCR6 ligands) proteins were found, with increases in only a limited number of patients (data not shown). In addition, CCL27 was present at higher levels, but the levels were not significantly different between healthy controls and pSS patients (data not shown). Increased CCR6 and a trend towards increased CXCR3 mRNA expression were found in the salivary glands of pSS patients (**Figure 2B**). No significant differences in CCR4 (p=0.34) and CCR10 (p=0.29) expression were observed (**Figure 2B**).

Since a decrease of CXCR3+CCR9+ Th cells was found in the circulation of pSS patients and both CCL25 and CXCL10 showed the strongest increased expression in the salivary glands on both mRNA and protein level, the chemotactic capacity of CXCL10 in combination with CCL25 was investigated. In a transwell system, chemotaxis of Th cells in response to different concentrations of CCL25 and CXCL10 was assessed. No differences in migration of Th cells or lymphocytes between HC and pSS was observed, hence the data of these groups were pooled. Interestingly, the combination of CCL25 and CXCL10 synergistically enhanced chemotaxis of CD4+ Th cells as compared to CXCL10 or CCL25 alone (**Figure 2D**).

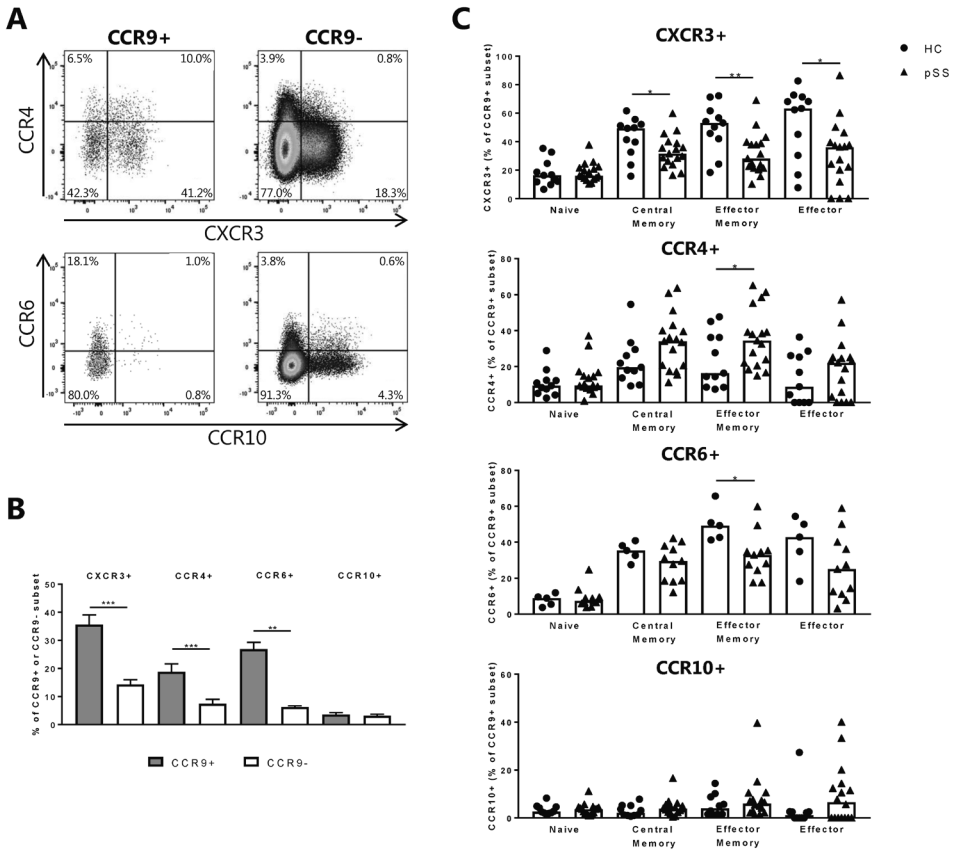

Figure 1. Circulating CCR9+ Th cells have enhanced CXCR3, CCR4 and CCR6 expression and pSS patients show decreased proportions of CCR9+ Th cells expressing CXCR3 and CCR6.

Representative flow cytometry images (**A**) and combined data of multiple healthy donors (n=5-11) (**B**) showing increased expression of CXCR3, CCR4 and CCR6, but not CCR10 on circulating CCR9+ versus CCR9- Th cells. (**C**) In pSS patients circulating CCR9+ central memory, effector memory and effector Th cells expressing CXCR3 and effector memory cells expressing CCR6 are significantly decreased. In addition, circulating CCR9+ effector memory Th cells from pSS patients show elevated percentages of CCR4-expressing cells. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 respectively.

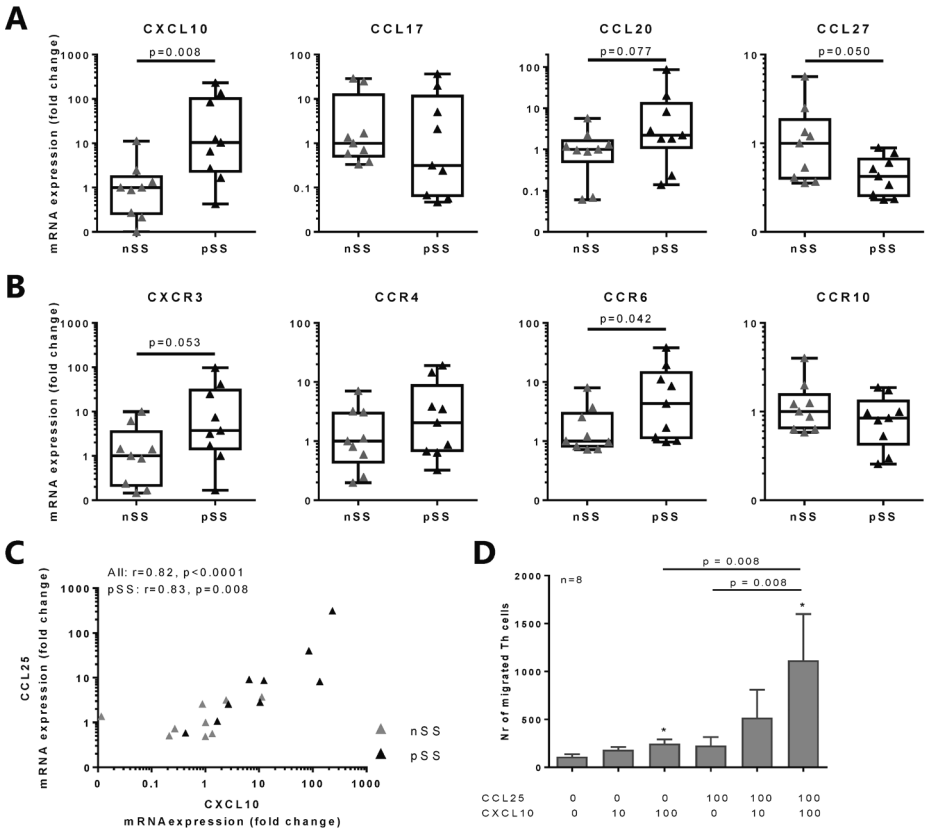

Figure 2. Elevated levels of CCL25 and CXCL10 in salivary glands from pSS patients correlate and in vitro these cytokines induce synergistic chemotaxis of Th cells.

(**A,B**) mRNA expression of the following chemokines and their receptors were evaluated in labial salivary gland biopsies from pSS and nSS patients: CXCL10 and CXCR3, CCL17 and CCR4, CCL20 and CCR6 and CCL27 and CCR10, showing elevated expression of CXCL10 and CCR6 and trends towards increases in CXCR3, CCL20 and a decrease of CCL27. (**C**) Expression of CXCL10 strongly correlated with CCL25 expression and (**D**) in a transwell chemotaxis assay, synergistic migration of Th cells is induced upon a combination of CCL25 and CXCL10 (n=8).

Discussion

In this study, increased co-expression of chemokine receptors CXCR3, CCR4 and CCR6 on circulating CCR9+ Th cells was found as compared to Th cells lacking CCR9. In the circulation of pSS patients decreased frequencies of CXCR3+CCR9+ and CCR6+CCR9+ Th cells were found, which may be due to a concerted action of overexpressed ligands at the site of inflammation. Corroborating this we found elevated and abundant expression of CXCL10 and CCL25 in the salivary gland and synergistic chemotaxis of Th cells for these cytokines *in vitro.*

This is the first study that shows co-expression and synergistic function of chemokine receptors on human circulating CCR9+ Th cells. Intestinal CCR9+ Th cells from mice have been found to have elevated expression of CCR6 and CXCR3 and lower expression of CCR10 than CCR9- Th cells.(16) Chemokine receptors play an important role in the positioning of antigen experienced Th cells in epithelial tissues. CCR4 and CCR10 are regarded as skin-homing chemokine receptors and CCR9 and CCR6 are involved in intestinal homing.(17, 18) The high co-expression of CXCR3, CCR4 and CCR6 we found on CCR9+ Th cells as compared to CCR9- Th cells potentially indicates enhanced homing to the gut but also to other tissues where their ligands are expressed. The chemokine receptor profile corresponds with the pleiotropic cytokine production by this subset, including IFN-γ, IL-4 and IL-17.(6)

In accordance with previous studies by others as well as our group, elevated local expression of the chemokines CCL25 and CXCL10 was found in pSS.(3, 6) Corresponding with the elevated chemokine levels, increased numbers of CCR9 and CXCR3-expressing cells have previously been found in pSS salivary glands.(3, 6) In this study, we demonstrated a significant correlation of CCL25 and CXCL10 protein expression in labial salivary gland biopsies. A similar correlation was not observed between CCL25 and other chemokines supporting coordinated responses to CCL25 and CXCL10 by CCR9+ Th cells co-expressing CXCR3. In this study, we demonstrated a significant correlation of CCL25 and CXCL10, which was not observed for CCL25 and the other chemokines on protein level, indicating that effects of CCL25 and CXCL10 on CCR9+ Th cells co-expressing CXCR3 can coincide. This was corroborated by CCL25 and CXCL10 inducing synergistic chemotaxis of Th cells. In our assay it was not possible to quantify the number of migrated CCR9-expressing cells, since CCL25 induces downregulation of CCR9 (Takeda Pharmaceuticals and Blokland et al. unpublished data). However, for synergistic chemotaxis of Th cells these cells need to co-express CCR9 and CXCR3. Hence, we are confident that both cytokines can contribute to enhanced migration of CCR9+CXCR3+ Th cells.

The important role of CXCR3 in pSS is supported by the recent finding that inhibition of CXCR3 impedes development of pSS-like disease in a sialadenitis mouse model.(19) Considering the observation that 40-50% of CCR9+ Th cells express CXCR3 it is likely that part of these

effects are mediated by blockade of CXCR3+CCR9+ Th cells. The finding that CCR9+ Th cells have shown to be crucial for experimental Sjögren's like disease in mice supports this notion. Although synergistic migration is facilitated by CCL25 and CXCL10, positioning of CCR9+ Th cells is likely regulated by much more complex expression patterns of other chemokines and chemokine receptors and adhesion molecules. This is corroborated by the observation that next to the decreased frequencies of circulating CXCR3-expressing CCR9+ Th cells in pSS patients frequencies of CCR6-expressing CCR9+ Th cells were reduced. Although this was only significant for effector memory cells, it should be noted that the number of tested samples was low and addition of more donors may reveal decreased frequencies of CCR6-expressing cells in the central memory and effector CCR9+ Th cell subsets as well. Despite the fact CCL20 on mRNA and protein level in the salivary gland was not robustly increased, we did observe significantly enhanced CCR6 mRNA in the salivary gland of pSS patients. This suggests that CCL20, which is the only CCR6 ligand, contributes to migration of CCR6-expressing CCR9+ Th cells. Whether this also induces synergistic migration or sequential or differential spatial migration in the inflamed tissue remains to be studied, but this interaction may play a role in the recruitment of CCR6-expressing Th17 polarized CCR9+ Th cells in pSS salivary glands. (20) In support of a role for CCL20/CCR6 recently it was shown that induction of Sjögren's like disease is associated by increase in CCL20 concentrations.(21) Positioning of CCR9 cells is unlikely mediated by CCR10 ligands. A low percentage co-expresses CCR10, and CCL27 is reduced in the labial salivary gland. The decrease of CCL27 mRNA expression in pSS is in accordance with the decrease of CCL28 – the other ligand for CCR10 – in saliva of pSS patients.(22)

In conclusion, this study indicates that coordinated elevated expression of chemokines in the salivary glands of pSS patients may have synergistic effects on migration of pathogenic CCR9+ Th cells towards the salivary glands. This concept also helps to explain why the CXCR3+CCR9+ Th subset may be reduced in the circulation of pSS patients.

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Supplementary material

Supplementary tables

	nSS (n=17)	pSS (n=24)
Female gender, n (%)	15 (88)	21 (88)
Age, years (mean ± SD)	49 ± 16	55 ± 12
Anti-Ro/SSA+, n (%)	0 (0)	16 (67)
Anti-La/SSB+, n (%)	0 (0)	4 (17)
ANA+, n (%)	9 (53)	17 (71)
Lymphocytic focus score (foci/4mm^2)	0 0 – 0)	1.6 (1.0 – 2.6)
IgA positive plasma cells (%)	77 (71 – 80)	59 (49 – 64)
Schirmer (mm/5min)	4 (1 – 7)	7 (3 – 11)
Serum IgG (g/L)	11.0 (6.5 – 12.3)	13.9 (10.1 – 18.3)
ESR (mm/h)	7 (6 – 10)	15 (7 – 29)
ESSDAI score (0-123)	NA	3 (1 – 7)
ESSPRI score (0-10)	NA	5.3 (2.3 – 6.0)
Immunosuppressants, n (%)	1 (6)	3 (12.5)

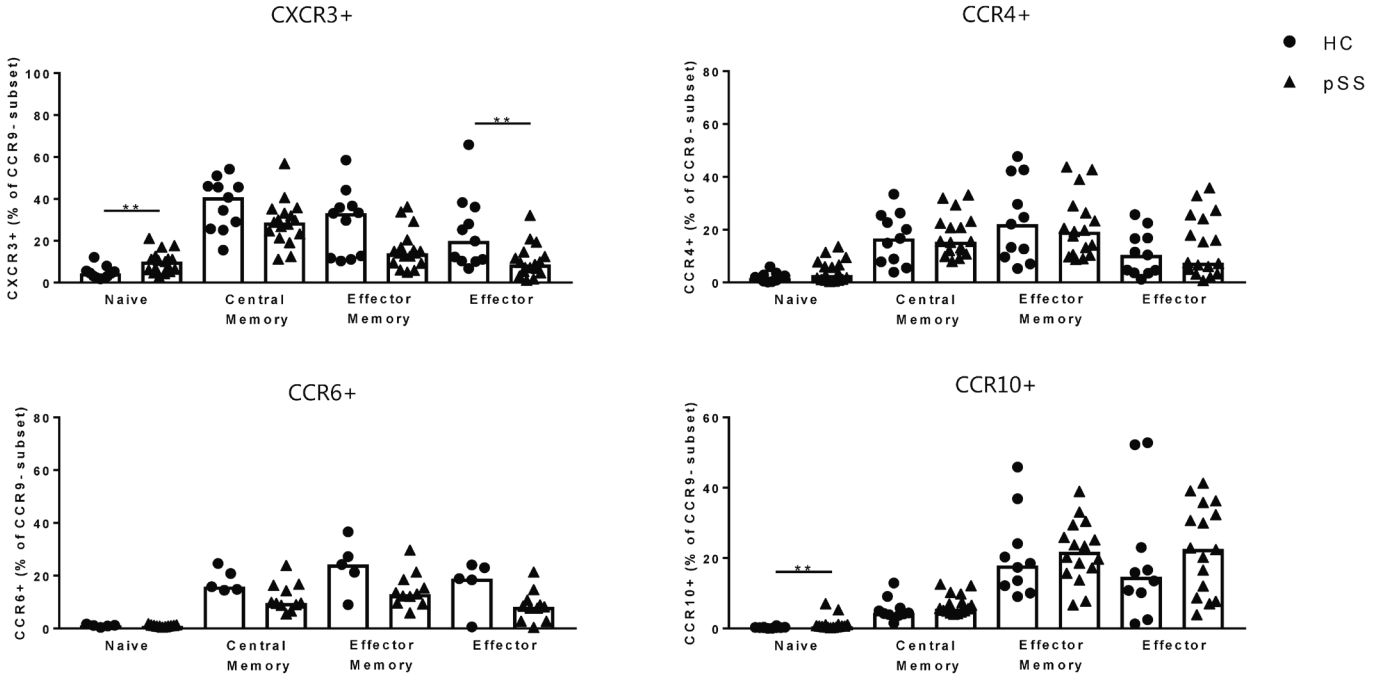
Supplementary table 1. Patients' characteristics salivary gland biopsy supernatants.

Median (IQR, interquartile range) are shown unless specified otherwise. nSS: non-Sjögren's sicca; pSS: primary Sjögren's syndrome; HC: healthy controls; ESR: erythrocyte sedimentation rate; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index.

Marker	Fluorochrome	Company	Clone
CD3	AF700	Sony Biotechnology	UCHT1
CD4	PerCP-Cy5.5	Sony Biotechnology	RPA-T4
CD45RO	BV711	Biolegend	UCHL1
CD27	APC-ef780	eBioscience	O232
CCR9	PE	Bio-Techne/R&D	248621
CXCR3	FITC	Biolegend	G025H7
CCR4	BV605	BD	1G1
CCR6	PE-Cy7	eBioscience	R6H1
CCR10	APC	R&D systems	314305

Supplementary table 2. Antibodies used for flow cytometry.

Supplementary figures



Supplementary figure 1. Chemokine receptor expression analysis on circulating CCR9- Th cells in HC versus pSS.

Similar to CCR9+ Th cells, trends towards decreased percentages of CXCR3 and CCR6 expressing cells are found in circulating CCR9- Th cells from pSS patients.

CHAPTER 9

Increased mTORC1 activation in salivary gland B cells and T cells from Sjögren's syndrome patients: mTOR inhibition as a novel therapeutic strategy to halt immunopathology

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Under review (RMD Open)

Abstract

Introduction. To investigate mTOR activation in patients with primary Sjögren's syndrome (pSS) and the potential of mTOR inhibition to halt B and T-cell activation.

Methods. Expression of mTOR pathway-related genes was measured in circulating B-cells from pSS and non-Sjögren's sicca (nSS) patients and controls. Salivary gland tissue was stained for CD20, CD138, CD3, CCR9, and phosphorylated S6 ribosomal protein (indicating mTOR activity) using immunofluorescence. Rapamycin was used to inhibit mTOR signalling *in vitro*.

Results. RPTOR and *IGF1R* expression and mTORC1-activity were decreased in circulating B-cells from pSS patients and inversely correlated with serum IgG. In contrast, numbers of salivary gland lymphocytes (T, B, plasma cells, and CCR9+ cells) with mTORC1-activity were elevated in pSS and correlated with B-cell hyperactivity. *In vitro,* mTOR activation in T and B-cells was associated with proliferation and production of IFN-γ and IgG, which were strongly inhibited by rapamycin.

Conclusion. pSS patients have decreased mTORC1-activity in circulating B-cells and increased mTORC1 activity in salivary gland lymphocytes, correlating with B-cell hyperactivity. Proliferation of T and B-cells and production of IFN-γ and IgG, associated with increased mTOR signalling, are robustly inhibited by rapamycin *in vitro*, pointing towards mTOR inhibition as a potential novel therapeutic strategy in pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disorder characterized by lymphocytic infiltration and dysfunction of exocrine glands. The lymphocytic infiltrates in exocrine glands mainly consist of Th cells and B cells. A hallmark feature of pSS is B cell hyperactivity, including formation of autoantibodies, elevated serum IgG levels, increased numbers of B cells and IgG+/IgM+ plasma cells in salivary glands, and formation of germinal centre-like structures in the salivary glands associated with an increased risk of lymphoma development(1,2). Th cells and in particular T follicular helper (Tfh) cells play an important role in activation of B cells and formation of germinal centre-like structures(1).

The mammalian/mechanistic target of rapamycin (mTOR) pathway is essential for growth, survival, and proliferation of T and B cells and integrates multiple signals from the immune microenvironment, including growth factors, nutrients, and T cell receptor (TCR)/B cell receptor (BCR) engagement(3,4). The serine/threonine kinase mTOR is the catalytic subunit of two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), characterized by the incorporation of the proteins Raptor and Rictor, respectively. mTORC1 is generally described to play a role in protein translation, cell growth, proliferation and metabolism, whereas mTORC2 regulates metabolism, cell survival, rearrangement of the cytoskeleton, and cell cycle progression (**Figure 1A**)(4).

Inhibition of mTORC1 by rapamycin (sirolimus) and its analogues effectively suppresses T and B cells in kidney transplant patients and multiple trials show promising results in treatment of lymphoid malignancies(5,6). Immunosuppression by mTOR inhibition also has been shown in animal models of rheumatic diseases and patients with RA and SLE(4,7,8). However, mTOR activity in T and B cells has not been studied in pSS patients. In this study we assessed whether the mTOR pathway plays a role in the activation of B cells and T cells of pSS patients and whether mTOR targeting has potential in pSS treatment.

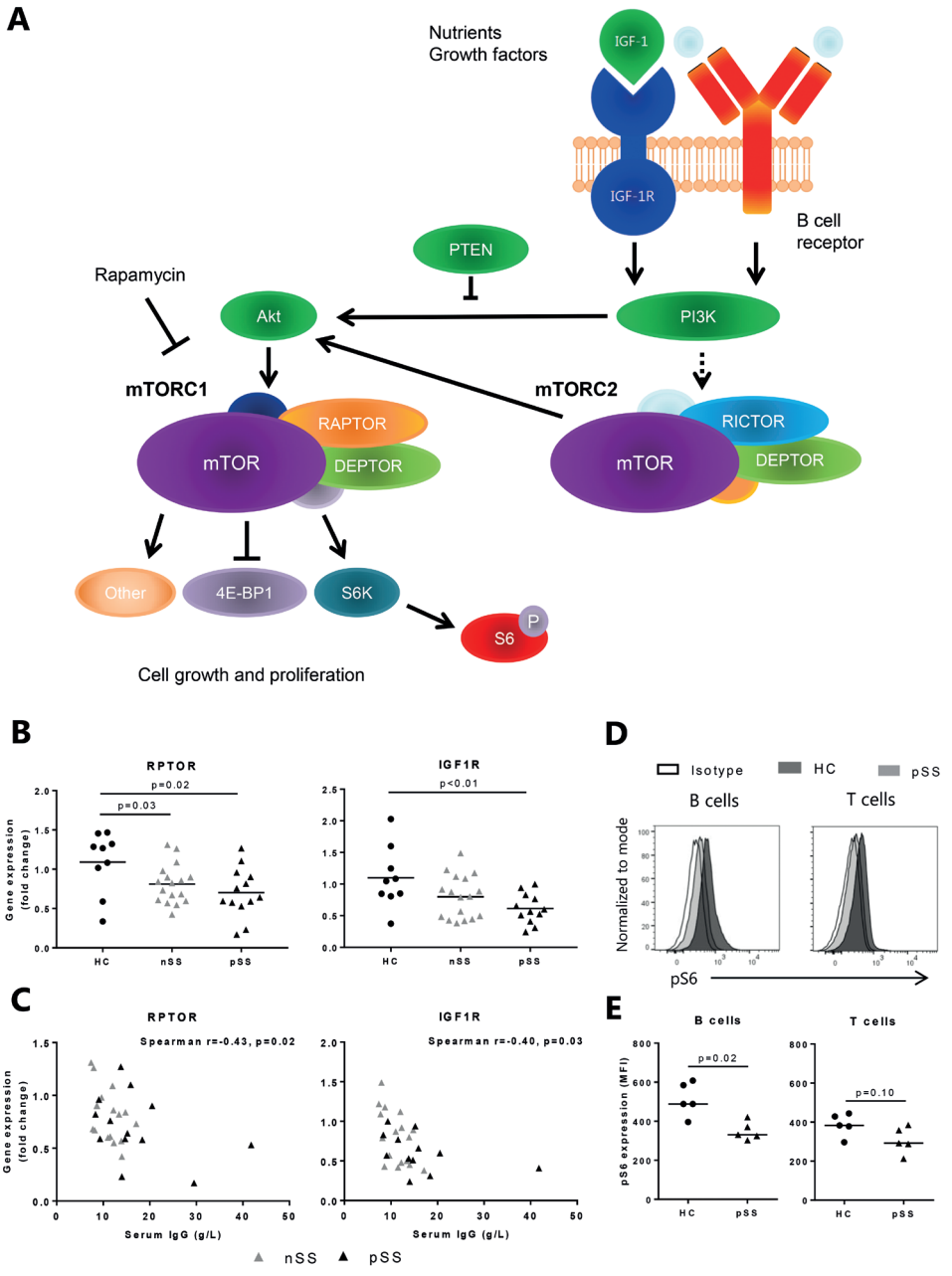
A
Nutrients
Growth factors
IGF-1
IGF-1R
B cell
receptor
PTEN
Rapamycin
Akt
PI3K
mTORC1
mTORC2
RAPTOR
mTOR
DEPTOR
RICTOR
mTOR
DEPTOR
Other
4E-BP1
S6K
S6
P
Cell growth and proliferation
B
RPTOR
p=0.02
p=0.03
IGF1R
p<0.01
Gene expression (fold change)
HC
nSS
pSS
C
RPTOR
Spearman r=-0.43, p=0.02
IGF1R
Spearman r=-0.40, p=0.03
Serum IgG (g/L)
nSS
pSS
D
Isotype
HC
pSS
B cells
T cells
Normalized to mode
pS6
E
B cells
T cells
p=0.02
p=0.10
pS6 expression (MFI)
HC
pSS

Figure 1. Decreased mTOR pathway-related gene and protein expression in circulating B cells from pSS patients.

(**A**) The mTOR pathway is activated by growth factor and B cell receptor signaling and consists of two complexes: mTORC1 and mTORC2. mTORC1 activation leads to phosphorylation of S6 ribosomal protein. (**B**) Decreased expression of *IGF1R* and *RPTOR* in circulating B cells from pSS patients, (**C**) correlates with serum IgG levels. (**D** representative histograms**, E**) Phosphorylation of S6 ribosomal protein (indicating mTOR activity) is decreased in circulating B cells from pSS patients as compared to healthy controls. A similar trend is seen in circulating T cells. Medians are shown. pSS: primary Sjögren's syndrome; nSS: non-Sjögren's sicca; HC: healthy controls.

Patients and methods

Patients and controls

pSS patients (n=13 B cells, n=12 labial salivary gland (LSG) tissue), non-Sjögren's sicca patients (nSS, n=17 B cells, n=6 LSG tissue), and healthy subjects (n=9 B cells) were included. All pSS patients were diagnosed by a rheumatologist and fulfilled the AECG criteria (2002) (9). When applied in retrospect all pSS patients fulfilled the 2016 EULAR-ACR criteria.(10) The nSS patients presented with dryness complaints without a known cause, were not clinically considered to have any generalized autoimmune disease including pSS, and did not fulfil classification criteria. The study was approved by the hospital's medical ethics committee and all subjects signed informed consent. Patient characteristics are shown in **Table 1**. At the time the biopsies of the immunofluorescence cohort were taken, ESSDAI and ESSPRI scores were not available.

Gene expression of isolated B-cells

CD19+ B-cells were isolated from fresh peripheral blood mononuclear cells using MACS. Expression of the following mTOR pathway-related genes was assessed using a custom qPCR-based array: *MTOR, RPTOR, RICTOR, DEPTOR, AKT1, IGF1R, IGF1* and *PTEN.*

Flow cytometry

Antibodies against CD45, CD19, CD20, CD27, CD38, IgG, CD3, CD4, CD14 and CCR9 were used for *ex vivo* staining and PBMC culture experiments. Activation of the mTORC1-pathway was assessed by intracellular detection of phosphorylated S6 ribosomal protein (pS6) expression, indicating kinase (S6K) activity downstream of mTORC1. Viability and cell proliferation were assessed.

Immunofluorescence

To study local mTOR-activation in salivary glands, immunofluorescence staining was performed on frozen tissue sections from pSS and nSS patients using anti-CD20, anti-CD138, anti-CD3 or anti-CCR9 in combination with anti-pS6 antibodies.

Cell culture

PBMCs ($0.5^{*}10^{6}$) were cultured in the presence or absence of 1-100 nM rapamycin to study the effect of mTOR-inhibition on T and B-cell proliferation (6 days) and production of IFN-γ (measured by Luminex) and IgG (measured by ELISA) (9 days). T and B-cell activation was induced with a combination of 0.1 ng/mL superantigen Staphylococcal Enterotoxin B (SEB) and 3.3 μg/mL of TLR9 agonist CpG-C.

Statistical analysis

Statistical analyses were performed in Prism 6 software and SPSS version 21. Student's t-test, paired sample t-test, Mann-Whitney U test and Wilcoxon signed-rank test were used where appropriate. For correlations with disease parameters, Pearson's and Spearman's correlation were used where appropriate. Differences and correlations were considered statistically significant at $p<0.05$.

More detailed information on methods is described in **Supplementary File 1**

	Gene expression peripheral blood B cells			Immunofluorescence labial salivary gland	
	HC (n=9)	**nSS (n=17)**	**pSS (n=13)**	**nSS (n=6)**	**pSS (n=12)**
Gender (female %)	100	100	77	100	100
Age (yr)	57±5	53±15	52±12	40±18	48±11
Anti-Ro/SSA+ (%)	NA	41	62	0	100
Anti-La/SSB+ (%)	NA	0	38	0	50
LFS (foci/4mm^2)	NA	0	1.8±1.1	0	3.7±2.5
IgG and IgM+ plasma cells in LSG biopsy (%)	NA	31±14	52±19	20±7	54±18
Serum IgG (g/L)	NA	11.2±2.9	17.1±9.3	9.7±2.6	20.1±9.1
ESR (mm/h)	NA	14±12	15±12	17±9	38±25
ESSDAI (0-123)	NA	NA	4±6	NA	NA
ESSPRI (0-10)	NA	NA	5±3	NA	NA

Table 1. Patients' characteristics.

HC: healthy controls; nSS: non-Sjögren's sicca; pSS: primary Sjögren's syndrome; LFS: lymphocytic focus score; LSG: labial salivary gland; ESR: erythrocyte sedimentation rate; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index. Data represent Mean±SD unless specified differently. At the time that the biopsies of the immunofluorescence cohort were taken, ESSDAI and ESSPRI were not published, and therefore these are not shown.

Results

Decreased expression of mTOR pathway-related genes and mTOR activity in circulating B cells from pSS patients correlates with B cell hyperactivity

Contemplating the prominent B cell hyperactivity in pSS we investigated mTOR-associated genes in circulating B cells. Unexpectedly, *RPTOR,* an important component of the mTORC1 complex, and *IGF1R,* cellular survival receptor activating the mTOR pathway, expression were significantly decreased in B cells from pSS patients as compared to HC (**Figure 1B**). In the B cells from nSS patients, *RPTOR* expression was significantly decreased. These results were technically validated by RT-qPCR (**Supplementary Figure 1**). Decreased expression of *RPTOR* and *IGF1R* in all sicca patients significantly correlated with increased serum IgG (**Figure 1C**). Correlations of the separate groups were as follows; pSS: *RPTOR* r=-0.44, p=0.15, *IGF1R* r=-0.55, p=0.08, nSS: *RPTOR* r=-0.49, p=0.03, *IGF1R* r=-0.43, p=0.08. No significant correlations with other clinical parameters were found. The remaining mTOR-related genes measured were not differentially expressed between the groups. Since *RPTOR* but not *RICTOR* was decreased, we further investigated the mTORC1 pathway. Downregulation of mTORC1 activation in circulating B cells from pSS patients was confirmed by decreased phosphorylated S6 protein (pS6) expression (**Figure 1D,E**), which was found in both naïve (CD27-) and memory (CD27+) B cells (**Supplementary Figure 2**). A similar trend was seen in T cells (**Figure 1D,E**). Gating strategy is shown in **Supplementary Figure 3.** We hypothesized that reduced mTOR activity in circulating B cells may reflect migration of activated B cells to the salivary glands and next studied labial salivary gland (LSG) mTOR activity .

Increased numbers of B cells and T cells with mTORC1 activation in the salivary gland of pSS patients correlates with B cell hyperactivity

Immunofluorescent co-localization showed presence of increased numbers of B cells and plasma cells with mTORC1 activation (pS6 expression) in the LSG from pSS patients (**Figure 2A,B,** nSS shown in **Supplementary Figure 4**). Observing that numerous non-B cells expressed pS6, we next identified CD3+ T cells as another major cell type expressing mTORC1 activity (**Figure 2C,** nSS shown in **Supplementary Figure 4**). In light of our recent identification of the CCL25/CCR9-axis as a potential driver of B cell activity(11), we also assessed mTORC1 activity in CCR9+ cells and observed an increase in pSS patients (mean±SD 13.7±24.5 in pSS versus 1.1±0.2 in nSS). Numbers of T cells and plasma cells, but not B cells or CCR9+ cells with mTORC1-activity, correlated with increased percentages of IgM and IgG-expressing plasma cells (**Figure 2D**). In addition, percentages of T cells, B cells and plasma cells expressing pS6 were calculated. The percentage of pS6+ T cells, but not B cells nor plasma cells were elevated in pSS patients (**Supplementary Figure 5**). The percentages did not correlate with clinical parameters including LFS (not shown).

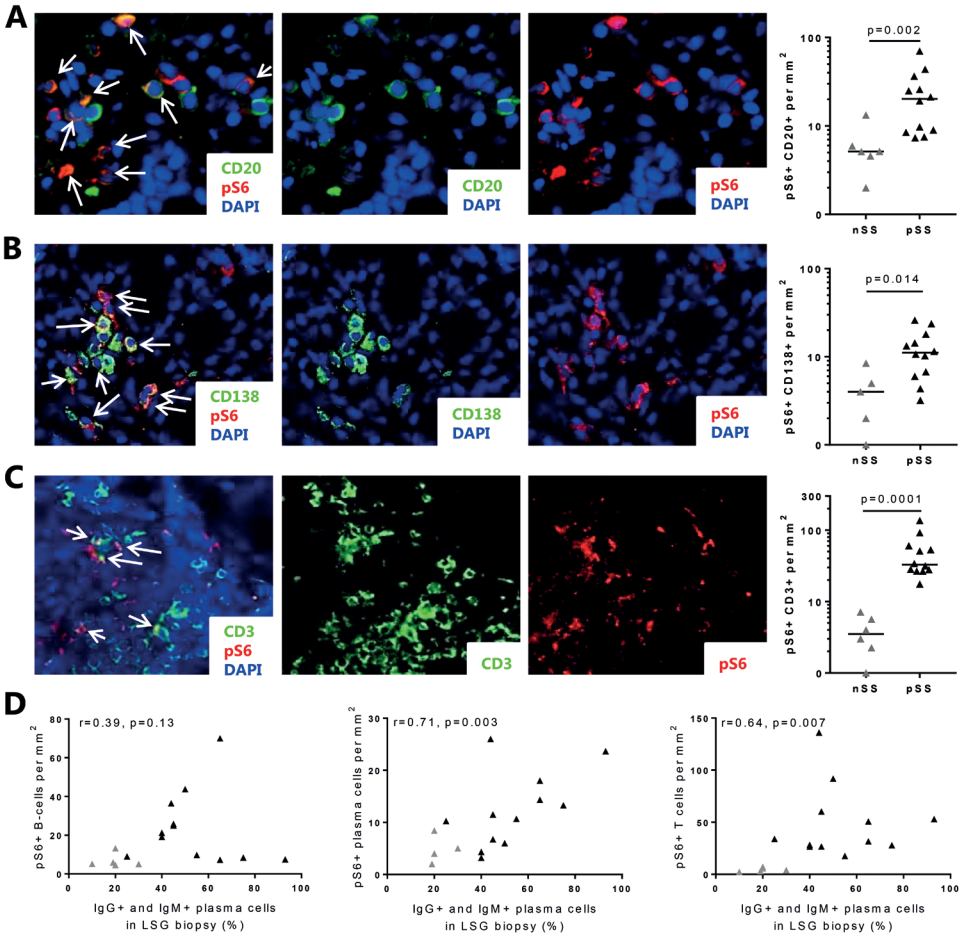

Figure 2. Elevated numbers of B cells, plasma cells and T cells with activated mTORC1 in salivary glands of pSS patients correlate with B cell hyperactivity.

In the salivary glands of pSS patients, increased numbers of B cells (**A**), plasma cells (**B**) and T cells (**C**) with activated mTORC1 were observed as compared to nSS patients. (**D**) Numbers of T cells and plasma cells with activated mTORC1 (pS6+) as assessed by immunofluorescence correlate with IgG+ and IgM+ plasma cells. Medians are shown. pSS: primary Sjögren's syndrome; nSS: non-Sjögren's sicca. Magnification: 400x.

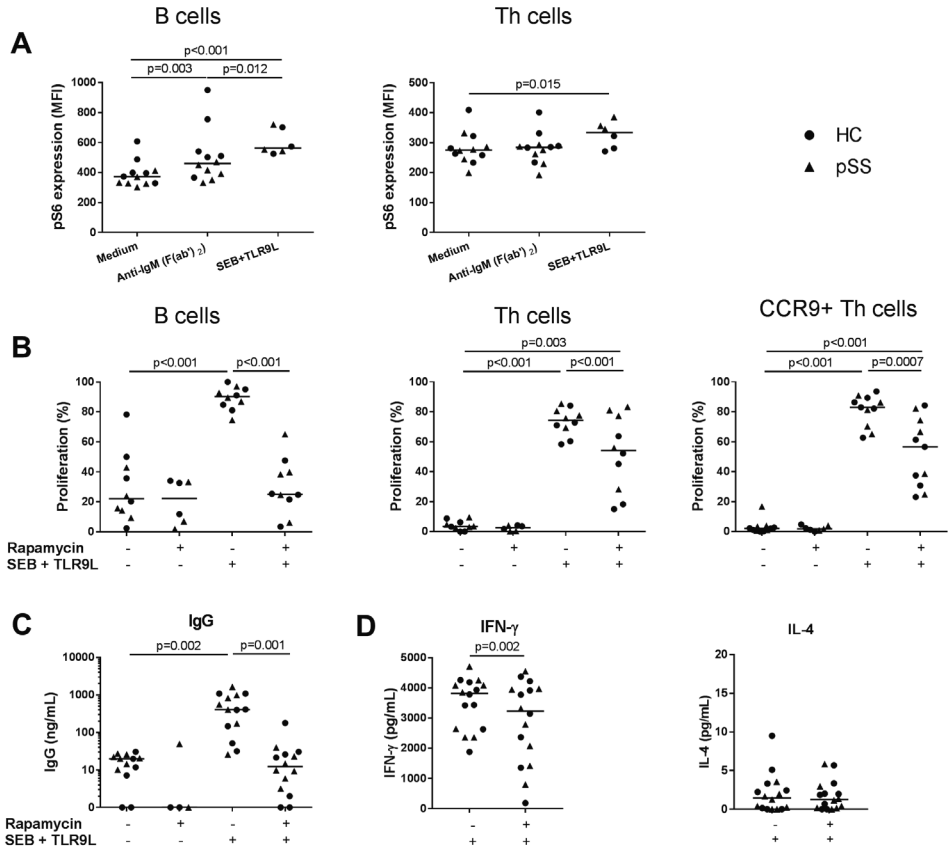

Figure 3. B cell and T cell proliferation as well as production of IgG and IFN-γ are inhibited by mTOR targeting *in vitro*.

(**A**) B cell receptor crosslinking results in increased mTORC1 activation (phosphorylation of S6) in B cells. Activation of T cells, including CCR9+ Th cells and B cells by a combination of superantigen SEB and TLR9-ligand CpG-C induces mTORC1 activation and is associated with proliferation of these cells (**B**) and IgG and IFN-γ production (**C**), which is inhibited by rapamycin (100nM). For all graphs: healthy controls (HC, circles), primary Sjögren's syndrome patients (pSS, triangles). Medians are shown.

mTOR inhibition robustly decreases B cell and T cell activation

Activation of PBMCs with a combination of TCR crosslinking superantigen SEB and TLR9 ligand resulted in increased phosphorylation of S6 in CD4+ T helper (Th) cells and B cells (**Figure 3A**), associated with Th cell and B cell proliferation (**Figure 3B**) and production of IFN-γ and IgG (**Figure 3C,D**) in both HC and pSS. BCR crosslinking by anti-IgM was used as a control condition and induced mTORC1 activation in B cells but not in Th cells (**Figure 3A**)(12). Rapamcyin most optimally inhibited T and B cell proliferation at 100 nM (**Supplementary Figure 6**). At this concentration rapamycin inhibited mTOR activity and reduced Th cell and B cell proliferation (**Figure 3B**) and production of IFN-γ and IgG (**Figure 3C,D**), of both HC and pSS patients. All inhibitions by rapamycin were significant in both HC and pSS ($p<0.05$), for Th cells and CCR9+ Th cells in pSS (both $p=0.08$) and IFNy in HC ($p=0.09$), similar trends were found. IL-4 production was low and unaffected by rapamycin (**Figure 3D**). This corroborates the large body of literature in which inhibition of T and B cell activity by rapamycin is shown (5,6,13,14). Proliferation of CCR9-expressing Th cells and CD3+CD4- (CD8/γδT/NKT) cells were inhibited similarly to total Th cells (**Figure 3B** and **Supplementary Figure 7**). Rapamycin did not significantly affect viability of lymphocytes (**Supplementary Figure 6**).

Discussion

We here for the first time studied mTOR activity in circulating and salivary gland lymphocytes from pSS patients and found increased mTORC1 activity in salivary gland B cells and T cells, which was associated with local and systemic B cell hyperactivity. Proliferation of B cells, Th cells, Tc cells and CCR9+ Th cells and production of IgG and IFN-γ could be effectively halted *in vitro* with mTOR inhibition using rapamycin, affecting proliferation and IgG production more strongly than IFN-γ production.

The downregulation of mTOR pathway-related genes in circulating B cells of pSS patients inversely correlated with increased serum IgG levels. As elevated numbers of B cells with activated mTORC1 pathway were found in salivary glands of pSS patients, this could indicate that activated B cells with higher mTOR activity have migrated to involved organs. Supporting this hypothesis, decreased numbers of memory B cells have been found in the peripheral blood of pSS patients(15). In our small cohort we did not observe significant reduction of frequencies of memory B cells (not shown), but we did observe reduced pS6 expression in both naïve and memory B cells in pSS patients as compared to healthy controls. This could mean that both activated memory and naïve B cells migrate to the inflamed tissues or alternatively lymph nodes. Reduced expression of mTOR related genes might alternatively be caused by systemic mediators causing down-regulation. The factors inducing such downregulation have not been identified.

Also in patients with nSS, downregulation of *RPTOR* in circulating B cells was found, possibly since some of these patients show signs of immune activation. In this respect, we observed local B cell hyperactivity in some nSS patients, like pSS patients. In addition, recently we demonstrated that in serum and circulating antigen-presenting cells like cDCs and pDCs molecular aberrances were significantly overlapping with pSS patients (Hillen et al., manuscript in preparation). Alternatively, B cells (and T cells) with reduced pS6 expression in nSS patients may also be caused by increased migration of B and T cells to the salivary gland. Recently, using a novel method to count cells in salivary gland tissue (epigenetic cell counting) we demonstrated that nSS patients have equal numbers of B and T cells in their salivary glands as a large proportion (~50%) of pSS patients (in both groups ~ 20% of all cells)(16). In this respect nSS patients may represent a group with signs of inflammation, but lacking lymphoid organisation (1 focus/4mm^2) that is associated with significant less mTOR activation in the glands. Future studies on the molecular and cellular characteristics of nSS patients may add to characterization of this poorly defined group both in the clinics and in research.

IGF-1 receptor triggering previously has been demonstrated as one of the upstream activators of mTOR (17-20). Furthermore, IGF-1 has shown to play role in B cell activation and function (21). Interestingly, we found *IGFR1* to be expressed in B cells from pSS patients at lower levels than in B cells from healthy controls. This was in line with decreased activation of the mTOR pathway. Downregulation of *IGF1R* may be one of the mechanisms by which decreased mTOR activation is found in peripheral blood B cells of pSS patients. Other receptors upstream of mTOR potentially involved in downregulation in pSS still remain to be studied.

To indicate mTOR pathway activation, we measured the phosphorylation of ribosomal protein S6 by flow cytometry in circulating lymphocytes and immunofluorescence microscopy in salivary gland tissues. Antibodies against two different positions of S6 phosphorylation for the two techniques were measured for technical reasons. Although both indicators of mTOR activity, phosphorylation of S6 at serine 240/244 (FACS) is considered to be more specific mTOR activation marker than at serine 235/236 (IF) as also kinases RSK1 and RSK2 downstream of the ERK pathway can in some conditions phosphorylate the latter position(22). Nonetheless, the phosphorylation status of both positions of S6 strongly correlate. Furthermore, B cells from mice deficient for *Rptor*, an essential component of mTORC1 complex (23,24), have distinguished loss of S6 phosphorylation at serine 235/236. Furthermore, phosphorylation of S6 at serine 235/236 is sensitive to rapamycin in BAFF-stimulated primary B cells (13) indicating that S6 phosphorylation at serine 235/236 largely reflects mTOR pathway activation in B cells.

The mTOR pathway plays an important role in the development and function of B cells, including in germinal centres(3). In addition, mTOR is critical for differentiation of Tfh cells, which are essential for germinal center reactions(3). Development of germinal centre-like structures in the exocrine glands is associated with an increased risk of developing B cell lymphoma(2). Although we did not investigate the association of mTOR and germinal centres we did find a clear correlation of CD3 T cells and B cell hyperactivity in the salivary glands. This clear correlation was not observed for CD20 B cells and B cell hyperactivity, which supports the fact that T cell-driven B cell activation and mTOR activation play a crucial role in pSS immunopathology.

We recently described increased CCR9-expressing Th cells and their ligand CCL25 in pSS salivary glands(11). CCR9+ Th cells are potent drivers of B cell hyperactivity and in mice were shown to induce salivary gland inflammation and CD8+ T cell-dependent immunopathology(25). Hence, CCR9+ Th cells that have Tfh-like characteristics and possibly play a role in germinal centre formation, may play a significant role in pSS pathogenesis(11,25). In this study we found increased CCR9+ cells with activated mTORC1 pathway in the LSG of pSS patients and demonstrated inhibition of proliferation of blood CCR9+ Th cells by rapamycin. This, next to inhibition of mTOR activity of B cells and Tc cells, indicates that mTOR inhibition may limit immunopathology induced by both Tfh and CCR9+ Tfh-like cells. CCR9-expressing cells, other than Th cells, that migrate to the salivary glands in response to increased CCL25 may include B cells, plasma cells, CD8 T cells and pDCs. Corroborating this we demonstrated considerable proportions of pS6-expressing cells to co-express CCR9 (~22%).

Corroborating the potential of mTOR targeting for pSS, Shah et al. reported that local administration of rapamycin inhibits infiltration of lymphocytes in the exocrine glands in the NOD mouse model and restores tear production(26,27). This was associated with suppression of many inflammatory mediators, including CXCL13, CCL19 and CCL20, chemokines associated with formation of ectopic lymphoid structures. Treatment with rapamycin has been shown to be safe and effective in a phase II trial with SLE patients, decreasing disease activity without withdrawals due to adverse effects(8). However, adverse effects due to rapamycin treatment, including leukopenia and infections, are known. Interestingly, the antidiabetic drug metformin, which has a more favourable safety profile, inhibits mTOR and reduced B cell differentiation into autoreactive plasma cells and formation of germinal centres in a murine SLE model(28).

Thus, our data indicate a role for mTOR activity in B cell hyperactivity in pSS and identify mTOR inhibition as a novel potential therapeutic strategy for this disease. As such, studying the efficacy of (combination) therapy using mTOR inhibitors with favourable toxicity profiles in patients with pSS should be pursued.

Acknowledgements

We would like to thank Prof. Dr. R. Goldschmeding and R. Broekhuizen from the Pathology department of the University Medical Center Utrecht for advice and supply of reagents for immunofluorescence experiments, E.H.M. Otten-van der Heijden for advice on culture experiments and Dr. C.P.J. Bekker for performing RT-qPCR experiments.

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Supplementary material

Supplementary File 1. Methods

Gene expression of isolated B cells

PBMCs were freshly isolated from blood by Ficoll-Paque density centrifugation. Subsequently, CD19+ B cells were isolated by MACS following the manufacturer's instructions (Miltenyi Biotec) using positive selection using bead-labelled anti-CD19 antibodies, with a purity of 97±3%. Isolated cells were lysed in RLT+ (Qiagen) with β-mercaptoethanol and stored at -80°C until further use. RNA was extracted using the Allprep universal kit (Qiagen) according to the manufacturer's instructions. Subsequently cDNA was created using the Biorad iScript kit. Quantitative Polymerase Chain Reaction (qPCR) was performed on a Quantstudio QPCR apparatus, with Taqman Beadchip technology (Applied Biosystems) under conditions as specified by the manufacturer. As housekeeping genes *GUSB* and *GAPDH* were included. Expression of the following mTOR pathway-related genes was assessed: *MTOR* (Hs00234508_m1), *RPTOR* (Hs00375332_m1), *RICTOR* (Hs00380903_m1), *DEPTOR* (Hs00961900_m1), *AKT1* (Hs00178289_m1), *IGF1R* (Hs00609566_m1), *IGF1* (Hs01547656_m1) and *PTEN* (Hs02621230_s1). Technical validation of *RPTOR* (Hs00375332_m1) and *IGF1R* (Hs00609566_m1) expression was performed by Taqman single gene qPCR assays. Expression was normalized to *GUSB* (Hs00939627_m1) and *GAPDH* (Hs02758991_g1) and subsequently was calculated as a fold change relative to the mean of the healthy control group set at 1, using the delta-delta CT method.

Immunofluorescence

To study local mTOR activation in salivary glands, immunofluorescence staining was performed on frozen salivary gland tissue sections from pSS and nSS patients using mouse monoclonal anti-CD20 (L26, Dako), anti-CD138 (MI15, Dako) anti-CD3 (F7.2.38, Dako) and anti-CCR9 (MAB1791, R&D) and rabbit anti-pS6 monoclonal antibody (Ser235/236, D57.2.2E, XP #4858, CellSignaling). Fluorescent labelled secondary antibodies were used: goat anti-mouse AF488 and goat anti-rabbit AF555 (Life Technologies). To assess specificity of the anti-pS6 antibody, as a negative control we followed the same protocol leaving out the primary antibody for pS6. No staining other than CD3, CD138, CD20 or CCR9 was observed (not shown). The surface area of each section was determined to quantify the number of positive cells per mm2, using a magnification of 400x, the mean size of the biopsies was 8mm2.

Flow cytometry

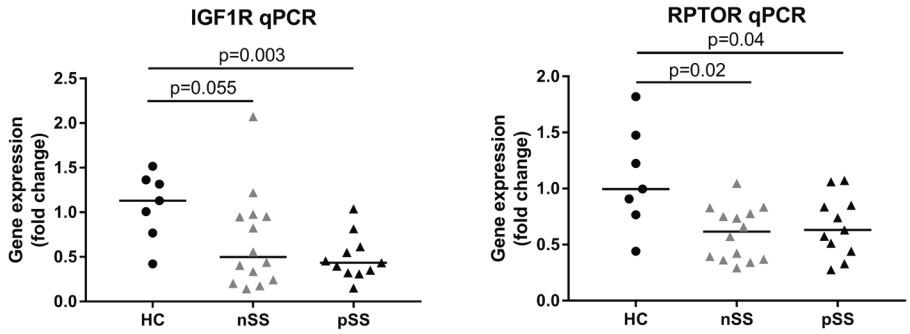
To analyse activation of mTORC1 in different cell subsets, antibodies against CD45 (PerCP, HI30, BioLegend), CD19 (BV605, SJ25C1, BD), CD20 (PE-Cy7, B9E9 Beckman Coulter), CD27 (PE, O323, Sony Biotechnology, APC-ef780 O323, eBioscience) , CD38 (APC, HIT2, eBioscience), IgG (FITC, Southern Biotec), CD3 (AF700, UCHT1, Sony Biotechnology), CD4 (BV711, OKT4,

Sony Biotechnology), CD14 (BV785, M5E2, BioLegend) and CCR9 (PE, 248621, Bio-Techne) were used. Fresh PBMCs were used, which were first stained with Fixable Viability Dye (eBioscience), after which antibodies for extracellular staining were used, followed by fixation and permeabilization and staining with intracellular antibodies. The eBioscience Fixation/ Permeabilization kit (#00-5123-43 and #00-5223-56) was used for intracellular staining and isotype control staining was performed (IgG1-FITC, REA control, Miltenyi Biotec). Activation of mTORC1 was assessed by intracellular detection of phosphorylated S6 ribosomal protein (pS6) expression (anti-S6 pS240 FITC-conjugated, clone REA420, Miltenyi Biotec), indicating kinase (S6K) activity downstream of mTORC1. Viability of PBMCs after culture experiments was assessed by Fixable Viability Dye (eBioscience) staining and proliferation of T and B cells was assessed using CellTrace Violet (ThermoFisher, Life Technologies), both according to manufacturer's instructions.

Cell culture

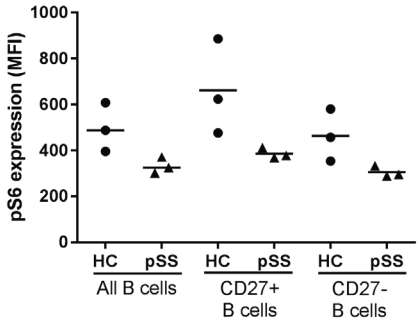
Peripheral blood mononuclear cells (PBMCs, $0.5x10^6$ per well in 48 well plates) from healthy controls (n=2-7) and pSS patients (n=2-8) were cultured in the presence of different concentrations of rapamycin (1-100 nM, Sellek Chemicals) to study the effect of mTORC1 inhibition on T and B cell proliferation (6 days) and production of IFN-γ and IgG (9 days). mTOR pathway activation in B cells was done by BCR crosslinking using anti-IgM $F(ab')_2$ fragments (Jackson ImmunoResearch Laboratories). To mimic the activation of lymphocytes observed in pSS patients, T and B cell activation was induced with a combination of superantigen Staphylococcal Enterotoxin B (SEB, 0.1ng/mL) and a TLR9 agonist (CpG-C, 3.3μg/mL). IgG production was measured in culture supernatants by ELISA (Bethyl Laboratories) and IFN-γ concentrations were assessed by Luminex.

Supplementary figures



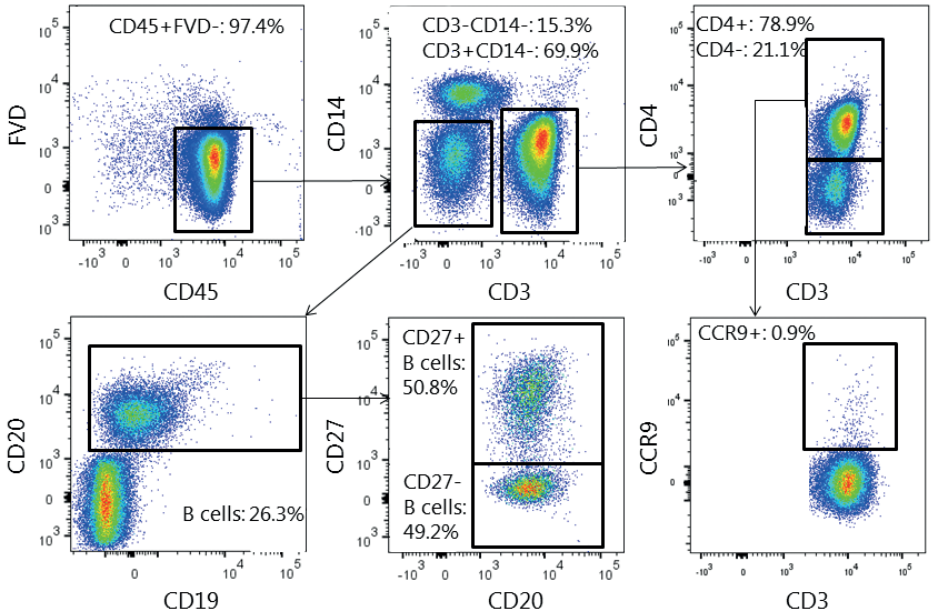
Supplementary Figure 1. Decreased expression of *RPTOR* and *IGF1R* in pSS patients is technically validated by single qPCR.

Single qPCR was performed which confirmed the decreased expression of *RPTOR* and *IGF1R* in pSS patients versus healthy controls as found using TaqMan Beadchip technology of which results are shown in Figure 1.



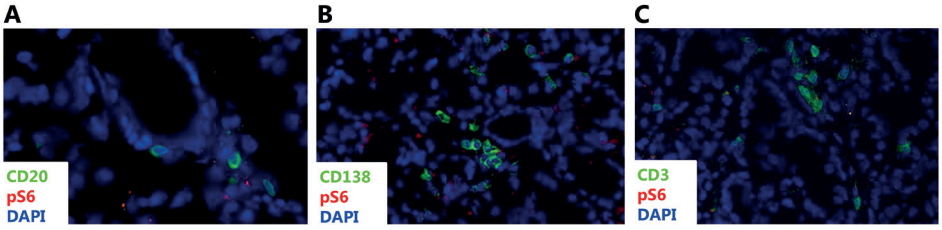
Supplementary Figure 2. Decreased pS6 expression in both naïve and memory B cells from pSS patients as compared to healthy controls.

pS6 expression (median fluorescence intensity) is shown for circulating total B cells, CD27- naïve B cells and CD27+ memory B cells. Healthy controls (HC, circles), primary Sjögren's syndrome patients (pSS, triangles).

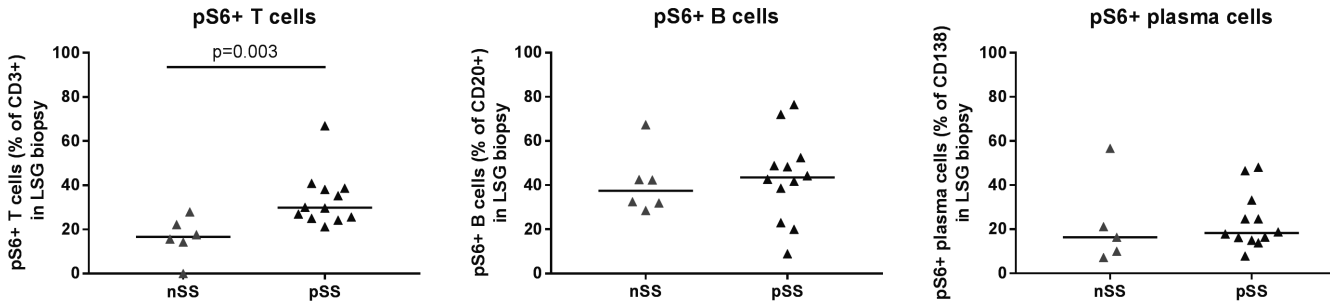


Supplementary Figure 3. Gating strategy for B cells, Th cells and CCR9+ Th cells.

The gating strategy used for flow cytometry experiments is shown. Within the PBMCs, singlets are selected (not shown) after which viable leukocytes are identified (Fixable Viability Dye negative, CD45 positive). Within the CD14-CD3- subset, B cells are identified based on CD19 and CD20 expression. B cells are divided in CD27- naïve and CD27+ memory B cells. The CD3+ CD14- subset is used to identify CD4+ Th cells and CD4- cytotoxic T cells (in which also NKT and γδ T cells are present). Within the CD4+ Th cells, CCR9+ Th cells are identified.

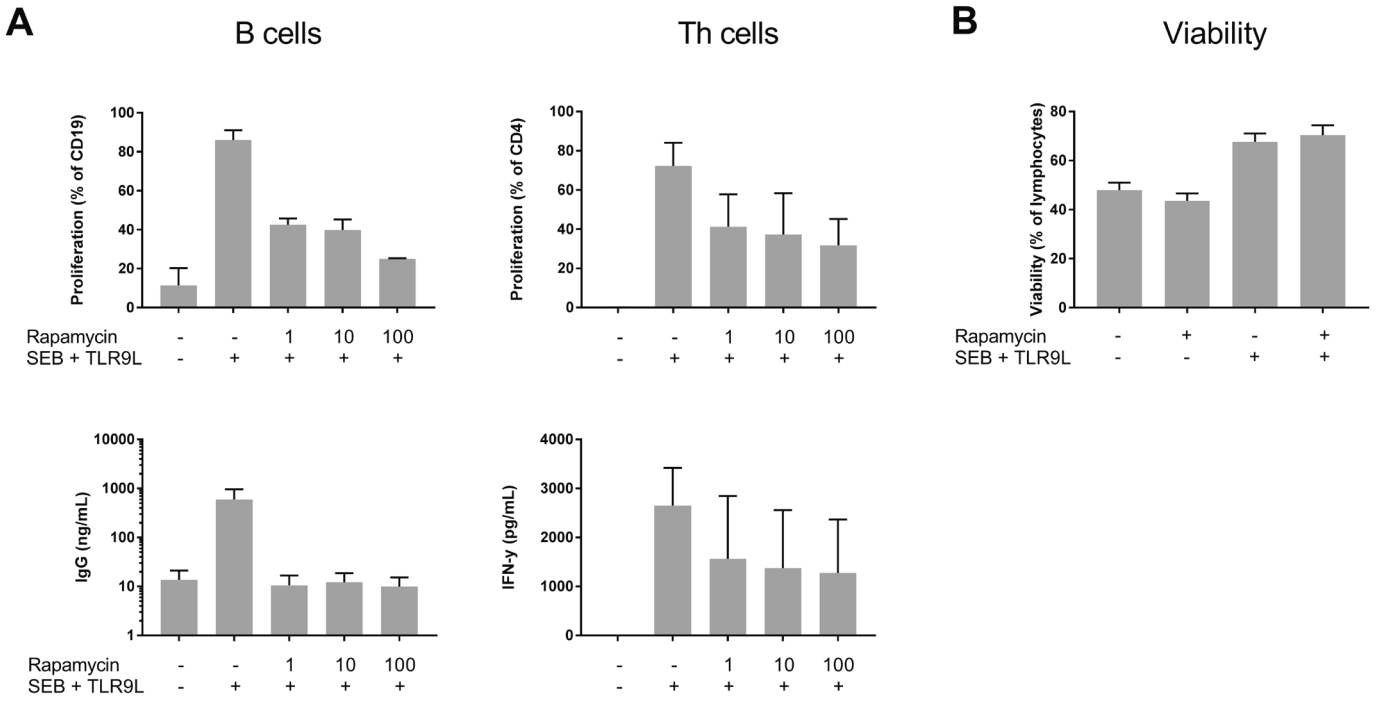


Supplementary Figure 4. Low numbers of lymphocytes with mTOR activity in nSS salivary glands.
Representative immunofluorescence images showing staining for (**A**) CD20 B cells, (**B**) CD138 plasma cells and (**C**) CD3 T cells with co-localisation of pS6 indicating mTORC1 activation. Data are shown in Figure 2. Magnification: 400x.



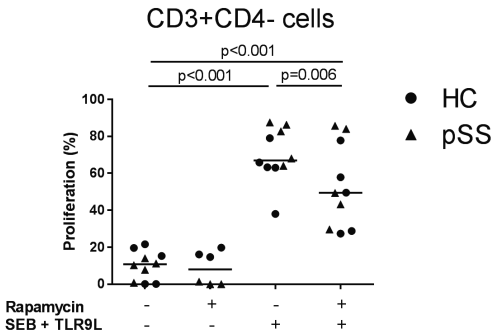
Supplementary Figure 5. Elevated percentages of pS6+ T cells, but not B cells or plasma cells in salivary glands of pSS patients.

Percentages of pS6-expressing T cells, B cells and plasma cells, were calculated as a percentage of the total number of T, B and plasma cells, respectively. Healthy controls (HC, circles), primary Sjögren's syndrome patients (pSS, triangles).



Supplementary Figure 6. Rapamycin inhibits T cells and B cells dose dependently without affecting viability.

(**A**) Activation of T cells and B cells by a combination of superantigen SEB and TLR9-ligand CpG-C results in proliferation and IgG and IFN-γ production, which is inhibited by rapamycin, in a dose dependent manner for IFN-γ (n=2-4) without affecting viability of cells (n=6) (**B**). Graphs depict Mean+SEM.



Supplementary Figure 7. Proliferation of CD3+ CD4- cells is inhibited by mTOR targeting *in vitro*.

Activation of T cells and B cells by a combination of superantigen SEB and TLR9-ligand CpG-C induces proliferation of CD4-CD3+ cells, which is inhibited by rapamycin in both healthy controls and patients (100nM). Medians are shown.

CHAPTER 10

Summary and discussion

Emerging roles of chemokines and cytokines as orchestrators of immunopathology in Sjögren's syndrome.

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Parts of this chapter are under review (Rheumatology):

Summary

Formation of lymphocytic infiltrates in exocrine glands and signs of systemic and local B cell hyperactivity are hallmark features of Sjögren's syndrome (SS). In this thesis, novel tools to study the contribution of immune cells and inflammatory mediators to immunopathology in the salivary glands of SS patients were explored. In particular, mechanisms potentially involved in lymphoid neogenesis and B cell hyperactivity were studied. With respect to the latter, the role of CCR9-expressing T helper cells was delineated, since these cells, have T follicular helper cell-like characteristics and play a major role in mucosal tissue homeostasis. Summarized below are the main findings reported in this thesis.

In **Chapter 2** the "salivary gland secretome" is explored as a novel tool to detect soluble mediators produced in the salivary glands of SS patients. Supernatants (that we termed 'secretomes') of diagnostic labial salivary gland (LSG) biopsies are collected without interference with clinical practice and were left for 1 hour in saline that was used as part of the routine surgical procedure. With Luminex technology n>100 analytes were measured in tissue supernatants of a discovery cohort. Surprisingly, using this simple method, many mediators were detectable in the tissue supernatants. Results were validated in an additional cohort demonstrating a number of robustly increased, partly newly identified, inflammatory mediators (elevated expression of 11 mediators: CXCL13, CCL19, CXCL10, TIM-1/KIM-1, IL-31, IL-2, GM-CSF, IL-23, sIL-2R, sIL-7Rα, and CCL25 in primary Sjögren's syndrome (pSS) vs non-Sjögren's sicca (nSS)), correlating with clinical parameters. To investigate potential diagnostic and prognostic value and a potential role in patient stratification in the future, classification tree analysis was performed, showing accurate separation of pSS vs nSS patients.

In **Chapter 3**, an additional novel tool to investigate local immunopathology in pSS immunopathology was assessed, called "epigenetic cell counting" (ECC). Using DNA extracted from diagnostic labial salivary gland biopsies immune cell frequencies were quantified based on cell subset-specific demethylated genetic loci. Immune cell frequencies were validated by mRNA expression and immunohistochemistry and correlated with lymphocytic focus scores (LFS), confirming reliable assessment of immune cell frequencies in LSG biopsies. Using ECC, a cluster of patients with the most severe immunopathology could be identified, indicating a potential role in patient stratification in the future.

In **Chapter 4**, using the secretome assay soluble IL-7Rα levels were measured in salivary gland biopsy supernatants (described in Chapter 2) from pSS and nSS patients and in serum from healthy individuals and pSS patients, for the IL-7/IL-7R axis play a role in ectopic lymphoid neogenesis and has been shown to play an important role in pSS immunopathology. Elevated levels of sIL-7R were observed in both tissue supernatants and serum from pSS patients correlating with clinical parameters. This study further substantiates the role of the

IL-7/IL-7R axis in pSS and indicates that sIL-7R may be a biomarker for patients with more severe disease.

In **Chapter 5**, frequencies and phenotype of circulating IL-7R+ innate lymphoid cells (ILCs) were studied in pSS and systemic lupus erythematosus (SLE) patients in relation to the interferon (IFN) signature. ILCs play an important role in epithelial tissue homeostasis and group 3 ILCs are involved in both the initiation of development of secondary lymphoid organs and ectopic/tertiary lymphoid structures, which is dependent on IL-7. These characteristics indicate a potential role for these cells in pSS and SLE, in which epithelial cells in the target tissues are involved. In addition,, ELS develop and IL-7 has been implicated to play a critical role in the disease pathogenesis. Recently, presence of ILC3-like cells is described by others in pSS salivary glands. In our study, increased circulating frequencies of ILC1s were found in SLE patients and in pSS patients with high disease activity. In addition, decreased frequencies of ILC2s and ILC3s were observed in patients with an interferon (IFN) signature. Recently, it has been shown that survival of ILCs is dependent on IFN induced susceptibility to apoptosis. Hence, the decreased frequencies of circulating ILC2s and ILC3s might be caused by IFN activation in part of pSS and SLE patients.

The CCL25-CCR9 interaction plays an important role in mucosal homeostasis and is involved in inflammatory bowel diseases. Recently, CCR9+ T helper (Th) cells have been described to have Tfh-like characteristics, expressing PD-1, ICOS, IL-21 and Bcl6, but not CXCR5 which is also characteristic for Tfh cells. CCR9+ Th cells in addition produce high levels of IFNγ and IL-10 and can potently stimulate B cells to produce immunoglobulins and can activate CD8+ T cells. They have been shown to play a role in NOD (non-obese diabetic) mice spontaneously developing sialadenitis and type 1 diabetes. In addition, elevated frequencies of CCR9+ Th cells were observed in the circulation of pSS patients. In **Chapter 6**, we demonstrate that CCL25 is overexpressed in pSS salivary glands correlating with B cell hyperactivity and markers of lymphoid neogenesis. In accordance with the increased expression of CCL25, elevated numbers of CCR9-expressing cells are present in pSS as compared to nSS salivary glands. In addition, we found that both in HC and pSS, CCR9+ Th cells are characterized by high expression of ICOS, PD1 and IL-7Rα and production of high levels of cytokines upon stimulation with IL-7 and upon antigenic triggering. These findings indicate that the CCL25-CCR9 axis potentially plays a role in pSS immunopathology.

In **Chapter 7**, we further investigated CCR9+ Th cells by performing transcriptome analysis of sorted cells from the circulation of HC and pSS patients, comparing them to CXCR5+ Th cells and CCR9-CXCR5- Th cells. Gene network analysis and pathway enrichment analysis revealed differential regulation of effector T cell functions between the three subsets. CCR9+ Th cells were characterized by elevated expression of genes involved Th1 functions and adhesion and migration. Target genes were validated in an additional cohort by qPCR or on protein

level using flow cytometry. Interestingly, the chemokine CCL5 was found as a novel effector molecule of CCR9+ Th cells.

In **Chapter 8**, co-expression of the chemokine receptors CXCR3, CCR4, CCR6 and CCR10 was assessed on circulating CCR9+ vs CCR9- Th cells, in combination with measurement of expression of these receptors and their ligands CXCL10, CCL17, CCL20 and CCL28 in LSG tissue. Elevated percentages of CCR9+ Th cells express CXCR3, CCR4 and CCR6 and in pSS patients a relative decrease of circulating CCR9+CXCR3+ and CCR9+CCR6+ Th cells is observed. In LSG tissue an increase of CXCL10 expression was observed, correlating with CCL25. In vitro, synergistic effects of CCL25 and CXCL10 on migration of Th cells was found. Altogether, these findings indicate a concerted role for CCL25 and CXCL10 in facilitating migration of CCR9/CXCR3 expressing Th cells towards exocrine glands in pSS.

In **Chapter 9**, activation of the mTOR pathway is studied in circulating and salivary gland lymphocytes of SS patients as compared to nSS patients and/or HC. The mTOR pathway plays an important role in activation of T and B lymphocytes, including in germinal center reactions and mTOR inhibition is immunosuppressive. Decreased expression of mTOR-related genes and decreased mTOR activation was found in peripheral blood B cells and from SS patients, while elevated mTOR activation of local lymphocytes, including CCR9+ Th cells, was found in the salivary glands, correlating with B cell hyperactivity. *In vitro* induced activation of T cells (including CCR9 Th cells) and B cells is successfully halted by mTOR inhibition in both HC and SS. These findings suggest that the mTOR pathway is a potential therapeutic strategy to consider for SS treatment.

Discussion

Innovative techniques to study local immunopathology in Sjögren's syndrome

Salivary gland (SG) biopsy has an important place in diagnostics of SS. The lymphocytic focus score (LFS), as enumerated in H&E-stained salivary gland tissue sections, forms part of routine clinical diagnostics and has an important position in the classification criteria for SS. In addition, the amount of lymphocytic infiltration and the presence of GC-like structures form risk factors for development of lymphoma and extraglandular manifestations. However, this method of analyzing salivary gland inflammation has several drawbacks, including lack of standardization of acquisition and processing of the salivary gland tissue, subjective histological interpretation and poor correlation of the scored abnormalities with dryness assessments. Limited information from SG biopsies is used in clinical practice, the LFS is scored as a number of infiltrates (foci) per 4 mm^2, and in some centers a plasma cell shift towards relatively less IgA-expressing plasma cells is scored, and presence of GC-like structures is determined. The number of lymphocytes, the size of the infiltrates or the distribution of immune cell subtypes are not taken into account. Efficient techniques to extract more information from SG biopsies may increase the diagnostic and prognostic value, and may increase knowledge of SS immunopathology.

In this thesis, two novel tools were explored to potentially aid in this process. Using the 'salivary gland secretome' a large number of soluble mediators were identified simultaneously (**Chapter 2**). This new assay could serve to better comprehend immunopathology, monitor therapeutic efficacy and facilitate classification and potentially prognosis and diagnosis. In addition, a big advantage as compared to saliva proteomics is that it can also be applied to the driest patients that produce no saliva. Using 'epigenetic cell counting' many cell subsets with functional properties can be quantified. This novel method seems promising to quantify tissue infiltrating immune cells objectively and in a standardized manner using a relatively small amount of tissue (**Chapter 3**). In both cases, subgroups of patients with high levels of cytokines or high numbers of immune cells and immune cell subsets were identified. In addition, a subset of patients was identified which was not diagnosed as SS, but did have aberrant cytokine levels or immune cell frequencies. Longitudinal studies in larger cohorts may reveal whether these findings are relevant for the prognosis of these patients. If the value of these techniques will be confirmed, these may form a more objective and standardized analysis of biopsies, independent of experienced technicians and pathologists. Also, future research regarding these techniques may show that smaller amounts of tissue are needed than are currently used for diagnostic/prognostic histopathological analysis, which may decrease the burden of the biopsy procedure for patients. In addition, these novel techniques may be useful tools for clinical trials to monitor treatment response and to identify biomarkers, contributing to patient-tailored treatments.

Chemokines and chemokine receptors as orchestrators of Sjögren's syndrome immunopathology including formation of ectopic lymphoid structures

Chemokines are chemotactic cytokines that regulate the migration and positioning of cells of the immune system. They constitute the largest family of cytokines, with currently more than 40 endogenous human ligands. Based on their structure and binding properties these are divided in 16 CXCL, 28 CCL, 2 XCL members and CX3CL.(1) Chemokines signal cells via 18 chemokine receptors that are differentially expressed on leucocytes to tightly control their function, in particular migratory properties. These are divided in 6 CXCR, 10 CCR, 1 XCR receptors and CX3CR1, that respectively bind CXCL, CCL, XCL and CX3CL ligands. In addition, chemokines can bind to atypical chemokine receptors that do not signal but that can significantly impact immune functions by chemokine scavenging, chemokine transcytosis or shaping chemokine gradients.(1) Through their complex and dynamic expression patterns chemokines and their receptors have been shown to be key mediators in acute inflammation and in generation of primary and secondary adaptive cellular and humoral responses. Hence, these mediators of inflammation are critical for host defence during infection but also immunity against cancers.(1,2) In patients with primary Sjögren's syndrome (pSS) roughly 15 chemokines have been studied on protein and RNA level. Here we review our current understanding of their role in pSS and we discuss the findings presented in this thesis regarding chemokines and chemokine receptors.

Hallmark features of pSS are formation of lymphocytic aggregates in the inflamed exocrine glands and B cell hyperactivity as witnessed by e.g. autoantibody formation (including anti-Ro/SSA and anti-La/SSB, rheumatoid factor), increased serum IgM and IgG levels and elevated risk of B cell lymphoma development.(3,4) Numerous molecular and cellular processes have been demonstrated to contribute to the homing, positioning and function of inflammatory cells in the exocrine glands. These cells include T and B cells that mediate acquired immunity and innate cells such as macrophages and dendritic cells that orchestrate the inflammatory response by presenting antigen, provision of co-stimulatory signals and production of numerous pro-inflammatory mediators, including cytokines and chemokines.(5) Many studies have captured the association of several inflammatory mediators with key processes in the inflamed glands. However, the initiating events and sequence of actions that builds up the organized lymphoid structures and immunopathology, including autoimmunity, B cell hyperactivity and dryness are largely unclear. Nonetheless, data from ex vivo measurements in biological fluids such as saliva and serum, salivary gland tissue (**Chapters 3** and **8**) and secretome (**Chapter 2** and **6**), in vitro experiments using cells from the glands and Sjögren-like disease models in mice have shed light on the important role of chemokines as immunopathological orchestrators.

The role of CCL19, CCL21, CXCL12 and CXCL13 in directing lymphoid neogenesis and B cell hyperactivity

Ectopic lymphoid structures (ELS), also known as tertiary lymphoid organs, containing germinal centre (GC)-like organization, develop in approximately 25-30% of pSS patients.(5,6) In the majority of the remaining patients lymphocytic infiltrates without these highly organized structures are present. The development and maintenance of ELS is indicated to follow many features of secondary lymphoid organs (SLOs) and formation of ELS in other inflammatory conditions as is excellently reviewed previously.(7) Although some key steps are less clear, in pSS inflamed tissues many of the ELS features are recapitulated. For instance, the role of the typical lymphoid tissue inducer (LTi) cells, contained in group 3 ILCs, that express IL-7Rα, RORγt, lymphotoxin (LT) α1β2 and RANK to initiate ELS by stimulating lymphoid tissue organizer cells (LTOs) is unclear.(8,9) LTO cells are thought to stimulate development of high endothelial venules (HEVs), necessary for entry of inflammatory cells from the systemic circulation into the exocrine glands. Although these have not been identified in pSS, activated stromal cells that acquire LTO-like properties including chemokine release and adhesion molecule expression, are likely to be present in pSS, like in other inflammatory conditions.(7,10-12). Alternatively, HEVs that are part of mucosa-associated lymphoid tissues (MALT, which is a SLO) such as present in the salivary gland (SG) may trigger recruitment of inflammatory cells. Indeed, PNAd+ HEV have been described in areas without lymphocytic infiltrates. and strongly correlate with the number of lymphocytes.(13) In addition, increased IL-7 and IL-7R-expressing cells in pSS correlate with the lymphocytic foci and the number of activated proliferating lymphocytes in the glands(14-16). In this thesis (**Chapter 4**), it was demonstrated that local and circulating levels of sIL-7R, which are indicative of the increased IL-7/IL-7R axis and potentiate function of IL-7 were associated with B cell hyperactivity and dryness in pSS patients.(17) IL-7 is a potent inducer of LTα1β2 as well as IFNγ, IL-17, IL-21, TNF and IL-4 by CD4+ T cells, all known to initiate ELS development and found to be expressed in the inflamed exocrine glands of pSS patients.(7,10,18) IL-7-activated tissue resident Th cells could initiate migration via activation of HEV, e.g. via mediators such as LTα1β2 and IFNγ that increase PNAd expression and other adhesion molecules such as VCAM-1 and ICAM-1 expression(19).

The subsequent production of the chemokines CCL19, CCL21 and CXCL13 is indicated to play pivotal roles in attracting and positioning of immune cells in the glands. Lymphocytes that enter LSG can be present in small non-segregated aggregates which can develop into larger foci where T and B cells become segregated to finally develop into ELS, associated with networks of CD21+ follicular dendritic cells (FDCs).(20)

Increased expression of CCL19, CCL21 and CXCL13 has been demonstrated in SG of pSS patients (13,21). Increased CXCL13 and CCL19 have been found in serum, saliva and in secretomes (**Chapter 2**) of SG tissue samples(22). All three chemokines correlate with

increased lymphoid organisation in pSS patients (13)(**Chapter 2**) and in one study were associated with increased xerostomia(22). The source of CCL19 and CCL21 secretion in SG has not been fully revealed but CCL21 has been found in association with HEV-like structures and in other ELS they were found to be produced by myofibroblast cells that surround the HEVs(13,20,23). Both chemokines bind to the CCR7 receptor that directs naïve T cells, antigen-experienced central memory T cells and dendritic cells to the SG. Together with CCR7, naïve B cells that express CXCR4, the receptor for CXCL12, have been shown to enter into SLOs and potentially ELS. In the germinal centre (GC), CXCL12 guides the B cells to the dark zone where somatic hypermutation takes place and it retains CXCR4-expressing T cells. CXCL12 was found to be produced by epithelial cells in salivary glands of pSS patients and was associated with CXCR4+ B and T cells. Elevated expression of CXCL12 was found in lymphomatous lesions. In addition, CXCL12 can attract plasma cells and is an important survival factor for these cells. These findings indicate a role for the CXCL12-CXCR4 interaction to attract lymphocytes in pSS and suggest a role in MALT lymphoma development.(24-26)

CXCL13 in the SG of pSS patients is produced by endothelial cells, macrophages Tfh cells and FDCs.(24,25,27) CXCL13 directs CXCR5-expressing B-cell and T follicular helper (Tfh) cell chemotaxis. In mouse models for Sjögren's disease, Cxcl13 expression in salivary tissue increases with disease progression, and its blockade modestly reduced glandular inflammation in experimental SS. In pSS patients inhibition of disease activity by abatacept and anti-CD40 blockade was associated with reduction of serum CXCL13 levels (International Symposium on Sjögren's Syndrome 2018 O-9 B Fisher et al. The novel anti-CD40 monoclonal antibody CFZ533 modulates biomarkers relevant to disease and CD40 pathways in patients with primary Sjögren's Syndrome, (28)). We recently demonstrated that CXCL13 expression in pSS patients strongly correlates with Tfh cells, associated with LFS, autoimmunity and B cell hyperactivity (**Chapter 2** and **3**). Hence, these data suggest that disease inhibition is partly mediated by prevention of Tfh migration that play a critical role in induction and maintenance of lymphoid organisation and ELS formation and B-cell activation. Tfh cells are potent B-cell stimulating cells and reside in GCs in lymph nodes. They express Bcl-6, CXCR5, ICOS, PD-1 and cytokines such as IL-21, IL-4 and CXCL13.(29,30) Activated Tfh-cells are elevated in the blood of pSS patients and the frequency of Tfh-cells correlate with autoantibodies, disease severity and aberrant memory B-cell and plasma cell subsets. Expression of IL-21, IL-4 and CXCL13 as well as Tfh numbers are increased in the salivary glands of pSS patients.(31-34) Altogether these findings support a role for CXCL13 and CXCR5+ Tfh cells in pSS.

CCL25/CCR9: a potential new axis to drive B cell hyperactivity

A novel Th cell subset was recently described that shares features with Tfh cells including production of IL-21 and ICOS and Bcl-6 expression but displays the chemokine receptor CCR9, and not CXCR5. These cells are therefore called "Tfh-like" cells.(35) In mice and humans, CCR9+ Th cells are present in SLOs.(36,37) They migrate to mucosal sites in response to the chemokine CCL25(38,39), which is the sole ligand for CCR9. CCR9+ Th cells play key roles in maintenance of mucosal immune homeostasis but are also involved in mucosal inflammation, potentially contributing to inflammatory bowel disease and primary sclerosing cholangitis.(40-42) In the inflamed intestinal tissue of Crohn's disease patients CCR9-expressing cells and CCL25 production are increased.(43,44) Inhibition of CCR9 decreased intestinal inflammation in an ileitis mouse model.(45-47) The Tfh-like features of CCR9+ Th cells and the important role in mucosal immunity makes them interesting candidates to study in pSS immunopathology. In non-obese diabetic (NOD) mice, CCR9+ T cells mediate immunopathology in mucosa-associated tissues in accessory organs of the digestive tract, including the pancreas and salivary glands. The NOD mice spontaneously develop pSS-like symptoms with infiltrating IL-21-expressing CCR9+ Th cells in the salivary glands.(35) In addition, CCR9+ Th cells were found to be increased in the circulation of pSS patients, expressing elevated levels of PD-1 and ICOS ((35,48) **Chapter 6**). A role for the CCL25/CCR9 axis in pSS is further supported by the following findings described in **Chapter 6** of this thesis. Elevated CCL25 protein and mRNA levels are found in the LSG from pSS versus nSS patients correlating with B-cell hyperactivity, autoimmunity and levels of IL-21 and soluble IL-7Rα. In accordance with the elevated CCL25 levels, the frequency of CCR9-expressing cells is increased in the LSG of pSS patients. In addition, their functional properties including their response to IL-7, a key factor in pSS pathogenesis, makes them potentially relevant in this disease. CCR9+ Th-cells have high IL-7Rα expression and secrete higher levels of IFN-γ, IL-17, IL-4 and IL-21 as compared to CXCR5+ Th-cells, both ex vivo and upon triggering with antigen or IL-7. Both CCR9+ and CXCR5+ Th cells induce IgG production by B-cells more potently than CCR9-CXCR5- Th cells. These data suggest that increased CCL25 expression in pSS LSG facilitates attraction of CCR9+ Th cells, secreting high levels of pro-inflammatory cytokines when triggered with antigen or IL-7. The associations with B-cell hyperactivity, autoimmunity and markers of lymphoid neogenesis indicate the CCL25/CCR9-axis to play a significant role in pSS immunopathology.(48)

Recent transcriptomic profiling of CCR9+ vs CXCR5+ Th cells (**Chapter 7**), indicating novel coherent gene networks, has identified high CCL5 expression by CCR9+ Th cells from pSS patients. In vitro, IL-7 and antigen stimulation strongly upregulated CCL5 secretion. Data on CCL5 in pSS immunopathology are scarce, but immunohistochemical and RNA microarray analysis have reported increased expression in SG of pSS patients(21,49). Interestingly, CCL5 and its receptor CCR5 were increased in inflamed gland in Sjögren-like disease and blockade of CCL5 can significantly reduce disease(50). As CCL5 induces chemotaxis of CCR5-expressing macrophages, NK cells and DCs, secretion of CCL5 by pathogenic CCR9+ Th cells could

significantly contribute to SG inflammation (1). In addition, CCL3 and CCL4, which are elevated in pSS serum and saliva, might add to the attraction of CCR5-expressing inflammatory cells as these ligands also bind CCR5.(51,52) However, apart from CCR5-expressing cells these chemokines mediate migration of CCR1 and CCR3-expressing cells. These target however the same cells as CCL5, including DCs, macrophages, NK cells and T cells, potentially converging to the same final result.

CXCR3 and its ligands CXCL9/10/11 mediate salivary gland immunopathology

CXCR3 is highly expressed by effector CD4+ and CD8+ T cells, but also by NK cells, pDCs and B cells. High levels of CXCL9, CXCL10 and CXCL11 are expressed in the ductal epithelium of pSS salivary glands and the majority of T cells express CXCR3 in salivary gland tissue(53,54). Also in the tear film and ocular surface CXCR3 ligands are elevated.(55) The elevated expression of the interferon-induced chemokines CXCL9/10/11 indicates a role for interferons (IFN) and thereby Th1 cells, potentially resulting in a positive feedback loop attracting CXCR3+ Th1 cells producing IFNγ. In accordance with these data, we confirmed elevated expression of CXCL9/10/11 and CXCR3 in the salivary glands and observed a decrease of CXCR3-expressing Th cells in the circulation of pSS patients, potentially indicating migration towards the affected tissues (**Chapter 8**). The most pronounced decrease in blood was seen in the CCR9+ Th cell subset, which produces large amounts of IFNγ. Interestingly, CXCL10 and CCL25 strongly correlated in LSG and facilitated synergistic migration of Th cells (**Chapter 8**). In early stages of sialadenitis in a MRL/lpr model lymphocytic infiltration was successfully inhibited by a modified CXCL10-analog.(56) Using a CXCR3-antagonist in a NOD mouse model xerostomia improved, although efficacy could not be directly linked to lymphocytic infiltration.(57) Alternatively, this axis may be targeted by blocking IL-7/IL-7R, since in mice IL-7 induces CXCR3 ligands via IFNγ. Targeting the IL-7R results in a decrease of CXCL9/10/11 is association with improved salivation and diminished lymphocytic infiltration.(58,59) In particular, cells expressing high levels of IL-7R may be targeted including CXCR3-expressing ILC1s and CCR9+ Th cells which potentially play a role in lymphoid neogenesis in pSS.(35,48,60,61)

A role for Th2 and Th17-associated chemokines?

Although most studies point towards a role for Th1 associated immunopathology, some studies show Th2 chemokines to be elevated in pSS. Th2 cytokine producing cells have been shown to be present in salivary glands from pSS patients, however, were not significantly different from nSS patients.(62) Nonetheless, mRNA expression levels were suggested to be associated with GC formation(63). This seems to match data from a recent finding showing increases of mRNA of Th2 attracting chemokines CCL17 and CCL22 as well as CCR4 in the salivary gland of pSS as compared to patients with mucoceles lacking inflammation. Higher levels of IL-4, IL-5, CCL17, CCL22 and CCR4 are found in patients with higher LFS scores (64). Recently, in line with our previous studies we were not able to demonstrate significant differences in CCL17 or CCR4 mRNA expression in pSS vs nSS patients ((62), **Chapter 8**).

At present these inconsistent findings are not explained. Altogether, although chemokine-directed Th2 cells may play a role in the humoral responses in pSS the evidence is scarce and needs further proof.

Interestingly, we recently demonstrated increased CCR6 expression in the salivary glands of pSS patients as compared to nSS patients. In addition, a trend towards increased CCL20 mRNA expression was observed (**Chapter 8**). It is still unclear which cells are attracted by CCL20 in the glands, but CCL20 has been shown to mediate B cell and DC homing to gut-associated lymphoid tissue(1). In addition, it could mediate chemotaxis of CCR6-expressing Th cells. We have demonstrated significant reductions of CCR6+ CD4 T cells (both CCR9- and CCR9+) in the circulation of pSS patients as compared to healthy individuals (**Chapter 8**). More specifically, CCL20 might attract pathogenic Th17.1 cells that express both IL-17 and IFNγ and that are, known to co-express both CXCR3 and CCR6. In a small set of patients we observed a trend towards decrease, but this remains to be confirmed. Finally, CCL20 could mediate chemotaxis of Th17 cells that express CCR6. However, data on the role of Th17 have been inconsistent, as recently reviewed in (65). In pSS patients, clear correlations between increased Th17 cell activity and symptoms of the disease have not been found and targeting of T cells using abatacept does inhibit disease activity but does not change increased Th17 cells in the circulation(28). In addition, using epigenetic cell counting we found that Th17 cells although present are not significantly different in pSS and nSS patients (**Chapter 3**). These data indicate that CCL20/CCR6 could contribute to positioning of important players in SS immunopathology, but further evidence needs to support this.

In contrast to reduced circulating memory CXCR3+ and CCR6+ CD4 T cells, we found no significant difference in circulating memory CCR10+ CD4 T cells in pSS vs HCs. In addition, reduced levels of ligands (CCL27 and CCL28) of CCR10 are found in pSS. Reduced expression CCL28 in saliva of pSS patients as compared to healthy controls is reported.(66) In line with this we recently demonstrated reduced mRNA expression levels of CCL27 (**Chapter 8**). In support of this differential regulation, mRNA expression of CCR10 showed a trend towards decrease as compared to nSS patients in contrast to increased CXCR3 cells and CXCR3 mRNA expression in salivary glands (**Chapter 8**).

Potential role for CX3CL1 in ELS formation

Apart from CXCL and CCL chemokine members and their receptor several studies have shown increased expression of fractalkine (CX3CL1) in patients with pSS. CX3CL1 is the only member of the CX3C chemokine family and functions as an adhesion and chemotactic molecule. Increased serum levels of CX3CL1 protein are observed in SS patients compared with healthy controls and sicca patients. RNA expression of CX3CL1 and its receptor are tightly correlating with increased focus scores. In addition, using histology it is identified that the cells expressing CX3CL1 and CX3CR1 in salivary glands of SS patients localize within the ELS(67). B cells and

epithelial cells have been indicated to produce CX3CL1, the latter cell type produces this chemokine upon interaction with T cells and is dependent on IFNγ(68). In line with its role to attract T cells, NK cells, monocytes/macrophages and dendritic cells, expression of CX3CR1 is found on T cells and non T cells and non B cells. Finally, CX3CL1 accelerates the autoimmune exocrinopathy of thymectomized NFS/sld mice(69). Hence, these findings point towards a potential role of CX3CL1 and CX3CR in formation of ELS and immunopathology of pSS.

Summary of the role of chemokines in Sjögren's syndrome

Chemokine-chemokine receptor interactions are essential for migration of immune cells and their localization in tissues. Accumulating evidence indicates an important role for chemokines in the formation of lymphocytic infiltrates including ELS in exocrine glands from pSS patients. Especially the IFN-induced chemokines CXCL9/10/11 and their receptor CXCR3, CXCL13 with its receptor CXCR5 expressed on B cells and Tfh cells, CCL19/21 attracting CCR7-expressing cells, and CX3CL1 and CX3CR1-expressing cells are implicated in the disease. In addition, emerging data indicate a role for the pro-inflammatory CCR9-expressing Th cells attracted by CCL25, in pSS immunopathology. In Figure 1 the role of chemokines and chemokine receptors with an emphasis on CCR9+ Th cells is illustrated.

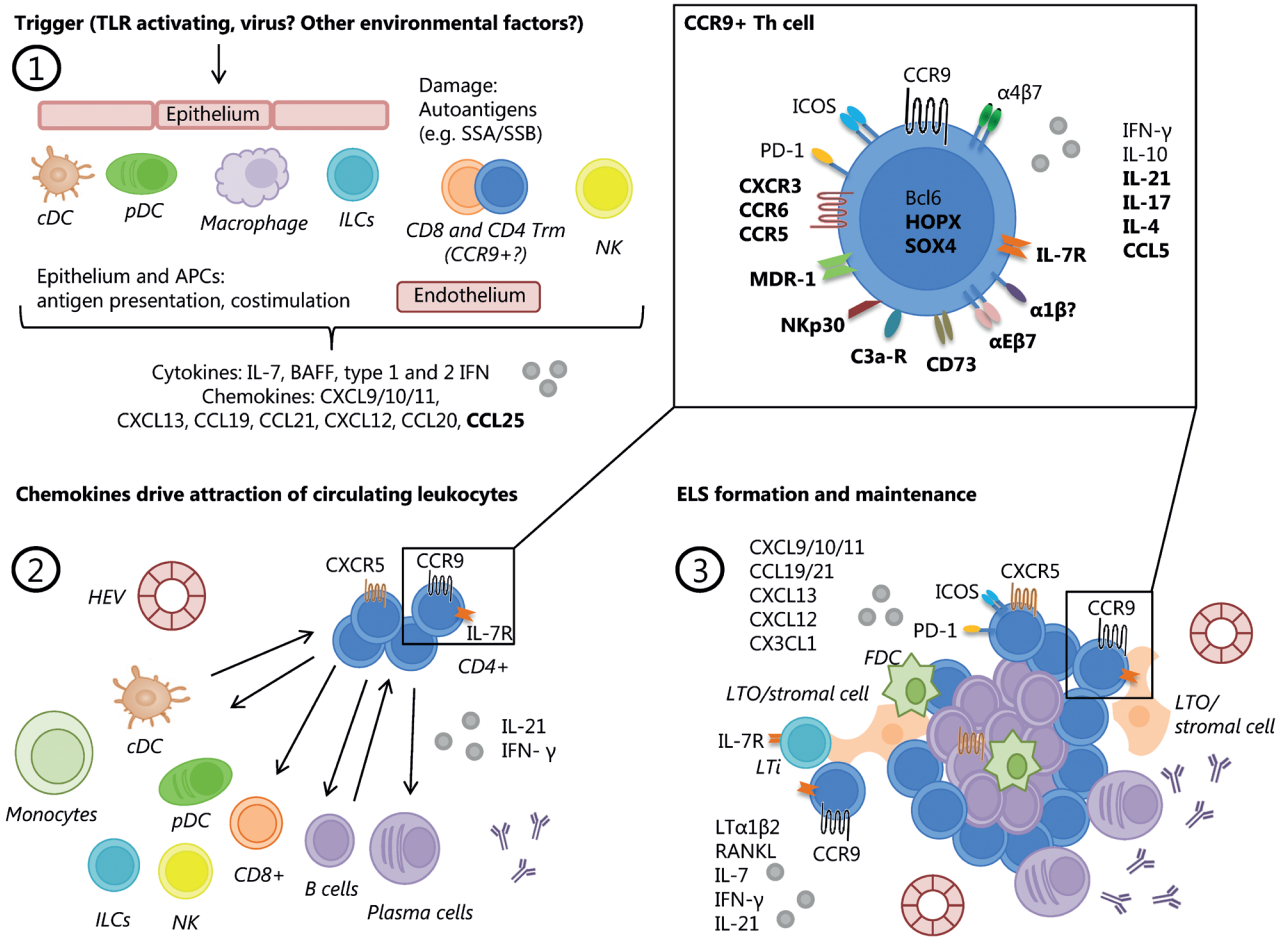
Trigger (TLR activating, virus? Other environmental factors?)
1
Epithelium
Damage:
Autoantigens
(e.g. SSA/SB)
cDC
pDC
Macrophage
ILCs
CD8 and CD4 Trm
(CCR9+?)
NK
Epithelium and APCs:
antigen presentation, costimulation
Endothelium
Cytokines: IL-7, BAFF, type 1 and 2 IFN
Chemokines: CXCL9/10/11,
CXCL13, CCL19, CCL21, CXCL12, CCL20, CCL25
CCR9+ Th cell
CCR9
α4β7
ICOS
PD-1
CXCR3
CCR6
CCR5
Bcl6
HOPX
SOX4
IL-7R
MDR-1
NKp30
C3a-R
CD73
αEβ7
α1β?
IFN-γ
IL-10
IL-21
IL-17
IL-4
CCL5
Chemokines drive attraction of circulating leukocytes
2
HEV
CXCR5
CCR9
IL-7R
CD4+
cDC
Monocytes
pDC
CD8+
B cells
Plasma cells
ILCs
NK
IL-21
IFN- γ
ELS formation and maintenance
3
CXCL9/10/11
CCL19/21
CXCL13
CXCL12
CX3CL1
CXCR5
ICOS
PD-1
CCR9
FDC
LTO/stromal cell
LTO/
stromal cell
IL-7R
LTi
LTα1β2
RANKL
IL-7
IFN-γ
IL-21
CCR9

Figure 1. The potential role of CCR9+ Th cells in Sjögren's syndrome immunopathology.

(**1**) Several triggers (e.g. viruses, other environmental factors) disturb tissue homeostasis by activation of tissue cells (epithelial cells, endothelial cells and fibroblasts) and tissue resident immune cells (including DCs, macrophages, ILCs, Trm, NK cells) and/or damaging the exocrine tissue. This is associated with induction of specific responses to autoantigens and development of autoimmunity characteristic for pSS. Activation of epithelial cells and APCs is associated with upregulation of MHC class I and II and costimulatory molecules on these cells to trigger T cell activation. In addition, cytokines including IL-7, BAFF and type I and II IFNs are produced, as well as chemokines inlcuding CXCL9/10/11, CCL19/21, CXCL13, CXCL12, CCL20 and CCL25. (**2**) The chemokines produced by tissue cells and activated immune cells cause influx of additional immune cells from the circulation, entering via HEV in the MALT tissue. The upregulation of MHC and costimulatory molecules on epithelial cells and APCs, and the cytokines produced (e.g. IL-7, IFNs) cause activation, proliferation and differentiation of CD4+ Th cells including differentiation towards CXCR5+ Tfh. In turn, cytokine (e.g. IL-21, IFN-γ) and cell-cell contact dependently, the Th cells activate CD8+ Tc cells and B cells leading to plasma cell differentiation and auto-antibody production. (**3**) Part of the patients with high IL-7 levels develop highly organized lymphocytic infiltrates (ectopic lymphoid structures) with GC-like morphology thought to follow many of the principles of lymphoid neogenesis in SLO. The interaction between IL-7R+RANK+ Lti cells and IL-7 and RANKL producing LTO/stromal cells leads to upregulation of LTα1β2 which activates LTβr on stromal cells promoting formation of HEV, upregulation of adhesion molecules, chemokine release (CCL19, CXCL12), leading to attraction of lymphocytes (CCR7+ naive and central memory T cells, mature DCs, CXCR4+ naive B cells). Alternatively, IL7R+ effector T cells producing RANK and LT α1β2 could stimulate these processes. The presence of FDCs and production of CCL21 and CXCL13 are crucial for segregation of T and B cell areas and maintenance of ELS. CXCR5+ Tfh and CCR9+ Tfh-like cells expressing IL-21, CXCL13, IL-4, ICOS and PD-1 support the maintenance of GCs and promote the generation of both long-lived plasma cells and memory B cells, contributing to class switching, affinity maturation and somatic hypermutation.

Inset: detailed representation of a CCR9+ Th cell, indicating the established and newly discovered (**bold**) transcription (co)factors, membrane molecules and cytokines they express.

Abbreviations: Th: T helper; Tfh: T follicular helper; DC: dendritic cell; cDC: classical dendritic cell; pDC: plasmacytoid dendritic cell; TLR: toll like receptor; ILC: innate lymphoid cell; Trm: tissue resident memory cell; NK: natural killer cell; APC: antigen presenting cells; MHC: major histocompatibility complex; HEV: high endothelial venule; MALT: mucosa associated lymphoid tissue; GC: germinal center; Tc: cytotoxic T cell; ELS: ectopic lymphoid structures; LT: lymphotoxin; IFN: interferon; RANK(L): receptor activator of nuclear factor kappa-B (ligand); LTO: lymphoid tissue organizer cell; Lti: lymphoid tissue inducer cell; SLO: secondary lymphoid organ.

Conclusions

In this thesis, we explored two innovative techniques to study Sjögren's syndrome (SS) salivary gland immunopathology. A large amount of soluble inflammatory mediators are detectable in salivary gland biopsy supernatants (termed 'secretomes'). In the future, this may be extended to markers of epithelial (dys)function and possibly dryness and fatigue, the key complaints of pSS patients. Some of these mediators that were elevated in the secretomes of SS patients correlated with disease parameters including lymphocytic focus scores and have previously been linked to lymphoid neogenesis. Hence this assay may appear to be of value to predict disease outcome as LFS and lymphoid neogenesis have been linked to lymphoma development. Future studies should reveal its potential in this respect. This also holds true for 'epigenetic cell counting' (ECC), which appeared to easily and robustly quantify several immune cell types in salivary glands of SS and other sicca patients. Both techniques may contribute to patient stratification in research and to diagnostics and prognostics in clinical practice in the future. Further research will be needed to validate the potential value of these novel techniques in SS. Typically international collaborations with large cohorts, such as HarmonicSS and Essential would be valuable for validation of these techniques. Combination of multiple layers of molecular information (secretome, epigenetic cell counting, RNA sequencing) may not only facilitate increased understanding of immunopathology but also patient-tailored therapeutic approaches. Importantly, ECC may contribute to more standardized analysis of salivary gland biopsies, which is currently lacking within and between hospitals.

In the second part, the potential role of CCR9+ pathogenic Th cells in SS immunopathology was studied. This thesis identified, in addition to T follicular helper-like characteristics, novel pathogenic features of CCR9+ Th cells that help to understand their pathogenic role in pSS. Considering their important role in mucosal tissues and their capacity to induce Sjögren's like disease this further supports the notion that targeting of CCR9+ Th cells may have therapeutic potential. The selective CCR9 antagonist vercirnon, a small molecule, showed promising results in phase II clinical trials in Crohn's disease and progressed to phase III clinical trials, but efficacy was limited, with the need for very high doses to block CCR9 activation.(45,47,70) Other strategies including specific immunotherapy e.g. by targeting CCR9 by monoclonal antibodies might be valuable alternative strategies. Alternatively, embarking on effector molecules (e.g. surface molecules or transcription factors) might significantly inhibit CCR9+ Th cell-induced immune responses. Future investigations should indicate the potential of such strategies.

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Nederlandse samenvatting
Dankwoord/acknowledgments
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Nederlandse samenvatting

Innovatieve technieken om speekselklierweefsel te onderzoeken en de rol van CCL25 en CCR9-positieve T-helpercellen in het syndroom van Sjögren

Het syndroom van Sjögren is, hoewel het na reumatoïde artritis de meest voorkomende reumatische auto-immuunziekte is, een relatief onbekende ziekte. De meeste patiënten met deze aandoening hebben last van droge ogen en een droge mond door verminderde traanproductie en verminderde speekselproductie. Een deel van de patiënten heeft wisselend last van zwelling van de speekselklieren. Daarnaast hebben de meeste patiënten last van vermoeidheid en spier- en gewrichtsklachten. In het speekselklierweefsel worden ontstekingen gevonden, maar bij een deel van de patiënten kunnen ook andere organen zoals de huid, longen of nieren ontstoken raken. Ook hebben Sjögrenpatiënten een verhoogd risico op het ontwikkelen van lymfeklierkanker. Meer dan 90% van de Sjögrenpatiënten is vrouw, en de diagnose wordt meestal op de leeftijd van 40-60 jaar gesteld. Hoe de ziekte ontstaat is onbekend en er is geen effectieve behandeling. Dit geeft aan hoe belangrijk het is dat er wetenschappelijk onderzoek wordt gedaan. De invloed van de ziekte op het leven van mensen kan het beste worden beschreven door patiënten zelf. Om een beeld te geven van hoe het is om met het (vaak aan de buitenkant onzichtbare) syndroom van Sjögren te leven, heb ik Sjögrenpatiënten gevraagd om een verhaal over hun ervaringen te schrijven voor in dit proefschrift. Ook een ander aspect wordt belicht in deze verhalen. De patiënten die deze stukken hebben geschreven werken samen met artsen en onderzoekers aan wetenschappelijk onderzoek en zorg, wat 'patiëntenparticipatie' wordt genoemd. Contact tussen patiënten en onderzoekers, en actieve deelname van patiënten aan wetenschappelijk onderzoek en zorg, zijn van grote waarde voor het verbeteren van de kwaliteit van onderzoek en zorg. Het gaat tenslotte niet alleen in de zorg, maar ook bij wetenschappelijk onderzoek – hoe 'basaal' ook, zoals in dit proefschrift – om de patiënt en het verbeteren van de toekomst voor patiënten. De patiënten zijn gevraagd om over hun ervaringen op dit gebied en over het belang van patiëntenparticipatie te schrijven. Deze verhalen zijn te vinden in het tweede deel van de introductie (**hoofdstuk 1**, "Ervaringen van patiënten: leven met het syndroom van Sjögren en het belang van patiëntenparticipatie in wetenschappelijk onderzoek").

Het onderzoek beschreven in dit proefschrift is gericht op het ontwikkelen van innovatieve technieken om speekselklierweefsel te bestuderen en op de rol van bepaalde witte bloedcellen (namelijk CCR9+ T-helpercellen) in het syndroom van Sjögren. Hier volgt een samenvatting van de resultaten.

Nieuwe technieken om speekselklieren van Sjögrenpatiënten te bestuderen

Het speekselklierbiopt is belangrijk voor het stellen van de diagnose het (primair) syndroom van Sjögren (hier verder afgekort als SjS, van Sjögren's syndrome in het Engels). Bij een patiënt met verdenking op SjS neemt een arts, onder plaatselijke verdoving, kleine stukjes speekselklierweefsel af uit de binnenkant van de onderlip of uit de oorspeekselklier (parotis); respectievelijk een lipbiopt of een parotisbiopt. In het laboratorium wordt het weefsel in dunne plakjes gesneden, bewerkt en vervolgens door de patholoog onder de microscoop bekeken. De patholoog beoordeelt of er ontstekingsreacties in het weefsel zijn. De mate van ontsteking wordt vastgesteld op basis van groepen immuuncellen in het speekselklierweefsel. Bij SjS past dat er, verspreid door het speekselklierweefsel, groepjes ontstekingscellen of ook wel immuuncellen genoemd, worden aangetroffen. Een groep ('focus' genoemd, meervoud 'foci') van minimaal 50 immuuncellen per 4mm^2 onderzocht weefsel, wordt in de meeste ziekenhuizen als minimaal afkappunt. voor passend bij SjS beschouwd. Er wordt hiermee een score berekend: het aantal van deze groepen (foci) per 4mm^2. Dit wordt de 'lymfocytenfocusscore' (LFS) genoemd. Het genoemde afkappunt van de minimale hoeveelheid ontsteking passend bij SjS, is een LFS van 1. De LFS is dus een (grove) maat voor de hoeveelheid ontsteking in de speekselklieren.

SjS-patiënten met een hoge LFS (meer dan 3 foci per 4mm^2, dit betekent een hoge mate van ontsteking) hebben meer risico op het ontwikkelen van ernstige verschijnselen in ook andere organen dan de speekselklieren, en meer kans op het ontwikkelen van lymfoom (lymfeklierkanker). Het bepalen van een LFS is dus niet alleen belangrijk voor het bevestigen van de diagnose, maar ook voor het bepalen van de prognose. Er zijn ook nadelen verbonden aan een speekselklierbiopsie. Het is niet alleen een belastend onderzoek voor patiënten, maar het bepalen van de LFS is een bewerkelijke procedure, die veel tijd vraagt. Om de LFS betrouwbaar vast te kunnen stellen, is namelijk niet alleen een bepaalde hoeveelheid weefsel nodig, maar ook een, met deze materie vertrouwde, ervaren patholoog die het weefsel beoordeelt. Daarnaast is de informatie die uit het weefsel verkregen wordt beperkt. Zo worden bijvoorbeeld niet routinematig de totale aantallen en alle aanwezige typen immuuncellen en de diverse ontstekingsstoffen (of ontstekingseiwitten) in het weefsel bepaald. Deze aanvullende informatie zou echter wel kunnen bijdragen aan de kennis over de ziekte, het in kaart brengen van de verschillen tussen patiënten op microniveau en het ontwikkelen van nieuwe behandelmogelijkheden. Bij de ene patiënt staan andere klachten op de voorgrond dan bij de andere patiënt, en ook op microniveau (aantal en type immuuncellen, soorten ontstekingsstoffen) zijn er verschillen tussen patiënten. Deze verschillen zouden kunnen betekenen dat de ene patiënt meer risico heeft op bepaalde ziekteverschijnselen dan de andere patiënt en dat de ene patiënt wel baat heeft bij een bepaald medicijn en de ander niet.

In dit proefschrift onderzoeken we twee technieken om op een efficiënte, objectieve manier meer informatie uit speekselklierbiopten te verkrijgen. De ene techniek betreft onderzoek van het 'secretome' (secretoom): alles wat uitgescheiden ('secreted', gesecreteerd) wordt door speekselklierweefsel (**hoofdstuk 2**). De andere techniek is 'epigenetic cell counting (epigenetische celtelling)': hierbij worden op basis van DNA-onderzoek diverse typen immuuncellen geteld (**hoofdstuk 3**).

Het vocht waarin het speekselklierbiopt gelijk na afname (nadat het eerst is afgespoeld) wordt bewaard voordat het naar de patholoog wordt gebracht, wordt gebruikt voor het secretoom. In dit vocht van biopten van patiënten waarvan een deel wel en een deel niet SjS bleek te hebben, hebben we ongeveer 100 ontstekingsstoffen (cytokines) gemeten. Er wordt onderscheid gemaakt tussen patiënten met SjS, nSjS en iSjS. De mensen met klachten van droogte en moeheid en/of spier-/gewrichtspijn, waarvoor geen andere verklaring werd gevonden, en die op basis van onderzoek in bloed en speekselklierweefsel geen SjS bleken te hebben, worden patiënten met 'non-Sjögrens sicca syndroom' (nSjS) genoemd. Er zijn ook patiënten met dezelfde klachten die wel enkele van de verschijnselen in bloed of weefsel vertonen die bij SjS passen, maar niet voldoende om de diagnose te kunnen stellen; dit wordt in dit proefschrift 'incompleet syndroom van Sjögren' (iSjS) genoemd. Ons onderzoek laat zien dat er verrassend veel ontstekingsstoffen aantoonbaar zijn in het vocht waarin de biopten worden bewaard, en dat we de concentratie van de ontstekingsstoffen kunnen bepalen. Een deel van deze stoffen blijkt in verhoogde concentratie aanwezig te zijn in het vocht van de biopten van SjS-patiënten. Hierbij hangt de concentratie samen met de LFS, dus met de mate van ontstekingsreactie in het speekselklierbiopt. Interessant genoeg zijn er ook iSjS-patiënten met verhoogde concentraties van een bepaald deel van deze stoffen; de toekomst zal uitwijzen of dit betekenis heeft voor het beloop van klachten en ziekteverschijnselen van deze patiënten.

In **hoofdstuk 4** bestuderen we een van de stoffen gemeten in het secretoom meer uitgebreid, namelijk 'soluble IL-7R'. Dit is de oplosbare vorm van de receptor (ontvanger) van het cytokine (ontstekingseiwit) IL-7, dat een belangrijke rol speelt in het ziektemechanisme van SjS.

De andere innovatieve techniek die we onderzoeken, is 'epigenetic cell counting' (ECC). Hiervoor wordt DNA uit speekselklierbiopten van nSjS-, iSjS-, pSjS- (primair syndroom van Sjögren) en sSjS- (secundair Sjögren, overlap met SLE, reumatoïde artritis of sclerodermie) patiënten geïsoleerd. Het DNA uit de biopten is dus afkomstig van speekselklierweefselcellen, maar ook van de immuuncellen die zich in de weefsels bevinden. Het DNA is de code van alle genen. In verschillende typen cellen staan verschillende genen 'aan'. Sommige van die genen zijn specifiek voor een bepaald celtype en zijn in andere celtypen niet actief (in een oogcel zijn dat andere genen dan in een huidcel, bijvoorbeeld). Op basis van deze celtype-specifieke genen wordt het aantal immuuncellen van een bepaald type berekend ten opzichte van

het totale aantal cellen in het weefsel. Dit getelde aantal immuuncellen op basis van DNA blijkt goed overeen te komen met het aantal geteld onder de microscoop. Belangrijk is dat deze DNA-meting veel minder tijdrovend en bewerkelijk is, zeker als er meerdere celtypen in het weefsel moeten worden geteld. Bovendien lijkt er voor ECC minder weefsel nodig te zijn. ECC lijkt daarmee een veelbelovende techniek om efficiënt en objectief immuuncellen te tellen in speekselklierweefsel. Op basis van deze techniek kan zo een groep patiënten met hoge aantallen immuuncellen worden geïdentificeerd. De hoge aantallen ontstekingscellen blijken samen te hangen met hoge concentraties van bepaalde ontstekingsstoffen in het weefsel van deze groep mensen. Ook waren er iSjS patiënten met relatief hoge aantallen immuuncellen. Nogmaals, in de toekomst zal blijken of deze bevindingen consequenties hebben voor het ziektebeloop van juist deze patiënten.

Vervolgonderzoek is nodig om te weten of deze nieuwe technieken een bijdrage kunnen leveren aan de diagnostiek van SjS en aan een betere voorspelling van het risico op het ontstaan van lymfeklierkanker en het optreden van (ernstige) orgaanbetrokkenheid. Zowel onderzoek naar het secretoom als het gebruik van ECC lijken veelbelovende technieken om bij toekomstig onderzoek naar de werkzaamheid van medicijnen te gebruiken: is een medicament in staat ontstekingsstoffen en immuuncelaantallen te doen dalen, kunnen we op basis van een profiel, gebaseerd op ontstekingsstoffen of immuuncellen, voorspellen welke patiënten wel of juist geen baat zullen hebben bij een bepaald medicament?

De rol van CCR9+ T-helpercellen in het syndroom van Sjögren

Het immuunsysteem bestaat vooral uit witte bloedcellen, immuuncellen, die zich in bloed, lymfeklieren en bijna alle organen bevinden. Ze spelen een belangrijke rol in de afweer tegen ziekteverwekkers. Er zijn veel verschillende typen immuuncellen met allemaal hun eigen taak. Deze immuuncellen communiceren met elkaar door cel-celcontact en ontstekingsstoffen (cytokines). Beschadigd weefsel en weefsel waarin een ziekteverwekker is binnengedrongen – maar ook immuuncellen die een signalerende functie hebben – kunnen ontstekingsstoffen maken, die andere immuuncellen aantrekken (chemokines). Op deze manier komt er een afweerreactie om de ziekteverwekker te bestrijden en het weefsel te herstellen. Bij een auto-immuunziekte zoals SjS, is het immuunsysteem te actief en keert het zich, behalve tegen ziekteverwekkers van buitenaf, ook tegen het eigen lichaam.

Voor SjS is, naast de eerder genoemde aanwezigheid van te grote aantallen immuuncellen in de speekselklieren, een tweede afwijking in het immuunsysteem karakteristiek, en dat is een te grote activiteit van B-cellen. B-cellen kunnen antistoffen produceren en ontwikkelen zich tot plasmacellen die in staat zijn nog grotere hoeveelheden antistoffen te produceren. Bij SjS-patiënten betekent dat de aanwezigheid van anti-Ro/SSA en anti-La/SSB antistoffen (dit zijn eiwitten) en ook dat het totaal aan immuunglobuline (serum IgG, eiwitten in het bloed) is verhoogd. In speekselklierweefsel van SjS patiënten komen te veel IgG en IgM producerende

plasmacellen voor. De verhoogde activiteit van B-cellen is ook verantwoordelijk voor het verhoogde risico op het ontwikkelen van lymfeklierkanker, dat met name in de grote speekselklieren kan ontstaan, en in de meeste gevallen als doorgeslagen activiteit van B-cellen kan worden beschouwd. Niet alleen de B-cellen spelen een belangrijke rol. Er zijn weliswaar veel B-cellen en plasmacellen in de speekselklieren van SjS-patiënten aanwezig, maar het grootste deel van de immuuncellen in de speekselklieren bestaat uit 'T helper (Th) cellen'. Zoals hun naam al zegt, helpen deze Th-cellen andere immuuncellen om te actief te worden, en zijn ze essentieel om ook B-cellen te activeren tot de eerder genoemde verhoogde B-celactiviteit. Dit doen ze door cel-celcontact met de B-cellen en door het produceren van cytokines. Naast B-cellen zijn dus ook Th-cellen belangrijk.

Ook andere soorten immuuncellen dan T- en B-cellen spelen een rol in SjS, maar hierop gaan we in dit proefschrift nauwelijks in, behalve dan in **hoofdstuk 5**. Daarin wordt nog een ander celtype beschreven dat geactiveerd kan worden door cytokine IL-7, namelijk 'ILCs' (innate lymphoid cells, 'aangeboren lymfoïde cellen'). Deze cellen bevinden zich vooral in slijmvliezen, reageren heel snel op ontstekingsactiviteit en zouden een rol kunnen spelen bij de vorming van de groepen immuuncellen in speekselklieren van SjS-patiënten. Meer onderzoek is nodig om de precieze rol van deze cellen te bepalen.

Er zijn binnen de Th-cellen ook weer verschillende typen. Een bepaald type dat in staat is B-cellen bijzonder effectief te activeren, is de 'T follicular helper cell' (Tfh, 'T folliculaire helpercel'). Deze cellen zijn in te hoge aantallen aanwezig in speekselklieren van SjS-patiënten, en in nog hogere mate als er meer ziekteverschijnselen zijn. Er wordt daarom gedacht dat ze een belangrijke rol spelen in SjS. Deze cellen zijn te herkennen aan een eiwit op hun oppervlak: CXCR5. Een aantal jaren geleden heeft een andere groep onderzoekers ontdekt dat een Th-celtype met een ander eiwit, namelijk het eiwit CCR9, functies gemeenschappelijk heeft met de CXCR5+ Tfh-cellen en dat ook CCR9+ Th-cellen in verhoogde aantallen aanwezig zijn in het bloed van SjS-patiënten. 'CXCR5+ Tfh-cel' betekent dat de Tfh-cel het molecuul CXCR5 op het celoppervlak heeft; 'CCR9+ Th-cel' betekent dat de Th-cel het molecuul CCR9 op het celoppervlak heeft. CCR9+ Th-cellen kunnen, evenals CXCR5+ Tfh-cellen, B-cellen krachtig activeren. Daarnaast heeft de CCR9+ Th-cel andere eigenschappen waardoor ze een rol zouden kunnen spelen in SjS. Deze cellen bevinden zich namelijk (ook) in (gezonde) slijmvliezen, en ze kunnen cytokines – zoals interferon-gamma (IFN-γ) – maken, waarvan wordt gedacht dat ze belangrijk zijn in SjS.

In **hoofdstuk 6** van dit proefschrift beschrijven we het verschijnsel dat de ontstekingsstof CCL25 verhoogd aanwezig is in speekselklieren van SjS-patiënten. CCL25 is het chemokine dat cellen die CCR9 op hun oppervlak hebben (CCR9+ cellen dus), aantrekt. En inderdaad, ook CCR9+ cellen zijn in verhoogde aantallen aanwezig in de speekselklieren van SjS-patiënten. Verder hebben we gevonden dat CCR9+ Th-cellen op hun oppervlak veel IL-7

receptoren hebben en sterk geactiveerd kunnen worden door het cytokine IL-7, waardoor ze hoge concentraties cytokines zoals IFN-γ maken. Uit eerder onderzoek is gebleken dat IL-7 een belangrijke rol in SjS speelt en wij denken dat dit deels via de activatie van CCR9+ Th-cellen kan verlopen. Een ander chemokine dat in hoge concentraties in speekselklieren van SjS-patiënten voorkomt, is CXCL10 en dit chemokine trekt cellen aan die CXCR3 op hun oppervlak hebben. In bloed van SjS-patiënten vinden we, in vergelijking met bloed van gezonde proefpersonen, een afname van cellen die zowel CCR9 als CXCR3 op hun oppervlak hebben (**hoofdstuk 8**), waardoor we denken dat CCL25 en CXCL10 samen een sterke aantrekkingskracht uitoefenen op deze Th-cellen om vanuit het bloed naar de klieren van SjS-patiënten te migreren.

Om de CCR9+ Th-cellen meer in detail te bestuderen, hebben we deze gesorteerd uit bloed van zowel SjS-patiënten als gezonde vrijwilligers, om te meten welke genen er actief zijn in deze cellen (**hoofdstuk 7**). Zo hebben we ontdekt dat er genen actief zijn, die er mogelijk voor zorgen dat deze cellen zich gemakkelijker in speekselklierweefsel gaan nestelen. Andere genen zouden een rol kunnen spelen bij de hoge cytokineproductie van deze cellen. Vervolgonderzoek zal kunnen duiden hoe essentieel deze genen zijn voor de functie van de CCR9+ Th-cellen in SjS. Zo gedacht zou het ontwikkelen van medicijnen waarmee CCR9+ Th-cellen (bijvoorbeeld via hun activator IL-7) kunnen worden geremd in de toekomst een interessante optie kunnen zijn.

In **hoofdstuk 9** bestuderen we een mechanisme van activatie van T en B-cellen: het 'mTOR' activatie-mechanisme in bloed en speekselklierweefsel van SjS-patiënten. We vinden verhoogde activiteit van dit mechanisme in de klieren van SjS-patiënten. In een kweekopstelling in het laboratorium hebben we deze activatie in cellen van bloed van SjS-patiënten effectief kunnen remmen. Uit onderzoek met muizen met SjS-achtige verschijnselen, dat is verricht door andere onderzoekers, is gebleken dat het remmen van mTOR gunstige effecten heeft op de droogte en ontstekingsverschijnselen van de ogen. Toekomstig onderzoek is nodig om uit te wijzen of het remmen van mTOR zou kunnen worden toegepast als behandeloptie voor SjS-patiënten.

In **hoofdstuk 10** wordt het onderzoek, dat wereldwijd gedaan is naar de rol van chemokines en hun receptoren in SjS samengevat, aangevuld met een samenvatting van de bevindingen van dit proefschrift.

Conclusie

Het onderzoek in dit proefschrift is hoofdzakelijk gericht op het ontwikkelen van nieuwe technieken om speekselklierweefsel te onderzoeken en op het bestuderen van de rol van bepaalde immuuncellen (namelijk CCR9+ Th-cellen) in het syndroom van Sjögren. Deze nieuwe technieken lijken veelbelovend om grote hoeveelheden

informatie op een objectieve, gestandaardiseerde, manier uit relatief kleine delen van speekselklierbiopsiemateriaal te verkrijgen. Dit zou een grote bijdrage kunnen leveren aan toekomstig geneesmiddelenonderzoek bij SjS-patiënten. Hoe groot de betekenis is van deze technieken voor de klinische praktijk, zal vervolgonderzoek moeten uitwijzen. CCR9+ Th-cellen lijken bijzondere en krachtige immuuncellen: hun vermogen tot productie van hoge concentraties ontstekingsstoffen – en daardoor sterke activatie van B-cellen – en hun aanwezigheid juist in slijmvliezen, maken nadere bestudering van deze cellen in SjS bijzonder interessant. Toekomstig onderzoek zal uitwijzen of ze inderdaad een essentiële rol spelen in het ontstekingsproces in klieren van SjS-patiënten en daardoor een geschikt doelwit zouden zijn voor de ontwikkeling van medicijnen om SjS te behandelen.

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Curriculum vitae

Sofie Liny Marie (Sofie) Blokland was born on the 7th of February 1989 in Amstelveen. In the same year the family moved to Breskens, a tiny village in Zeeland, where 3 and 6 years later her two sisters were born. After 9 years they moved to Maarssenbroek, near Utrecht. In 2007 Sofie finished secondary school (VWO with Latin) at the Niftarlake College in Maarssenbroek. The last two years of secondary school she was enrolled in the Junior College Utrecht talent program, which includes education in biology, science, physics and mathematics and contains extracurricular modules. She chose modules on astrophysics and HIV (during which she discovered her passion for immunology) and wrote her thesis on cancer cell migration. Afterwards she moved to Utrecht to study Medicine at Utrecht University. She chose subjects on immunotherapy, neuroscience, immune inhibitory receptors and a clinical internship at the Rheumatology & Clinical Immunology department of the University Medical Center Utrecht. In the final year of Medicine she followed a clinical internship on Infectious Diseases at the University Medical Center Utrecht and a research internship under supervision of Dr. Maarten Hillen, Dr. Joel van Roon and Prof. dr. Timothy Radstake on the role of dendritic cells in Sjögren's syndrome. After finishing Medicine in 2013 she started her PhD in Prof. dr. Radstake's lab under supervision of Dr. Joel van Roon and Dr. Aike Kruize. During her PhD some of the work in this thesis was selected for oral presentation at the conferences of EULAR (European League Against Rheumatism), ACR (American College of Rheumatology), NVVI/BSI (Nederlandse Vereniging voor Immunologie/British Society for Immunology) and ISSS (International Symposium on Sjögren's syndrome). She received travel grants from EULAR, NVSP (Nationale Vereniging Sjögrenpatiënten), NVVI/BSI, Reumafonds and ISSS. In 2015 she moved to Arnhem, to live closer to her partner, and in 2018 she moved to Amersfoort for her current job. Since June 2018 she is enrolled in the training to become a rheumatologist at the UMC Utrecht (supervisors Prof. dr. Jaap van Laar/drs. Evelien Ton), starting as a resident in Internal Medicine in the Meander Medical Center in Amersfoort (supervisor Dr. Rob Fijnheer).

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