Role of dendritic cells and TSLP in rheumatoid arthritis and primary Sjögren's syndrome

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De rol van dendritische cellen en TSLP in reumatoïde artritis en het primair syndroom van Sjögren

(met een samenvatting in het Nederlands)

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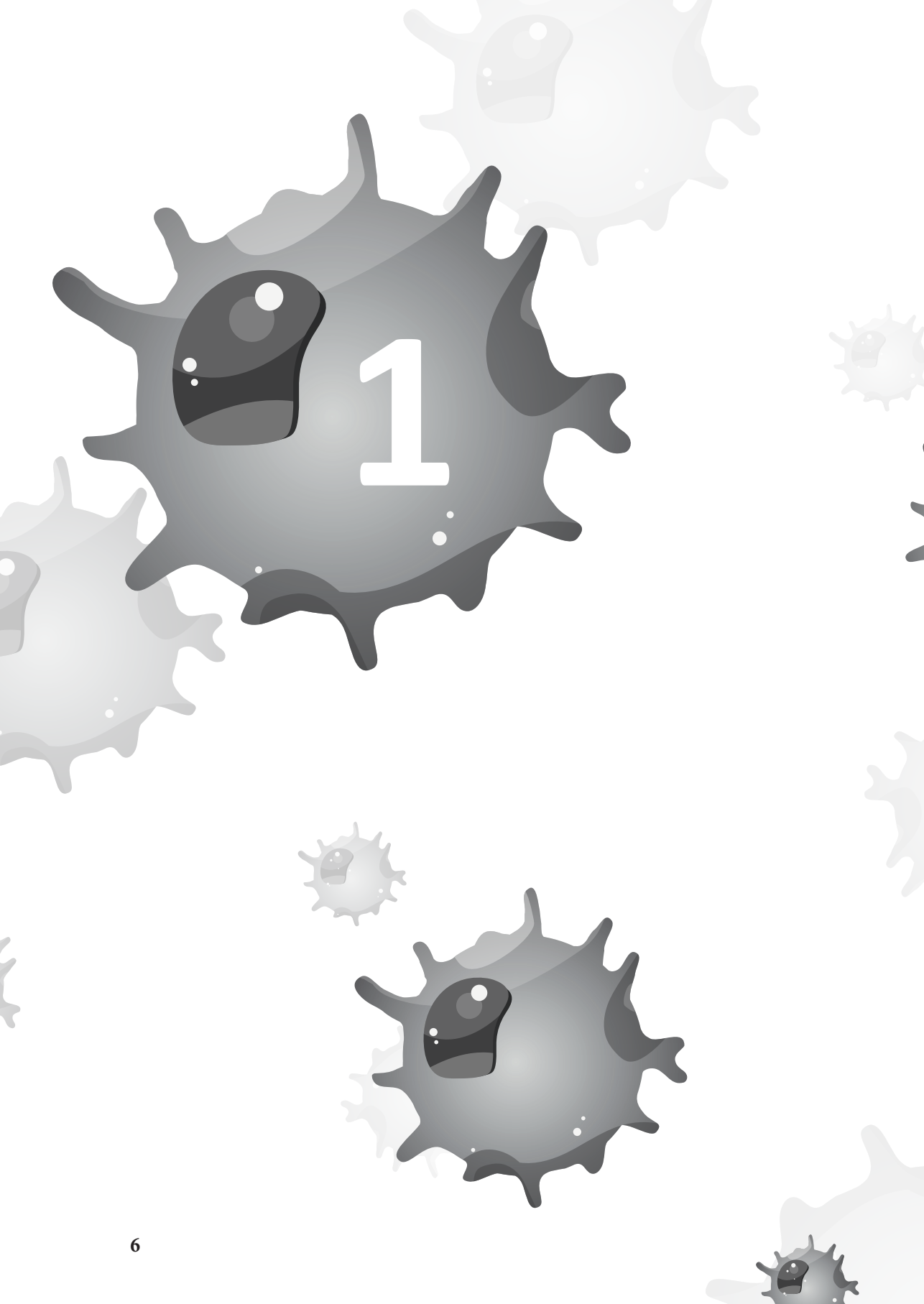
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Introduction

Parts of this chapter have been published in:
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Autoimmune responses are characterized by a close interplay between cells from the innate and adaptive immune systems, which disturbs tissue homeostasis and contributes to chronic inflammation. Dendritic cells (DCs) have gained considerable interest because of their potent immunostimulatory capacity and play critical roles in many experimental *in vivo* animal models and *in vitro* models with human cells. Interestingly, naturally occurring DCs are rarely studied as many researchers opt to instead investigate DCs *in vitro* differentiated from human peripheral blood $CD14^+$ monocytes, which are a more accessible source. This is also true for DC research in rheumatoid arthritis (RA) and primary Sjögren's syndrome (pSS), autoimmune diseases in which T-cells and B-cells are thought to play an important role. Naturally occurring DCs are potent activators of these cells and key regulators of a range of autoimmune phenomena and thus are the subject of this thesis. In particular type-1 classical DCs (cDC1) and the role of a key activating factor of these cells, thymic stromal lymphopoietin (TSLP), were studied. In addition, we performed miRNA profiling of the two most abundant circulating subsets of DCs in pSS patients.

Dendritic cells

Dendritic cells (DCs) were first described some 40 years ago by 2011 Nobel Laureate in Physiology or Medicine Ralph Steinman and colleagues[1]. Within the cells of the mouse spleen that adhere to plastic surfaces they observed a cell-type that behaved differently from monocytes, granulocytes and lymphocytes. These differences were especially clear under a microscope, where the cells assumed a variety of branching forms constantly extending and retracting, creating the dendrites to which these cells owe their name.

Today, DCs are perceived to be at the center of the immune system and are considered to play a central role in the initiation of immune responses.

DCs are bone marrow-derived specialist antigen presenting cells that are present in the blood, lymphoid, interstitial and epithelial tissues of the human body. They play a central role in initiating antigen-specific immunity and tolerance and are responsible for determining the type, magnitude and specificity of adaptive immune responses[2]. Though DCs have constitutively high expression of MHC class-II, maturation signals are needed for effective antigen presentation[3]. DCs can be subclassified based on their location in the body and function. Resident DCs are localized in lymphnodes and other lymphoid tissues where they take up antigen and present peptide fragments to T-cells. Migratory DCs are present in most tissues and constantly scour the environment for potential pathogens or other antigens of interest[2]. When an antigen is taken up, these DCs migrate towards the lymphnode where they present the antigen to T-cells. During inflammation, secondary stimuli including toll-like receptor (TLR) ligands and cytokines will ensure upregulation of co-stimulatory molecules on the DC, resulting in activation of T-cells that recognize the presented antigen. In the absence of infection or altered tissue homeostasis, migrating DCs will not be fully maturated and will transduce tolerogenic signals to cognate T-cells instead[4].

Blood dendritic cells

Several types of dendritic cells are described in blood, characterized by a lack of lineage markers CD3, CD19, CD14, CD20, CD56 and glycophorin A (Lin^-) and high expression of MHC class-II. Peripheral blood DCs are further divided into plasmacytoid dendritic cells (pDCs) and two types of classical dendritic cells (cDCs)[5,6]. Though several other subsets have been proposed including monocyte-derived DCs and 6-Sulfo LacNac (slan) DCs[7], their phenotype in blood and exact function are still under discussion. Currently, the blood dendritic cell antigen (BDCA) molecules are the most important markers to distinguish blood DCs from each other. BDCA-1, -2, -3 and -4 have been described so far and all four are specific for DCs within the Lin^- population. Other than BDCA-2 and -4, which are both expressed on pDCs, the BDCA markers are non-overlapping and can thus be used with great accuracy[8,9].

Classical DCs, also often referred to as myeloid DCs (mDCs), are highly specialized cells that are found in lymphoid and non-lymphoid tissues. They capture environmental- and cell-associated antigens and migrate via the lymphatic system to the T-cell zone of secondary lymphoid organs. cDCs present the captured antigens to prime naive T-cells and an adaptive immune response is initiated or inhibited[4,6]. The classical DCs are often characterized as $Lin^{-}MHC\text{-}II^{+}CD11c^{+}$, though CD11c is not a marker exclusively for cDCs as it is also expressed on most human monocytes and macrophages[6]. The cDCs are more accurately identified and subdivided by expression of CD1c $(BDCA\text{-}1)^{+}$ and CD141 $(BDCA\text{-}3)^{+}$, distinguishing type-1 and type-2 cDCs respectively[8]. $BDCA\text{-}1^{+}$ cDC1s are potent attractors and activators of $CD4^{+}$ T-cells and important for peripheral tolerance. $BDCA\text{-}3^{+}$ cDC2s are potent cross-presenting cells capable of inducing $CD8^{+}$ T-cell activation with antigens taken up from the environment[6].

Plasmacytoid DCs are characterized as $Lin^{-}MHC\text{-}II^{+}BDCA\text{-}2^{+}BDCA\text{-}4^{+}$ and are known for their ability to secrete large amounts of type 1 interferon (IFN) after viral challenge. Activation of pDCs not only induces IFN production, but it also renders them more apt at antigen- presentation. However, pDCs are not phagocytic and express low levels of MHC class-II molecules with a high turnover, which makes them inefficient at antigen-presentation compared to cDCs. Instead, pDCs induce early activation of NK cells and mediate T cell survival via IFN production, making pDCs essential in controlling chronic viral infections[10].

Thymic stromal lymphopoietin: potent activator of type-1 cDCs

TSLP is a potent immunomodulatory cytokine that belongs to the Interleukin (IL)-2 cytokine family[11]. In humans, TSLP is produced by a wide variety of cells including epithelial cells, fibroblasts, smooth muscle cells, keratinocytes, mast cells, macrophages, granulocytes and *in vitro* monocyte-derived DCs[12,13] [14-17]. *In vitro* assays with human cells demonstrated that TSLP is regulated by range of stimuli including tumor necrosis factor (TNF)-α, IL-1β, IFN-β, transforming growth factor (TGF)-β, IL-4, IL-13, and ligands for TLR-2, -3, -4, -5, -8 and -9[17-22]. In mast cells, TSLP expression is induced upon cross-linking of surface IgE molecules[12]. Given these observations, TSLP is considered to play a crucial role in the fine-tuning of theimmune response.

TSLP binds to a heterodimer formed by the unique common-γ (γ_c) chain-like TSLP receptor (TSLPR) molecule and the IL-7 receptor alpha chain (IL-7Rα), while IL-7 uses the γ_c chain together with the IL7Rα to signal[23-25]. Upon ligation of the TSLPR/IL-7Rα complex, cross-phosphorylation of Janus kinase (Jak)-1 and -2 is induced, resulting in phosphorylation of signal transducer and activator of transcription (STAT)-5 which transduces the signal towards the nucleus[26]. TSLP acts mainly on human cDCs, but has also been demonstrated to activate mature T-cells, natural killer T (NKT)-cells, mastcells, monocytes, eosinophils and basophils[13,14,27-31]. Thus, TSLP mediates communication between tissue cells and immune cells but also between cells of the innate and adaptive immune system.

In humans, TSLP does not directly activate naive T-cells, attributable to the fact that TSLPR is not expressed on these cells. However, TSLP can directly stimulate *in vitro*-activated $CD4^+$ T-cells that upregulate TSLPR[28]. TSLP also has an effect on T-cell development. TSLP is expressed in the thymus and plays a role in T-cell selection and Treg induction there[32,33]. Though TSLPR deficient mice do not show defects in T-cell development, mice deficient for both TSLP and γ_c chain show more severe impairment of T-cell development compared to γ_c chain deficient mice[34]. Furthermore, overexpression of TSLP in an IL-7 deficient mouse can fully rescue T-cell development in the thymus and peripheral lymphoid organs[35]. This suggests that TSLP and IL-7 are partly redundant, with TSLP being able to take over the role of IL-7 when its signalling is disturbed. Currently, IL-7 or TSLP deficiency in humans has not been described. Conversely, IL-7Rα deficiency has been described in humans and has been shown to lead to impaired T-cell development[36]. Subjects with this deficiency do not show impairment in B-cell numbers or function. Therefore, IL-7 and TSLP do not appear to be involved in B-cell lymphopoiesis in humans. This is different in mice, where IL-7R signalling is vital for B cell development[37].

Though in humans TSLP cannot directly activate naive T-cells, it acts strongly on these cells via cDCs. TSLP stimulation of $CD11c^+$ DCs *in vitro* confers a license for migration and induces upregulation of T-cell-activating and –costimulatory molecules including MHC class-II CD40, CD80 and CD86[12]. When stimulated with TSLP, cDCs are able to present self-peptides on MHC class-II molecules, which is critical for homeostatic proliferation of $CD4^+$ and $CD8^+$ T-cells[32,38]. This seems partly mediated by the transcription factor Autoimmune regulator (AIRE), which is involved in presentation of

self-peptides by stromal cells in the thymus and is upregulated by CD11c$^+$ cDCs upon TSLP stimulation[32,39]. Indeed, cDCs induce strong autologous CD4$^+$ T-cell proliferation when stimulated with TSLP *in vitro*, dependent on self-peptide-MHC complex-mediated activation[32]. Both naive and central memory T-cells respond to the DCs and expand in polyclonal fashion. Naive cells differentiate into central memory cells while central memory cells retain their phenotype. Both subsets retain the ability to further expand and differentiate into different T-helper subsets. Effector memory cells do not respond to TSLP-DCs[32]. This is corroborated in mice where T-cell pool recovery in TSLPR deficient mice is defective upon sub-lethal irradiation, and when CD4$^+$ T-cells from TSLPR-/- mice were transferred to a healthy irradiated wild-type host the TSLPR-/- cells proliferated less compared to the wild-type cells, indicating impaired homeostatic proliferation[34].

While T-helper cell skewing is not observed during homeostatic expansion, TSLP-stimulated cDCs can specifically induce Th2-activity, which explains the postulated role of TSLP in allergic diseases[12,40]. TSLP-activated CD11c$^+$ DCs can secrete high levels of Th2-attracting chemokines such as thymus and activation regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22)[12]. In addition, in context of T-cell activation by non-self-antigens, TSLP-activated CD11c$^+$ DCs induce production of Th2-related cytokines including IL-4, IL-5 and IL-13 by CD4$^+$ T-cells. However, there is evidence that Th1- and Th17 activity can also be induced by DCs upon TSLP stimulation depending on the immunological environment. In a Th1-skewed milieu with high IL-12 levels present, Th1 cytokines are produced by TSLP-stimulated CD11c$^+$ cDCs in addition to Th2 cytokines[41]. Moreover, strong IL-12-dependent Th1 activation in conjunction with Th2-downmodulation by TSLP-stimulated cDCs was shown upon exposure to tuberculosis vaccine Bacillus Calmette-Guérin (BCG), which is a potent Th1-activator[42]. Furthermore, IL-23 production by CD11c$^+$ cDCs and Th17 activation was shown when stimulated with both TSLP and TLR3L[43]. Finally, though TARC and MDC are generally regarded as Th2-cell attracting chemokines and their common receptor CCR4 is primarily expressed on Th2-cells[44], CCR4 is also expressed on Th17-cells[45], Th22-cells[46], and Tregs[47].

Together, the abovementioned studies indicate that the influence of TSLP on T-cell activation and skewing is variable depending on the immunological environment and cellular interactions. TSLP is a strong Th2 activating mediator involved in atopic diseases and TSLP-stimulated DCs triggered

by allergens induce potent Th2 activity[44]. However, in a strong Th1-skewed environment with high IL-12 levels, Th1- and Th17 activity are also induced[41]. Alternatively, TSLP induces homeostatic expansion of T-cells via DCs without triggering T-cell skewing. Naive cells become effector memory cells and become respondent to low levels of TCR triggering while retaining the potential to differentiate into different Th-cell subsets. In a strong Th1/Th17-skewed environment as found in a range of autoimmune diseases, this allows for subsequent differentiation into Th1- and Th17-cells.

Type-1 classical DCs and TSLP in rheumatoid arthritis

RA is an autoimmune disease characterized by chronic synovitis associated with inflammation in the synovial lining of the joints. This results in cartilage- and bone damage by the cytokines and catabolic enzymes produced by inflammatory cells and transformed synovial fibroblasts[48]. $CD4^+$ T-cells that produce IFN-γ or IL-17, B-cells and macrophages each play an important role in this disease, as both cell numbers and activity of these cells in the synovial tissue correlate with clinical symptoms[49]. Therapeutic intervention targeting components of the inflammation and modulating the pro-inflammatory environment effectively decreases disease severity in many patients[50,51]. However, inflammation in RA, as in many other inflammatory diseases, is characterized by redundancy of multiple mediators that signal immune cells to initiate and maintain inflammation, causing lack of therapeutic efficacy in part of the patients. Enhanced understanding of crucial celltypes and mediators may help discern the patients that should be treated with certain types of medication and offer new therapeutic options.

Recently, TSLP has been implicated as a disease-exacerbating mediator in RA. Intra-articular concentrations of TSLP are upregulated in the majority of RA patients[52,53] and TSLP was shown to correlate with soluble IL-7 receptor, a marker of fibroblast activation[52]. Fibroblasts from RA patients were shown to express TSLP ex vivo and TSLP secretion was induced *in vitro* upon stimulation with proinflammatory cytokines IL-1β and TNF-α and ligands for TLR3 and TLR4[15], which are present in RA synovium and synovial fluid (SF) and are implicated in immunopathology[54].

cDC1s are increased in the SF of RA patients compared to the peripheral blood. These DCs display higher expression of CD1c, MHC class-II, CD80, CD86, CD40 and TSLPR[55]. When stimulated with TSLP, these cells induce T-cell proliferation and secretion of Th1- and Th17-associated cytokines

IFN-γ and IL-17 by T-cells. cDCs from the SF were able to induce more T-cell proliferation compared to cDCs from the peripheral blood[52]. In addition, these cells produce high levels of T-cell attracting chemokines including TARC[12] and MIP-1α[52]. MIP-1α is regarded as a Th1-cell attractor and signals through CCR1, CCR3 and CCR5. Though expression of CCR1 and CCR3 does not differ much between Th1- and Th2-cells, CCR5 is highly expressed on Th1-cells, which are abundantly present in the RA joint[49], while Th2-cells show little to no expression of this receptor[56,57]. TARC attracts cells that express CCR4, which includes CD4 T-cells with Th2, Th17, Th22 and Treg phenotypes[46,47,58,59], NKT-cells[60] and fibroblasts[61]. A range of these cells are capable of producing cytokines that are pro-inflammatory in RA[60,62,63]. Interestingly, CCR4 is present on the majority of IL-17 producing cells in the synovium of RA patients[45], but the implications of this finding have not yet been studied.

Supporting the role of TSLP in arthritis, several mouse experimental arthritis models show that TSLP increases inflammation. Administration of neutralizing antibodies against TSLP ameliorates disease in a TNF-α- and immune complex-dependent collagen type-II antibody-induced arthritis (CAIA) model[53]. In the collagen-induced arthritis model (CIA), which is dependent on both B- and T-cells, administration of recombinant TSLP caused more severe disease and tissue destruction as measured by clinical arthritis score and histology. Furthermore, TSLPR-deficient mice have less severe proteoglycan-induced arthritis (PGIA) when compared to wild-type mice[64].

In addition to TSLP, its family member IL-7 plays a pivotal role in T-cell development and homeostasis in mice and humans[65,66]. Like TSLP, IL-7 signals via the IL-7R in complex with the γc chain and mainly acts on T-cells, inducing potent T-cell proliferation and cytokine production. IL-7 is increased in RA SF and stimulates T-cells to produce various cytokines including TNF-α, IFN-γ, and IL-17[67,68]. IL-7 mediates pro-inflammatory processes in experimental arthritis mouse studies, as injections with IL-7 in a CIA model induced increased arthritis severity[69] while administering a monoclonal anti-IL-7R antibody in the same model reduced disease severity[70]. Though IL-7 and TSLP signal through the same receptor subunit and have synergistic functions in disease, their combined effects have not yet been studied in autoimmunity.

Dendritic cells and TSLP in primary Sjögren's syndrome

pSS is a systemic autoimmune disease characterized by lymphocytic infiltration of the salivary and lachrymal exocrine glands that leads to dryness of mouth and eyes. Infiltrates mainly consist of activated T- and B-lymphocytes in conjunction with a range of antigen presenting cells, including monocytes, macrophages and dendritic cells. The infiltrating leukocytes and activated local epithelial cells produce a range of proinflammatory mediators including cytokines and proteases that interfere with glandular cell-function and drive tissue destruction, resulting in dryness symptoms[71].

$CD4^+$ T cells are thought to be crucial for disease, as they are essential for efficient activation of autoantibody-producing B cells and production of proinflammatory cytokines. In addition, genetic association studies showed an important role in disease for MHC class-II and IL-12 signalling, indicating that presentation of antigen to T-cells and CD4 T-cell differentiation are critical in disease[72,73]. Indeed, SS-like symptoms develop in mice upon injection with auto reactive CD4+ T cells[74]. $CD4^+$ T cell subsets Th1 and Th17 cells producing the proinflammatory cytokines IFN-γ and IL-17 respectively, are both associated with tissue damage in pSS[75]. Though pSS is associated with increased Th1/Th17 activity like RA and increased numbers of cDCs are present in the salivary glands[76,77], the factors that mediate local cDC activation are unknown. Considering that the main target cells for TSLP are present locally, expression of TSLP in the salivary glands of pSS patients may play an important role in DC activation and driving of T-cell activation.

As classical dendritic cells are potent activators of T-cells, they are candidate key players in pSS pathogenesis. However, they are rarely studied in the disease. A number of studies have reported on expression of maturation markers by the DCs present in the salivary glands. Expression of MHC class-II, CD1a, CD83, DC lysosome-associated membrane glycoprotein (DC-LAMP, CD208) and DC-specific ICAM-3 grabbing non-integrin (DC-SIGN, CD209) on DCs was shown in pSS salivary glands[76,78,79]. DC-LAMP is described as a marker for mature DCs[80] while DC-SIGN is expressed at high levels by immature cDCs in non-lymphoid tissues and mature cDCs in lymphoid tissues[81,82]. DC-SIGN is involved in a range of processes including DC migration, recognition of pathogens by DCs and activation of T-cells[83]. CD1a is involved in presentation of lipids to T-cells and is used as marker for immature DCs[84]. In addition, CD83, CD86 and MHC class-II are often used

as markers for DC maturation. In pSS, DC-SIGN$^+$, DC-LAMP$^+$ and CD1a$^+$ cells were present in the salivary gland[76,85]. Expression of DC-SIGN, DC-LAMP and CD1a correlated with expression of the IL-7 receptor, which in turn is associated with lymphocytic infiltration and activation[79]. Thus, both immature and mature DCs are present in the salivary glands and all three markers are indirectly related to T-cell activity. Although cDCs are present in the pSS salivary glands and there are signs of their mature state and capacity to potently activate lymphocytes, their exact function remains unclear.

Of the different DC subsets, the role of pDCs has been most thoroughly studied in pSS. pDCs are extremely potent IFN-α producing cells and are thought to be responsible for the majority of IFN-α produced in these patients[10,86]. pSS patients have an activated type-1 IFN system and genes activated by the IFN produced are detectable in a range of celltypes in these patients.[86-89]. IFN-regulated gene upregulation, which is referred to as IFN signature, is correlated with disease activity and production of autoantibodies in pSS[90]. pDCs in pSS blood appear to be activated and their activation status correlates to the presence of an IFN signature, suggesting a link between activation of the pDCs in blood and type-1 IFN production[89]. In addition, a collection of studies on pDC levels in peripheral blood and in salivary glands support the hypothesis that pDCs migrated from the blood into the glands[86,89,91]. Thus, pDCs seem important in production of IFN-α and subsequent activation of several pro-inflammatory pathways, though the exact implications of this system need further investigation. Molecular profiling of these cells in pSS may help to understand the cause of enhanced IFN production and the determining pathways leading to IFN signature in a part of the patients. In addition, this may help to understand the initial triggers of pDC activation, potentially leading to novel targetable molecules and/or biomarkers for disease.

Thesis outline

In this thesis, several facets of dendritic cell function in rheumatoid arthritis and primary Sjögren's syndrome are studied, with a focus on the role of TSLP therein. In the first part (chapters 2-4), the role of TSLP in RA and pSS is investigated in the context of IL-7 and TARC. In the second part (chapters 5-7), DC biology in pSS is studied and the regulation of cDC1s and pDCs in pSS is investigated on the miRNA level.

Considering the pivotal role of TSLP in RA and the potential redundancy with IL-7 due to the shared use of the IL-7Rα, in **chapter 2** the effects of combined ablation of signalling of TSLP and IL-7 are studied in a mouse model. In addition, the combined effects of TSLP and IL-7 are assessed *in vitro* using primary human blood cDC1s and CD4 T-cells.

In view of the increased expression of CCR4 on T-cells and the role of TSLP in induction of TARC production by cDC1s, **chapter 3** describes the role of TARC in rheumatoid arthritis. This chemokine is produced at high concentrations by TSLP-stimulated cDC1s and is a potent attractor of CD4 T-cells.

As mature cDCs are present in pSS salivary glands and these cells are potential crucial mediators in T-cell activation, in **chapter 4** the changes in expression of TSLP in the salivary glands of primary Sjögren's syndrome patients are investigated in relation to disease parameters.

Chapter 5 reviews the current literature on the role of dendritic cells in the pathogenesis of primary Sjögren's syndrome, with a focus on type-1 classical dendritic cells.

As miRNAs are critical regulators of gene expression, dysregulated miRNAs can pinpoint novel dysregulated pathways in patient cells. To discovery novel pathways dysregulated in DCs of pSS patients and gain further understanding on the role of cDC1s in immune-activation and pDCs in enhanced type-1 IFN production in pSS, changes in expression of microRNAs were studied in these cells in **chapter 6** and **chapter 7**.

Chapter 8 summarises the findings in this thesis and discusses their implications.

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The additive inflammatory *in vivo* and *in vitro* effects of IL-7 and TSLP in arthritis underscore the therapeutic rationale for dual blockade

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Abstract

Introduction. The cytokines interleukin (IL)-7 and thymic stromal lymphopoietin (TSLP) signal through the IL-7R subunit and play proinflammatory roles in experimental arthritis and rheumatoid arthritis (RA). We evaluated the effect of inhibition of IL-7R- and TSLPR-signalling as well as simultaneous inhibition of IL-7R- and TSLPR-signalling in murine experimental arthritis. In addition, the effects of IL-7 and TSLP in human RA dendritic cell (DC)/T-cell co-cultures were studied.
Methods. Arthritis was induced with proteoglycan in wildtype mice (WT) and in mice deficient for the TSLP receptor subunit (TSLPR-/-). Both mice genotypes were treated with anti-IL-7R or phosphate buffered saline. Arthritis severity was assessed and local and circulating cytokines were measured. Autologous CD1c-positive DCs and CD4 T-cells were isolated from peripheral blood of RA patients and were co-cultured in the presence of IL-7, TSLP or both and proliferation and cytokine production were assessed.
Results. Arthritis severity and immunopathology were decreased in WT mice treated with anti-IL-7R, in TSLPR-/- mice, and the most robustly in TSLPR-/- mice treated with anti-IL-7R. This was associated with strongly decreased levels of IL-17, IL-6 and CD40L. In human DC/T-cell co-cultures, TSLP and IL-7 additively increased T-cell proliferation and production of Th17-associated cytokines, chemokines and tissue destruction factors.
Conclusion. TSLP and IL-7 have an additive effect on the production of Th17-cytokines in a human *in vitro* model, and enhance arthritis in mice linked with enhanced inflammation and immunopathology. As both cytokines signal via the IL-7R, these data urge for IL-7R-targeting to prevent the activity of both cytokines in RA.

Introduction

Interleukin (IL)-7 is a potent immunostimulatory cytokine of the IL-2 cytokine family that is mainly produced by stromal cells of primary lymphoid organs and plays a pivotal role in T-cell development and homeostasis in mice and humans[1,2]. IL-7 signals via the high affinity IL-7 receptor-alpha chain (IL-7R) in complex with the common gamma chain and mainly acts on T-cells, inducing potent T-cell proliferation and cytokine production. IL-7 enhances inflammation in various autoimmune disorders, including rheumatoid arthritis (RA) where it is increased in the synovial fluid (SF) and stimulates T-cells to produce various cytokines including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-17[3,4].

Thymic stromal lymphopoietin (TSLP) is an IL-7-related cytokine that shares the IL-7R for signalling, but only when in complex with the unique TSLP receptor (TSLPR) instead of the common gamma chain[5,6]. It is produced by fibroblasts, epithelial cells, mast cells, smooth muscle cells, macrophages, granulocytes, monocyte-derived DCs, and keratinocytes[7-12]. TSLP acts on classical DCs (cDCs, also referred to as myeloid DCs/mDCs), mast cells, monocytes, granulocytes, natural killer T (NKT)-cells, and activated T-cells[9,13-16]. Like is true for IL-7, TSLP levels are increased in RA SF[17,18]. Whereas IL-7 acts directly on T-cells, TSLP indirectly activates T-cells via stimulation of cDCs, which in turn become extremely potent T-cell activators promoting T-cell proliferation and production of proinflammatory cytokines TNF-α, IFN-γ, and IL-17 in the presence of Th2 mediators[18,19].

Our group previously demonstrated that IL-7 and TSLP separately play a pro-inflammatory role in experimental murine arthritis. Injections with IL-7 in a collagen-induced arthritis (CIA) model induced increased arthritis severity[20] while administering a monoclonal anti-IL-7R antibody in the same model reduced disease severity[21]. TSLPR-deficiency resulted in less severe disease in both CIA and proteoglycan-induced arthritis (PGIA) models along with reduced levels of proinflammatory cytokines, chemokines, and factors involved in tissue destruction[22]. Furthermore, we have shown with *in vitro* assays using cells from RA patients that both cytokines induce production of key proinflammatory factors involved in RA pathogenesis[3,18]. Though both IL-7 and TSLP induce T-cell activation and proinflammatory cytokine production via different target cells while signalling through the same receptor subunit, their combined effects have not been studied in arthritis. We here investigated the effects of

simultaneous blocking of TSLP and IL-7 activity in murine experimental arthritis and studied their combined effects in *in vitro* DC + T-cell co-cultures using RA patient material.

Materials and Methods

Proteoglycan-induced arthritis

TSLPR deficient (TSLPR-/-) mice were generated as previously described[23] and backcrossed to a BALB/c background. Proteoglycan (PG) was used to induce arthritis (PGIA) in 24-week-old female BALB/c mice (Charles River Laboratories Inc). Human PG was dissolved at 20 mg/mL in phosphate buffered saline (PBS) and emulsified in an equal volume of synthetic adjuvant dimethyl-dioctadecyl-ammoniumbromide (DDA; Sigma) in PBS. On day 0 and day 21, all mice were immunised with 200 µL PG emulsion (400 µg PG and 2 mg DDA) ip. Wildtype and TSLPR-/- mice were divided into two groups of 16 or 17 mice. The groups were treated with either 100 µg of monoclonal rat anti-mouse IL-7R antibody (IgG2b, Amgen Inc. Seattle, WA, USA) or PBS at days 21, 24, 27, 30, and 33. Mice were sacrificed by CO_2 inhalation at day 36. All animal experiments were approved by the institutional animal care and use committee of Amgen.

Arthritis assessment

Arthritis symptoms were graded as previously described[20]. In short, arthritis symptoms were graded from day 24 onwards using a scale from 0-4 based on the severity of arthritis. Each limb was graded, giving a maximum possible score of 16. Arthritis incidence was defined as grade 1 or higher. Researchers examining mice for onset and severity of arthritis were blinded to mouse genotype and treatment group.

Radiological joint damage was assessed as previously described[21]. Both ankles of all mice ankles were scored for severity of radiographic lesions by researchers blinded for mouse characteristics, using a scoring system ranging from grade 0 to grade 3. The mean value per ankle was averaged for each mouse.

Histological joint damage was assessed as previously described[20]. Briefly, tissue sections of 5µm were prepared and stained with hematoxilin-eosin (HE). Slides were scored for severity of cellular infiltrates, subchondral bone erosion, osteophyte formation and articular cartilage erosion. For osteoclast evaluation, slides were stained with tartrate-resistant acid phosphatase (TRAP). As control, sections were treated identically without presence of the

enzyme substrate, no staining was observed in these sections. Each histological parameter was graded on a scale from 0-4 and the mean value per ankle was averaged for each mouse.

Splenic and thymic cell preparation and flow cytometry

To check the T-cell compartment in primary lymphoid organs due to TSLPR deficiency and anti-IL-7R administration, spleen and thymus were collected from all mice at day 36. They were weighed and single cell suspension was prepared in Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (FBS; Invitrogen) using a 70 μm cell strainer (Falcon, BD Bioscience, San Jose, CA, USA). Red blood cells in spleen were lyzed by 5 minute incubation in lysis buffer (Invitrogen). The reaction was stopped by addition of 10 mL cold PBS. Cells were counted, pelleted and resuspended in HBSS with 5% FBS and 1 μg Fc block (2.4G2; CD32/16, BD). Cells were incubated with monoclonal anti-CD4 APC/Cy7 (clone Gk1.5; BD), anti-CD8 Pacific Blue (clone 53-6.7; BD), anti-CD19 PerCP/Cy 5.5 (clone 1D3; BD), anti-CD44 PerCP/Cy 5.5 (clone IM7; eBioscience), and anti-CD62L APC (clone mel-14; BD). 10^5 events were registered using FACS-LSRII (BD). Events were gated for viable lymphocytes based on forward and side scatter and specific staining using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Paw lysate and serum multi analyte profiling

Blood samples were taken on day 36 by heart puncture. Additionally, both front paws of each mouse were collected and frozen in liquid nitrogen directly upon removal. Skin was removed and paws were homogenized in a 50 mM Tris-HCL buffer supplemented with 0.1 M NaCL and Triton X-100 (pH 7.4) and mini-EDTA free protease-inhibitor tablets (Roche) using a tissue lyser (Qiagen, Valencia, CA, USA) and 5 mm stainless steel beads (Qiagen). Lysates were stored at -20°C until further analysis. Protein content of the lysates was measured with Pierce BCA total protein quantitation kit and each sample was brought to a total protein content of 1 mg/mL. For 10 mice per group with representative arthritis scores, luminex-based multi analyte profiling (MAP) was performed in the paw protein lysates and in the serum samples by Rules Base Medicine Inc. (Austin, TX, USA) using RodentMAP v2.0. IL-17 was measured with luminex.

RA patients

RA patients were classified according to the American College of Rheumatology criteria[24]. The University Medical Center Utrecht approved the *in vitro*

experiments with human material in compliance with the Helsinki Declaration. All patients gave their written informed consent.

Cell isolation

Mononuclear cells (MCs) from RA patients were isolated from heparinized peripheral blood (PB) by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to MC isolation, PB was diluted 1:1 in RPMI 1640 medium (Gibco, Life Technologies, New York, USA) containing penicillin (100 U/mL), streptomycin (100 µg/mL) and glutamine (2 mM) (all PAA Laboratories, Pasching, Australia). $CD19^-$ $CD1c^+$ cDCs and $CD4^+$ T-cells were isolated from PB MCs by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^+$ and $CD4^+$ isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions.

DC/T-cell co-cultures

Cells were cultured in RPMI glutamax (Gibco) supplemented with penicillin, streptomycin, and 10%, v/v, human AB serum (GemCell, West Sacramento, USA). Isolated CD1c-positive cDCs (2000-5000 cells/well) were co-cultured with 50.000 autologous CD4 T-cells per well in round-bottomed 96-well plates in the presence of 20ng/ml recombinant TSLP (R&D systems) and 0.3-10 ng/mL of recombinant IL-7 (Preprotech Inc, Rocky Hill, NJ, USA) where appropriate. Cells were cultured for 6 days at 37°C and proliferation and cytokine production were measured. Proliferation was measured (n=7) by ^{3}H-Thymidine incorporation assay at the end of the culture period. ^{3}H-Thymidine (1µCi/well; PerkinElmer, Waltham, USA) was added during the last 18 hours of the culture period. In separate cultures (n=7), supernatants of co-cultured T-cells were re-stimulated with ionomycin (500ng/ml) and phorbol myristate acetate (50ng/ml) (both from Sigma-Aldrich) during the last 24 hours of the culture period. Culture supernatants were collected and frozen at -80°C until cytokine production analysis with Luminex.

Cytokine analysis

Cytokine production of human *in vitro* cultures by multiplex immunoassays were performed at the MultiPlex Core Facility of the Laboratory for Translational Immunology (UMC Utrecht, Netherlands) using an in-house validated panel of analytes. Uniquely color-coded magnetic beads (MagPlex Microsphere, Luminex, Austin, Texas, USA) were conjugated to antibodies specific for the reported analytes and incubated with 50 uL of standard dilutions or samples for 1 hour (with continuous shaking in he dark). Plates were washed (Bio-Plex

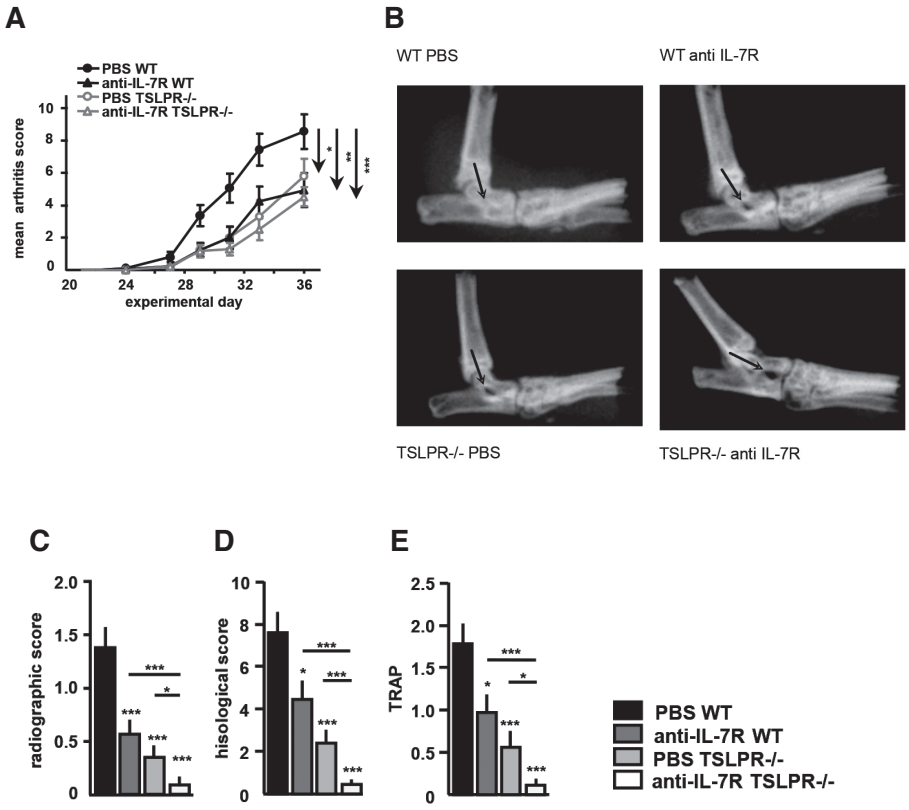

Figure 1. Arthritis severity is inhibited in TSLPR deficient mice and upon blockade of the IL-7R. WT or TSLPR-/- mice were treated with PBS or anti IL-7Rα antibodies (anti IL-7R, 100 μg ip. on day 21, 24, 27, 30, and 33) and arthritis severity was graded by visual examination of the paws. IL-7R blockade, TSLPR deficiency or a combination of both significantly decreased arthritis severity compared to PBS WT mice (**A**). The same was true for radiological joint damage (**B, C**), histology (**D**) and osteoclast formation as measured by the number of TRAP+ cells (**E**). Anti-IL-7R TSLPR-/- mice showed almost complete inhibition of joint damage. Values are mean ± SEM of 16 mice per group. *, **, and *** indicate statistical differences of $p<0.05$, $p<0.01$, or $p<0.005$ respectively.

Pro II Wash Station; Bio-Rad, Hercules, California, USA) and a corresponding cocktail of biotinylated detection antibodies was added for 1 hour. Repeated washings were followed by a 10 minute streptavidin-phycoerythin (PE) incubation. Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analyzed using Bio-Plex Manager software version 6.1 (Biorad) using 5-parameter curve fitting.

Statistical Analyses

For mouse experiments, Mann-Whitney U test with two-sided testing was used to examine differences between treatment and control groups of mice in arthritis score, radiographic- and histological joint scores. Pearson Chi-Square test was used for comparing arthritis incidence between treatment groups. For MAP data analysis, Kruskal Wallis test with post-hoc Mann Whitney U test using two-sided testing was used. For *in vitro* human proliferation experiments, Mann Whitney U test with two-sides testing was used. For human cytokine expression experiments, Mann Whitney U test with one-sided testing was used. P-values of 0.05 or smaller were considered statistically significant.

Results

Blockade IL-7- and TSLP signalling significantly decreases severity of arthritis

Treatment of WT mice with anti-IL-7R (25 ± 5.9, p=0.008; Mean area under curve ± SEM) or TSLPR-/- mice treated with PBS (24.1 ± 6.0, p=0.019) significantly reduced clinical arthritis score compared to PBS treated WT mice (50.7 ± 6.8). Strikingly, TSLPR-/- mice treated with anti-IL-7R displayed even lower clinical arthritis scores compared to the anti-IL-7R treated WT mice and the PBS treated TSLPR -/- mice (18.7 ± 3.6, p=0.003), though there was no statistically significant difference between anti-IL-7R treated TSLPR-/- mice and the anti-IL-7R treated WT mice or PBS treated TSLPR-/- mice (Fig.1A).

Anti-IL-7R treated WT mice (0.6 ± 0.1, p=0.002) and PBS treated TSLPR-/- mice (0.3 ± 0.1, p<0.001) showed a significantly decreased radiographic joint damage score per ankle compared to PBS treated WT mice (Mean ± SEM; 1.4 ± 0.2). In-line with the observed clinical arthritis scores, the anti-IL-7R treated TSLPR-/- mice showed an even lower radiological score (0.1 ± 0.1; p<0.001), which was significantly lower than the scores in all three other groups (Fig. 1B,C). This was confirmed with histopathological analysis (Supporting Information Fig. S1), demonstrating significantly decreased joint damage in anti-IL-7R treated

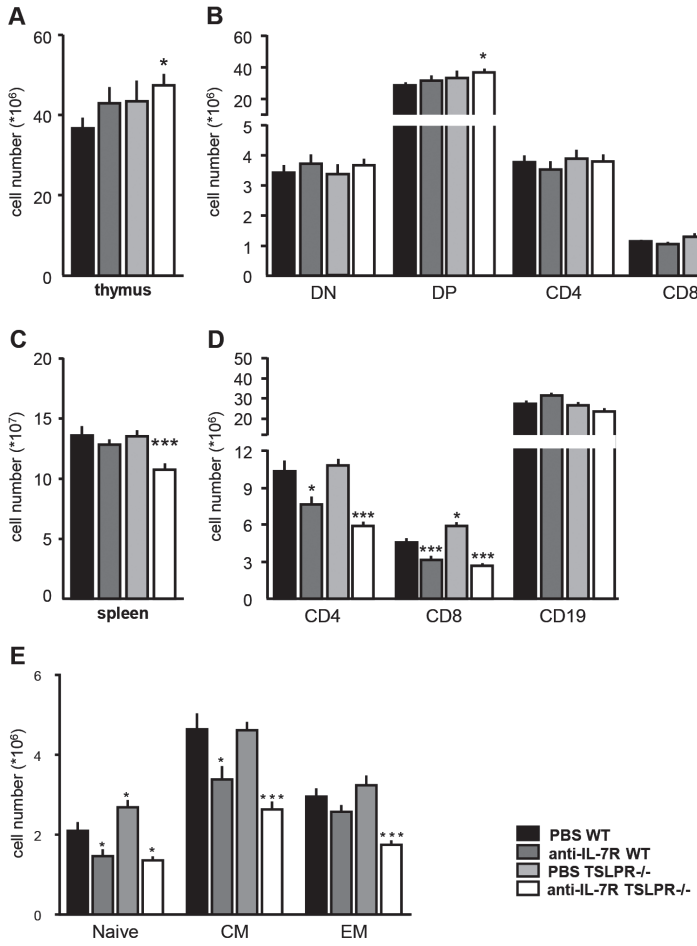

Figure 2. TSLPR-deficiency and IL-7R blockade alter numbers of thymocytes and splenocytes. Total numbers of thymocytes were modestly increased in anti-IL-7R treated TSLPR-/- mice as compared to PBS treated WT mice (**A**), associated with an increase in CD4+ CD8+ double positive (DP) thymocytes (**B**). Total numbers of splenocytes were decreased in anti-IL-7R treated TSLPR-/- mice compared to PBS treated WT mice (C), associated with a decrease in CD4+ and CD8+ T-cells in the anti-IL-7R treated TSLPR-/- mice (D). The number of CD4+ T-cells with characteristics of naive (Naive; CD44-CD62L+), central memory (CM; CD44+CD62L+) and effector memory (EM; CD44+CD62L-) T-cells were significantly decreased in anti-IL-7R TSLPR-/- mice as compared to PBS WT mice. Values are mean ± SEM of 16 mice per group. * and *** indicate statistical differences of $p<0.05$ and $p<0.005$ respectively.

TSLPR-/- mice compared to all three other groups (Fig. 1D). The same holds true for osteoclast activity as measured by TRAP staining (Fig. 1E).

The arthritis incidence at day 36 was similar in all of the groups. However, there seemed to be a delay in disease onset in all three experimental groups as they showed significantly lower arthritis incidence during observation days 27-31 compared to the PBS treated WT mice control group (Supporting Information Fig. S2).

IL-7R blockade and TSLPR-deficiency alter numbers of thymic and splenic T-cells in mice

As IL-7 and TSLP have been shown to affect T- and B-cell expansion[2,19,25], we investigated the effects of anti-IL-7R treatment and TSLPR deficiency on the number of T- and B-cells in spleen and thymus. Anti-IL-7R treatment and TSLPR deficiency separately did not have a significant effect on the number of thymic and splenic lymphocytes. However, anti-IL-7R treated TSLPR-/- mice showed significantly increased total number of thymocytes compared to PBS treated WT mice (Fig. 2A), which was associated with an increase in $CD4^+CD8^+$ double-positive thymocytes (Fig. 2B). The other thymocyte subsets were not different between any of the groups. In the spleens of anti-IL-7R treated TSLPR-/- mice, a decrease in total cell number was observed compared to PBS treated WT mice (Fig. 2C), associated with lower $CD4^+$ T-cell and $CD8^+$ T-cell numbers (Fig. 2D). In the anti-IL-7R treated WT mice, numbers of both CD4 and CD8 T-cells were lower compared to PBS treated WT mice. In the TSLPR-/- mice, only the number of $CD8^+$ T-cells was higher compared to WT mice. Splenic B-cell numbers were not different between any of the groups. In the spleen, naive (CD44- CD62L+) and central memory (CD44+ CD62L+) CD4 T-cell numbers were decreased in the anti-IL-7R treated WT mice compared to PBS treated WT mice (Fig. 2E). In the anti-IL-7R treated TSLPR-/- mice, the frequencies of naive, central memory and effector memory (CD44+ CD62L-) CD4 T-cells were decreased compared to PBS treated WT mice. In contrast, PBS treated TSLPR-/- mice showed higher numbers of naive CD4 T-cells compared to PBS treated WT mice (Fig. 2E).

Prevention of IL-7R and TSLPR signalling reduces expression of local and systemic pro-inflammatory mediators

Multi-cytokine analysis was performed on paw lysates and serum from all four groups studied. Compared to the PBS treated WT control group, the anti-IL-7R treated TSLPR-/- mice showed decreased CD40L, IL-17 and IL-12 levels

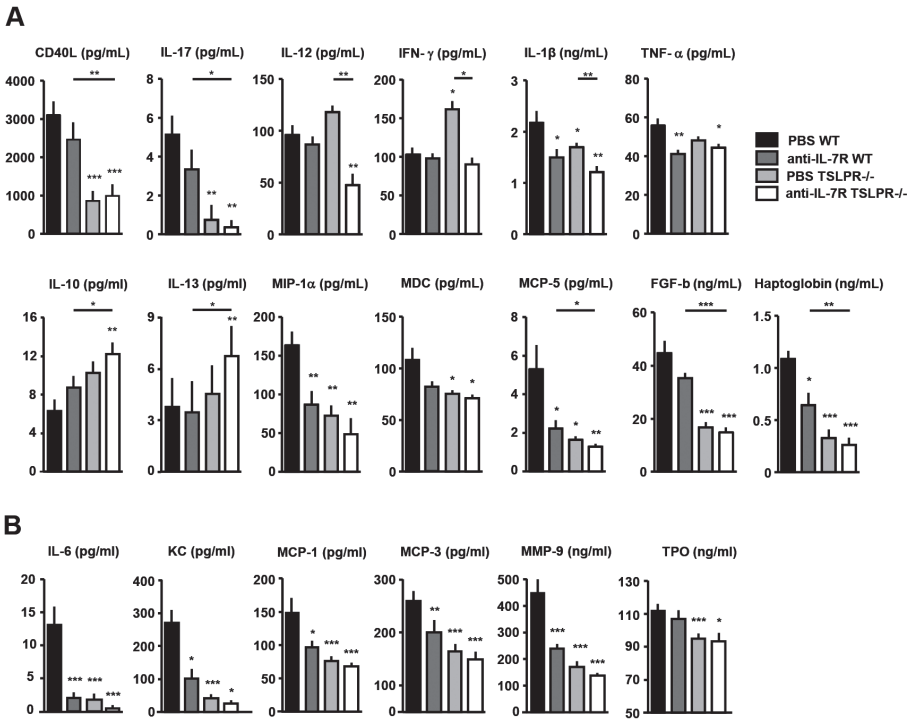

Figure 3. IL-7R blockade and TSLPR-deficiency additively reduce production of pro-inflammatory cytokines, chemokines and mediators involved in tissue destruction and angiogenesis. Cytokine concentrations in paw protein lysates (**A**) and serum (**B**) were measured by multicytokine analysis. Cytokines associated with T-cell and monocyte/macrophage activation, chemotaxis, angiogenesis and tissue destruction were reduced by IL-7R blockade and TSLPR deficiency. Values are mean ± SEM of 10 mice per group with representative arthritis scores. *, **, and *** indicate statistical differences of $p<0.05$, $p<0.01$, or $p<0.005$ respectively.

in the paw lysates (Fig. 3A) coinciding with lower IL-6 serum levels (Fig. 3B). Regulatory cytokines IL-10 and IL-13 were significantly increased in the anti-IL-7R treated TSLPR-/- mice. In addition, significant decreases in IL-1β and chemokines MIP-1α, MDC, MCP-1, MCP-3, MCP-5 and KC were observed upon combined prevention of IL-7R and TSLPR signalling, whereas IFN-γ did not show a difference in paw lysates or serum. Furthermore, significant decreases of factors involved in tissue destruction FGF-b, TPO and MMP-9 were found (Fig. 3A,B). IL-4 in all cases was not measurable (data not shown).

IL-7 and TSLP additively induce T-cell proliferation and Th17-associated cytokine production in cDC/T-cell co-culture of human primary cells

To study whether the combined effects of IL-7 and TSLP observed in our mouse model are also relevant in the human setting, we performed co-cultures with autologous $CD1c^+$ cDCs and $CD4^+$ T-cells, the main target cells for TSLP and IL-7 respectively. Combined addition of both IL-7 and TSLP significantly increased T-cell proliferation compared to either cytokine alone (Fig. 4A). IL-7 and TSLP significantly and additively increased secretion of Th17-associated cytokines IL-17, IL-21, IL-22 and IL-6 compared to either cytokine alone (Fig. 4B). In addition, T-cell attracting chemokines MIP-1α, MIP-1β and MDC were additively upregulated by IL-7 and TSLP compared to either cytokine alone (Fig. 4C). Furthermore, a range of other mediators showed a stronger increase in the presence of both cytokines compared to either cytokine alone, (Fig. 4C). Regulatory cytokines IL-10 and IL-13 (Fig. 4C) and IL-4 and IL-5 (not shown) were not significantly additively modulated.

Discussion

Here we show that combined ablation of IL-7R and TSLPR signalling strongly inhibits experimental arthritis and almost completely prevents immunopathology. This is associated with marked decreases in T-cell attracting- and co-stimulatory factors and levels of cytokines associated with Th17 activity. We further observed robust additive effects of IL-7 and TSLP on T-cell proliferation and Th17 activation in human DC/T-cell *in vitro* co-cultures, suggesting these data are relevant for the human setting.

In the anti-IL-7R treated TSLPR-/- mice, strongly ameliorated clinical arthritis and immunopathology were associated with decreased levels of IL-17-associated cytokines IL-17 and IL-1β locally and IL-6 systemically. This is consistent with previous data that describe PGIA to be strongly IL-17 dependent, especially in the later stages of disease[26]. As CD40L can trigger production of IL-17 by T-cells[27], the observed local decrease in CD40L may contribute to disease amelioration. In line with these mouse data, in the DC/T-cell co-cultures we observed potent additive effects of IL-7 and TSLP on production of Th17-associated cytokines IL-17, IL-21 and IL-22 that can strongly stimulate autoimmunity[28]. Though we have added the cytokines in our *in vitro* cultures while the *in vivo* model is based on abrogation of signalling, the effects on Th17-activity and immune activation

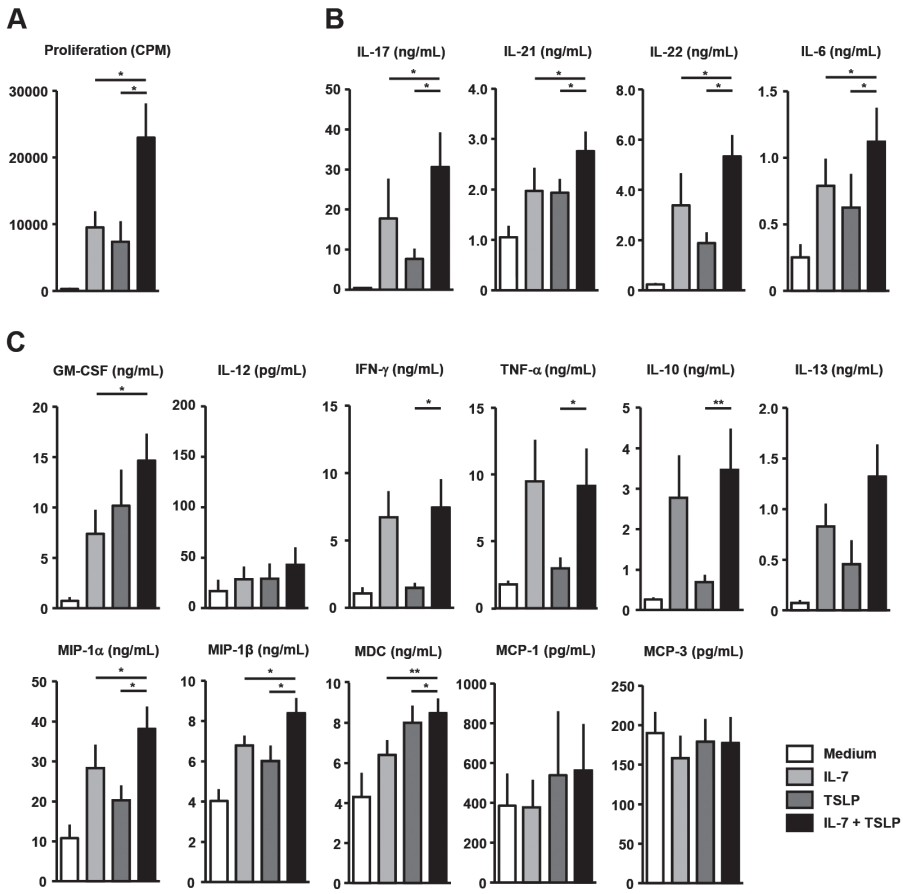

Figure 4. TSLP and IL-7 additively induce T-cell proliferation and Th17-activity in a human cDC/T-cell co-culture. Paired CD1c-expressing cDCs and CD4+ T-cells were isolated from rheumatoid arthritis patients. CD4 T-cells were co-cultured with cDCs for 6 days in the presence of thymic stromal lymphopoietin (TSLP), IL-7 or both cytokines. TSLP and IL-7 additively induce T-cell proliferation as measured with tritium thymidine incorporation (**A**). TSLP and IL-7 show additive induction of Th17-associated cytokines (**B**). Additive effects were also found on expression of other proinflammatory cytokines and T-cell attracting chemokines (**C**). * and ** indicate statistical differences of $p<0.05$ and $p<0.01$ respectively.

are clear in both settings. This indicates that the additive effects of TSLP and IL-7 are not restricted to the mouse setting and underscores the potential of their combined blockade in patients.

The anti-IL-7R antibody used in our mouse experiments could potentially inhibit TSLP signalling in addition to IL-7 signalling, but we have shown *in vitro* that the antibody is 100-fold more effective at blocking IL-7-induced chemokine secretion by murine DCs compared to TSLP-induced secretion (C.R. Willis, unpublished data). Additionally, the differential effect of anti-IL-7R antibody treatment and TSLPR-deficiency on thymic- and splenic cell numbers as well as expression of IFN-γ and IL-12 production strongly suggests a selective inhibition of IL-7-mediated immune responses. Though we have previously observed a larger decrease in thymocytes and splenocytes upon treatment with anti-IL-7R[21], the previous study was performed in young mice while the present study was performed in retired breeders of at least 6 months old. At this age the number of IL-7-producing stromal cells in thymus and bone marrow is dramatically reduced compared to young mice[29,30], which likely causes the less pronounced effects of blockade of IL-7 signalling on T-cell numbers that we observed in this study.

In mice TSLPR-deficiency results in strong reduction of IL-4 production[31]. We could not verify this as IL-4 was not detectable in our study. We did observe an increase in Th1 mediators IFN-γ and IL-12 in TSLPR-deficient mice. Though Th1-activity is known to promote PGIA, IFN-γ and IL-12 have also been demonstrated to down regulate Th17 activity [32,33], thus their increased levels may contribute to the marked decrease in IL-17 production we observe in the TSLPR-deficient mice. In addition, the enhanced IFN-γ and IL-12 production in deficient mice was negated by anti-IL-7R treatment, which also further down regulated IL-17 expression.

TNF-α levels were modestly but significantly inhibited upon anti-IL-7R treatment in the mice. This is in line with our DC/T-cell co-cultures, where IL-7 upregulated TNF-α secretion. TSLP deficiency only minimally decreased TNF-α levels in our mice and TSLP induced expression of TNF-α to limited extent in the DC/T-cell co-cultures. However, in short-term (24 hours) DC-priming experiments we previously showed that TSLP-stimulated cDCs clearly produce TNF-α and are able to induce TNF-α production by T-cells[34]. The cause of the less evident inhibition of TNF-α in mice in the present study is unclear, but may be related to species differences. Redundancy in arthritogenic cytokines could also play an important role, with IL-1β taking over the role of TNF-α as local

IL-1β concentrations were much higher than TNF-α.
A significant increase in regulatory cytokines IL-10 and IL-13 was only observed in the anti-IL-7R treated TSLPR-/- mice as compared to PBS treated WT control mice. Considering the inhibitory potential of these cytokines on many proinflammatory mediators like IL-17, IL-6 and IL-1β, they may contribute to the observed suppression of arthritis severity[35]. As IL-10 and IL-13 were also produced in the DC/T-cell co-cultures, these cells might be a major source of these cytokines in the mice as well, contributing to disease amelioration.

This is the first study to assess the combined roles of IL-7 and TSLP in autoimmunity and shows that both cytokines play a specific pro-inflammatory role during experimental arthritis. This emphasizes the additional benefits of TSLP-signalling blockade in conjunction with blockade of IL-7-signalling as a therapeutic strategy in rheumatoid arthritis and possibly other autoimmune diseases. As both cytokines signal via the IL-7R, the data described here emphasize the potential for antibodies or other compounds that target this subunit or abrogate activity of both cytokines simultaneously to prevent immunopathology and tissue damage in RA. Recently developed second-generation anti-IL-7R antibodies that inhibit signalling of both IL-7 and TSLP may prove to be a very beneficial treatment option.

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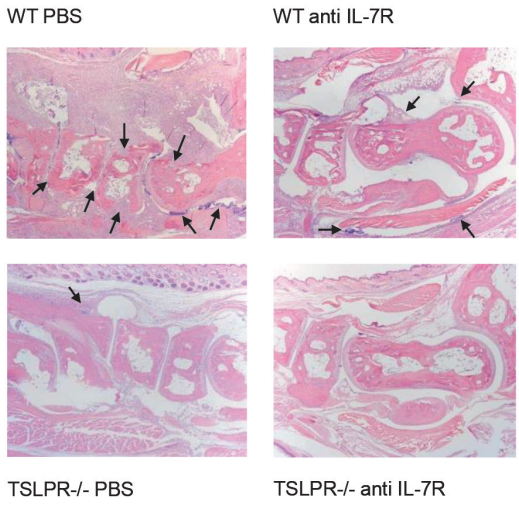

Figure S1. Joint damage and infiltration are inhibited in TSLPR deficient mice and upon blockade of the IL-7R. Photomicrographs of representative ankle joint sections. TSLPR deficiency and IL-7R blockade decrease the destruction of the articular surface and the amount of infiltrating cells in the connective tissue and joint space (arrows). Original magnification x40.

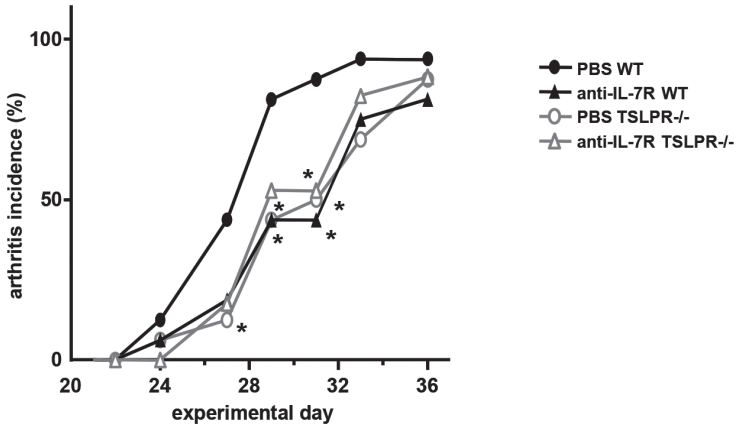

Figure S2. Abrogation of IL-7 and TSLP signalling results in delayed arthritis development. WT or TSLPR-/- Balb/c mice were treated with PBS or anti-IL-7Rα antibodies on day 21, 24, 27, 30, and 33. Arthritis severity was graded and arthritis incidence was defined as grade 1 or higher. On day 31 all three groups showed lower arthritis incidence as compared to the PBS treated WT mice. * indicates a statistical difference of $p<0.05$.


3

Targeting CD1c-expressing cDCs to prevent thymus and activation-regulated chemokine-mediated T-cell chemotaxis in RA

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Abstract

Objectives. Thymus and activation regulated chemokine (TARC) attracts cells that express the C-C chemokine receptor (CCR)4 including CD4 T-cells. As expression of CCR4 is increased on peripheral T-cells and intra-articular IL-17 producing cells in rheumatoid arthritis (RA) patients we investigated whether TARC plays a role in attraction of T-cells to the synovial compartment. In addition, we assessed the role of classical dendritic cells (cDCs) in the production of TARC in RA.

Methods. TARC was measured in synovial fluid (SF) from RA and osteoarthritis (OA) patients. Spontaneous and TSLP-induced TARC production by mononuclear cells (MC) and from peripheral blood (PB) and SF was assessed. The role of TARC in CD4 T-cell migration towards cDCs was assessed and the contribution of CD1c-expressing cells to TARC production by MCs were studied.

Results. TARC concentrations were higher in SF of RA patients compared to OA patients. MCs from SF produced TARC spontaneously and produced more TARC upon stimulation than paired PBMCs. Blocking TARC strongly inhibited CD4 T-cell chemotaxis by TSLP-stimulated cDCs, associated with decreased production of TNF-α, IL-17 and IFN-γ. Depletion of CD1c-positive cells from SFMCs strongly reduced TARC production.

Conclusions. TARC levels are increased in RA SF and our data indicate that this results from production by SFMCs and in particular CD1c cDCs. TARC attracts pro-inflammatory T-cells and TARC secretion by MCs is critically dependent on the presence of CD1c cDCs. Considering the potential of SF cDCs to activate T-cells and induce proinflammatory cytokine secretion, targeting intra-articular cDCs constitutes a novel therapeutic approach in RA.

Introduction

Thymus and activation regulated chemokine (TARC/CCL17) was first identified in the thymus and was shown to attract cells expressing C-C chemokine receptor (CCR)4[1], including NK-cells, fibroblasts and CD4 T-cells with Th2, Th17 and Treg phenotypes [2-6]. TARC is released by activated platelets and is produced by keratinocytes and specific subsets of activated T-cells[7, 8]. In addition, secretion was shown by epidermal Langerhans cells[9] and by activated $CD11c^+$ classical dendritic cells (cDCs; also referred to as mDCs) in allergic conditions[10].

TARC is described as a critical mediator in atopic diseases including atopic dermatitis (AD) and asthma[6, 11]. However, TARC expression has also been described in a range of autoimmune diseases, including primary Sjögren's syndrome, systemic lupus erythematosus, autoimmune blistering disease, juvenile idiopathic arthritis and rheumatoid arthritis (RA)[12-16]. In RA, TARC protein has been detected in plasma[15] while TARC mRNA is elevated in RA synovial tissue[17]. In addition, RA patients have increased numbers of peripheral blood CD4 T-cells that express CCR4[18]. Moreover, the majority of IL-17 positive CD4 T-cells in RA synovial fluid (SF) is $CCR4^+$ and the percentage of these cells of all mononuclear cells is higher in the SF compared to the peripheral blood[5]. These observations suggest that TARC mediates migration of pro-inflammatory CCR4-expressing T-cells into the joint in RA. Furthermore, we have previously shown that thymic stromal lymphopoietin (TSLP), a strong TARC-inducer on cDCs, is increased in the RA SF[19] and that cDCs from the RA SF produce TARC *ex vivo*[20]. The aim of this study was to evaluate TARC levels in SF of RA patients, study the role of cDCs in TARC production in RA joints and investigate the role of TARC in attraction of pro-inflammatory cells in RA.

Materials and Methods

Patients

Synovial fluids were obtained from patients attending our outpatients' clinics. RA patients were classified according to the American College of Rheumatology criteria[21]. Synovial fluids from patients with osteoarthritis (OA) were used for comparison in TARC ELISA. The medical ethical committee of the University Medical Center Utrecht approved collection of samples in accordance with the Helsinki declaration. All patients gave their informed consent. Characteristics of all patients are depicted in Table 1.

Flow cytometry

Fluorescence associated cell sorting (FACS) and a FACSCanto II flow cytometer (BD Bioscience, San Jose, USA) were used to analyze expression of extracellular markers on all samples. Results were analyzed using FlowJo software (Tree star, Ashland, USA). To assess the number of CD1c-expressing cDCs in SF of RA patients (n=17), SFMCs were stained with CD1c-PE (Biolegend, San Diego, USA) and CD19-PerCP-Cy5.5 (Biolegend). $CD1c^+$ cDCs were gated as CD19 negative and CD1c positive cells within the live gate. The number of $CD1c^+$ cDCs was calculated using the number of isolated SFMCs.

Cytokine analyses

TARC content in synovial fluids of RA patients (n=100) and OA patients (n=50) was measured with ELISA (R&D Systems, Minneapolis, USA). Samples were pre-treated with hyaluronidase (20U/mL; type IV, Sigma-Aldrich, St. Louis, USA). TARC ELISA was also used to measure TARC production in MC and CD1c-depleted MC culture supernatants. TARC production by isolated $CD1c^+$ cDCs and T-cell cytokine production after chemotaxis were analyzed by multiplex immunoassay as described elsewhere[22].

Cell isolation

Mononuclear cells (MC) were isolated from lithium-heparinized PB and SF by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Prior to isolation, PB or SF was diluted 1:1 in RPMI 1640 medium (Gibco, Life Technologies, New York, USA) containing penicillin (100 U/mL), streptomycin (100µg/mL) and glutamine (2mM) (all PAA Laboratories, Pasching, Australia). For assessment of TARC production by cDCs and chemotaxis experiments, CD19-$CD1c^+$ cDCs and $CD4^+$ T-cells were isolated from PBMCs by magnetic-activated cell sorting (MACS) using CD1c (BDCA-1)$^+$ and $CD4^+$ isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. To confirm efficacy of CD1c-isolation and CD1c-depletion, isolated cells were stained with CD45-PerCP (Biolegend), CD1c-PE (Biolegend) and CD19-FITC (BD Bioscience, San Jose, USA).

Cell cultures

Cells were cultured in RPMI Glutamax (Gibco) supplemented with penicillin, streptomycin (both PAA laboratories, Pasching, Austria) and 10%, v/v, human AB serum (GemCell, West Sacramento, USA). To compare TARC production by SFMCs with that of PBMCs, MCs were purified from paired PB and SF samples from RA (n=6) patients. Cells were seeded at a concentration of $1.0 * 10^6$/mL in

	SF TARC Analysis		RA *in vitro*
	RA	OA	cultures
N (M/F)	100 (32/68)	50 (12/38)	35 (13/22)
Age (yr.)	56 ± 15	64 ± 10	53 ± 16
Disease duration (yr.)	17 ± 17	-	11 ± 6.3
Rheumatoid factor (no. positive)	61	-	16
ESR (mm/hour)	44 ± 34	NA	21 ± 22
No. treated with Corticosteroids/ DMARDs/Biologics	9/51/26	NA	5/14/9

Table 1. Patients' characteristics. RA: Rheumatoid arthritis; OA: Osteoarthritis; ESR: Erythrocyte sedimentation rate; DMARD: Disease modifying anti-rheumatic drug; NA: Not applicable; SF: Synovial fluid. Numbers depicted are Mean ± standard deviation unless stated otherwise.

96-wells round-bottomed plates and cultured in triplicates for 72 hours at 37°C in the presence of 20ng/mL recombinant human TSLP (R&D) and/or 10 ng/mL of recombinant human IL-7 (Preprotech, Rocky Hill, USA). Supernatants were harvested and stored at -80°C. To investigate TARC production by cDCs, $CD1c^+$ cDCs were isolated from RA patients' PB (n=6) and cultured in Sarstedt tubes at 37°C with 20ng/mL TSLP (R&D) for 20 hours ($5*10^5$ cells/mL). Supernatants were collected and stored at -80°C and analyzed by multiplex immunoassay.

Chemotaxis assay

$CD1c^+$ cDCs and $CD4^+$ Tcells were isolated from PBMCs of RA patients (n=6) and healthy controls (n=3) and cell purity of subsets was confirmed by FACS. Cells were taken up in RPMI Glutamax (Gibco) supplemented with penicillin, streptomycin (both PAA) and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Per condition $3*10^4$ cDCs were added to the lower compartment of a 24-wells transwell system (Corning, New York, USA), supplemented with 20 ng/mL TSLP (R&D) and where appropriate 10ug/mL of monoclonal anti-TARC (mouse IgG1; R&D) or an equal concentration of an isotype control antibody (mouse IgG1; Amgen, Seattle, USA). A well without cDCs and only culture medium was prepared as control for aspecific T-cell migration. $5*10^5$ CD4 T-cells were added to the upper compartment of the transwells (5.0 μm pore size, polycarbonate membrane; Corning) and cells were allowed to migrate for 20 hours at 37°C. The percentage of migrated cells was determined by staining the cell fraction in the lower compartment with anti-CD3 FITC (BD), anti-CD19 APC (Biolegend), anti-CD1c PE (Biolegend) and anti-CD4 PerCP

(Biolegend). For analysis of the net T-cell cytokine production, the remaining cells were resuspended in 200uL RPMI Glutamax (Gibco) supplemented with penicillin, streptomycin (both PAA) and 10% fetal bovine serum (Invitrogen) and stimulated for 24 hours at 37°C with ionomycin (500ng/mL) and phorbol myristate acetate (50ng/mL) (both from Sigma-Aldrich). Cytokines were measured by multiplex immuno assay. The percentage of migrated T-cells was calculated using the number of cells counted in the lower compartment and the percentage of T-cells therein as measured by FACS, corrected for aspecific T-cell migration. Stimulation index (SI) of produced cytokines was calculated by dividing by the cytokine production of the culture medium control that contained no cDCs.

CD1c-depletion

MCs were isolated from PB (n=7) and SF (n=8) of RA patients, including 2 paired samples. CD1c-expressing cells were depleted using anti-CD1c biotin and anti-biotin beads (Miltenyi). As control, B-cells were depleted using anti-CD19 beads. In addition, the CD1c-depletion protocol was performed omitting the CD1c-biotin on SFMCs of RA patients (n=4) in a separate experiment. Depleted and undepleted MC fractions were cultured for 72 hours in the presence of 20 ng/mL TSLP (R&D) at 37°C. Supernatants were harvested and stored at -80°C until analysis of TARC levels with ELISA.

Statistical Analyses

Differences in TARC content in SF between groups were determined using Mann-Whitney U test. Correlation between TARC content in SF and number of cDCs was calculated with Spearman's rank correlation coefficient. Wilcoxon signed rank test was used to assess differences in TARC production, T-cell migration and T-cell cytokine production. Statistical analyses were performed using SPSS software version 20.0 (IBM, Armonk, USA). Differences were considered statistically significant at $p \leq 0.05$.

Results

TARC levels are increased in RA synovial fluid and correlate with numbers of cDCs

TARC concentrations in the SF of RA patients were significantly increased compared to OA patients (Figure 1A). In addition, TARC levels were significantly higher in RA patients with increased systemic inflammation, defined as having an erythrocyte sedimentation rate (ESR) of 30 or higher, compared to those

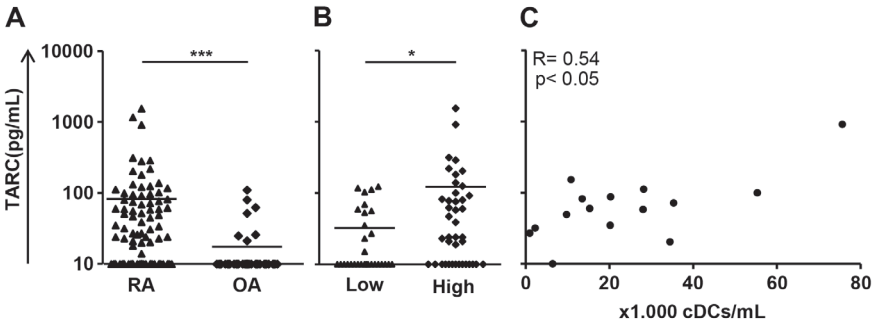

Figure 1. TARC is increased in RA SF and correlates with the number of CD1c cDCs. TARC levels in SF of RA (n=100) patients are higher than in OA (n=50) patients (**A**). Levels are significantly increased in RA patients with ESR ≥30 mm (High) compared to patients with ESR ≤30 (Low) (**B**). Numbers of CD1c+ cDCs in SF of RA patients correlate with TARC levels in SF (**C**). Levels below detection limit were indicated as 10 pg/mL to allow plotting on log axis, values were recorded as zero for analyses. * and *** indicate statistical differences of p<0.05 and p<0.0001 respectively.

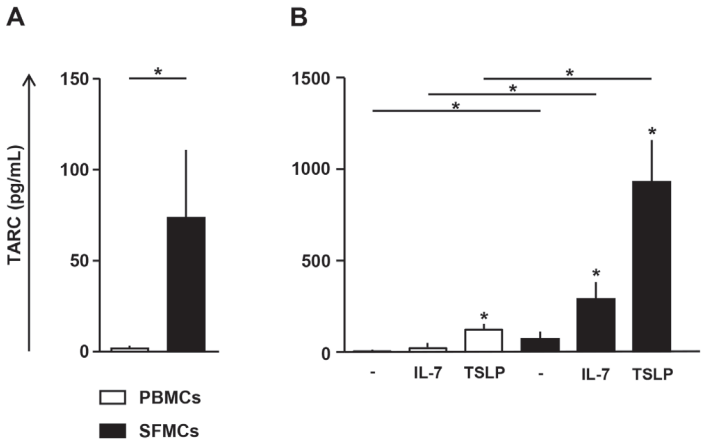

Figure 2. MCs from the synovial fluid spontaneously secrete TARC, which is increased by addition of TSLP or IL-7. Unstimulated mononuclear cells (MCs) from SF of RA patients produce increased TARC levels compared to paired MCs from the peripheral blood (PB) (**A**). Upon stimulation with TSLP, and to a lesser extent with IL-7, TARC production by PBMCs and SFMCs is greatly increased. SFMCs from RA patients produce more TARC than paired PBMCs (**B**). Bars indicate mean ± SEM. * indicates a statistical difference of p<0.05.

with low systemic inflammation (Figure 1B). Since CD1c cDCs are very potent TARC producers we investigated the relationship between TARC levels and local numbers of these cells. The number of CD1c-expressing cDCs in the SF correlated with TARC levels, suggesting a role for these cells in intra-articular TARC production (Figure 1C). As methotraxate was previously described to influence TARC expression in plasma of RA patients[15], we further examined the effects of therapy on intra-articular TARC levels in these samples. When the group of RA patients was divided based on therapy status, there were no significant differences in TARC levels between the untreated patients (Mean ± SEM; 42 ± 14 pg/mL) and those treated with biologics (29 ± 10 pg/mL, p=0.58) or those treated with DMARDs (80 ± 35 pg/mL, p=0.52). There was also no difference between patients treated with biologics and those treated with DMARDs (p=0.79).

Mononuclear cells from synovial fluid of arthritis patients spontaneously produce TARC

MCs from SF samples of arthritis patients spontaneously produced TARC while this was not observed for paired MCs from PB, indicating that immune cells produce TARC locally in the joint (Figure 2A). We used thymic stromal lymphopoietin (TSLP) and interleukin (IL)-7, both pro-inflammatory cytokines that are increased in RA SF, to investigate induction of TARC production by activating the cDCs or T-cells within the MCs respectively. SF MCs produced significantly higher TARC levels compared to PB MCs from the same donor when stimulated with TSLP or IL-7, indicating that local MCs are more prone to produce TARC than their peripheral counterparts. TSLP induced more TARC production than IL-7. The combination of TSLP and IL-7 did not further enhance the amount of TARC produced (not shown). This suggests that cDCs are important contributors to TARC-driven attraction of immune cells in RA. This was tested in two ways, by analyzing the capacity of RA cDCs to induce T-cell chemotaxis and by assessing the effect of cDC depletion on TARC production of MC populations from the PB and SF.

TARC produced by RA patients' cDCs induces chemotaxis of T-cells producing pro-inflammatory cytokines

We have previously shown that TSLP-stimulated CD1c cDCs from peripheral blood closely resemble CD1c cDCs that are present intra-articularly[19]. We thus used CD1c cDCs isolated from RA patients' PB to study the effect of TARC on attraction of autologous T-cells. cDCs from RA PB produced TARC when stimulated with TSLP while unstimulated PB cDCs did not (Figure 3A). When added to the lower compartment of a transwell system, TSLP-stimulated cDCs

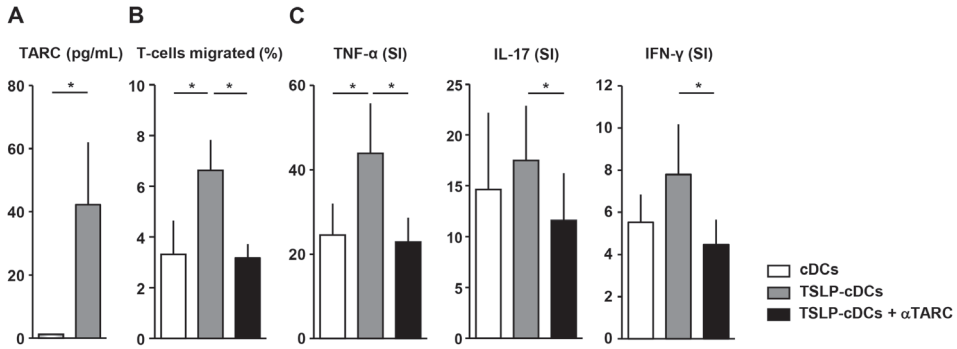

Figure 3. Chemotaxis of proinflammatory T-cells towards activated cDCs is critically dependent on TARC. When stimulated with Thymic stromal lymphopoietin (TSLP), cDCs show significantly increased TARC production (**A**). In a transwell chemotaxis assay TSLP-stimulated cDCs attract significantly more T-cells compared to unstimulated cDCs and anti-TARC neutralizing antibody completely negates this (**B**). TARC blockade inhibits attraction of T-cells that produce TNF-α, IL-17 and IFN-γ (**C**). Cytokine production depicted as stimulation index (SI) relative to negative control without any cDCs in the lower well. Bars indicate mean ± SEM. * indicates a statistical difference of $p<0.05$.

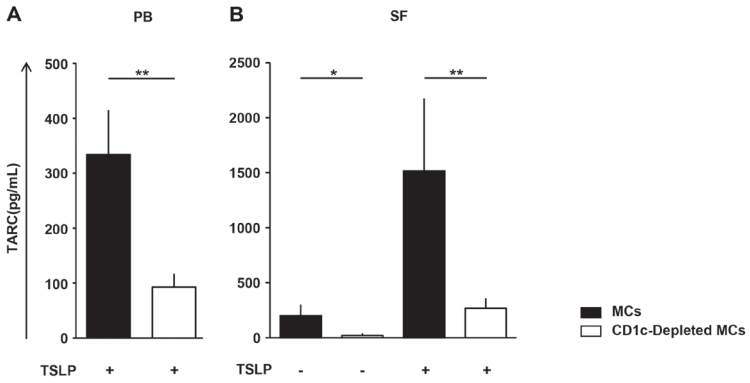

Figure 4. TARC production by PB and SF mononuclear cell fractions is critically dependent on CD1c-expressing cells. Depletion of CD1c-expressing cells from the PB mononuclear cell (MC) population of RA patients robustly inhibits TARC production upon TSLP stimulation (**A**). CD1c depletion almost completely prevents spontaneous TARC production by RA SFMCs and strongly reduces TARC production by TSLP-stimulated SFMCs (**B**). Bars indicate mean ± SEM. * and ** indicate statistical differences of $p<0.05$ and $p<0.01$ respectively.

induced significantly more autologous $CD4^+$ T-cell migration from the upper compartment compared to unstimulated cDCs. This T-cell attraction was robustly inhibited by a neutralizing antibody against TARC (Figure 3B). No significant difference was observed when adding an isotype control antibody instead (6.67 ± 1.69%, mean ± SEM, vs. 5.56 ± 1.73%, p=0.31). Attracted T-cells were analyzed for cytokine production and we observed a significant decrease in production of TNF-α, IL-17 and IFN-γ upon TARC neutralization (Figure 3C), which was absent when an isotype control was used instead (not shown). No differences in IL-10 production were observed, while IL-4 production was below detection limit in the majority of the samples (not shown).

Depletion of CD1c cDCs from intra-articular MCs robustly prevents TARC production

To confirm the critical role of cDCs in TARC production, we depleted MCs for these cells and assessed the effect on TARC production. Depletion of PBMCs from RA patients for CD1c-expressing cells resulted in a clear inhibition of TARC production upon TSLP stimulation compared to undepleted MCs (Figure 4A). Depletion of SFMCs for CD1c-expressing cells efficiently inhibited both spontaneous TARC production and TSLP- induced TARC production (Figure 4B). As B-cells can express CD1c we performed CD19-depletion to exclude that the effect of CD1c-depletion was B-cell-dependent. No differences were observed between undepleted SFMCs and CD19-depleted SFMCs in spontaneous TARC production (136 ± 40.2 pg/mL, mean ± SEM, vs. 136 ± 68.0 pg/mL, p=1.00) or TSLP-induced TARC production (1140 ± 376 pg/mL vs. 908 ± 369 pg/mL, p= 0.88). In addition, to control for influences of the MACS isolation procedure the CD1c antibody was omitted during depletion. No differences were observed compared to undepleted MCs upon TSLP stimulation (395 ± 213 pg/mL vs. 360 ± 205 pg/mL, p=0.63).

Discussion

We here for the first time show that levels of TARC are increased in SF of RA patients, are associated with systemic inflammation and mediate attraction of T-cells that produce cytokines that are pro-inflammatory in RA. TARC levels in SF correlate with CD1c numbers, suggesting that these cells are critical producers of TARC in SF. Indeed, TARC production was robustly inhibited by depleting CD1c-expressing cDCs from SFMCs.

Recently we demonstrated that CD1c cDCs from RA synovial fluid are extremely potent activators of CD4 T-cells, associated with strong proliferation and induction of Th1- and Th17 cytokine production[20]. TSLP, which is increased in RA synovial fluid, was shown to activate cDCs from PB and mimic functional and phenotypic features of intra-articular cDCs, including TARC production[19]. Here we demonstrate that increased TARC concentrations in SF are associated with cDC numbers and that TARC produced by TSLP-activated cDCs directs migration of CD4 T-cells with the capacity to produce pro-inflammatory cytokines including TNF and Th1- and Th17-associated cytokines. Interestingly, TARC can also attract fibroblasts and NK-cells[2, 3] which are implicated in RA immunopathology by production of proinflammatory cytokines in the joint[23] and induction of tissue damage by production of catabolic enzymes[24]. Together, these data clearly point towards a role for TARC as pro-inflammatory mediator in RA.

In the chemotaxis assay, TSLP-activated CD1c cDCs attracted TNF-α-producing cells, whereas activation of the cDCs with TSLP had no significant effect on attraction of IL-17- and IFN-γ-producing T-cells. Nonetheless, TARC blockade significantly reduced IL-17 and IFN-γ production. These data suggest that the minute amounts of TARC produced by unstimulated cDCs are sufficient to induce attraction of IL-17- and IFN-γ-producing cells.

Though expression of CCR4 is classically described as a feature of Th2, Th17 and Treg cells, recent data show that CCR4-expressing cells can contain considerable percentages of IFN-γ producing cells[25], explaining the reduction in IFN-γ secretion upon TARC blockade. Considering the fact that CD4 T-cells in RA patients are polarized towards Th1 and Th17 activity locally, and express large amounts of TNF-α, our data suggest that TARC blockade can contribute to a decreased influx of pathogenic T-cells producing TNF-α, IFN-γ- and IL-17.

A considerable proportion of FoxP3-expressing cells also expresses CCR4 and potentially can be attracted by TARC. Due to the low numbers of these cells, we have not been able to assess the attraction of Tregs specifically, though we did not observe differences in IL-10 production upon TARC blockade. Considering the downregulation of TNF-α, IL-17 and IFN-γ upon TARC blockade, our data suggest that TARC overrules Treg function. Furthermore, as TNF-α may mediate dysfunction local Tregs in RA[26] and is produced by TARC-attracted cells in our experiments, it is likely that TARC-mediated Treg attraction will be insufficient to suppress the pro-inflammatory T-cells attracted.

As TARC mediates in vitro attraction of pro-inflammatory T-cells, preventing TARC-mediated T-cell migration may prove to be beneficial for RA patients. A humanized anti-CCR4 antibody exists: Mogamulizumab, which is currently used for treatment of T-cell leukemia and lymphoma[27]. Alternatively, targeting the cDCs as the main source of TARC might be a successful method. CD1c cDC-depletion using an antibody would be an efficacious way to prevent TARC production as shown in our experiments. Considering the potent proinflammatory role of cDCs as sentinel cells of the immune system, targeting these cells is an attractive approach to inhibit pathways by which they attract and activate T-cells.

Acknowledgements

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Decreased expression of thymic stromal lymphopoietin in salivary glands of patients with primary Sjögren's syndrome is associated with increased disease activity

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Abstract

Objectives. Thymic Stromal Lymphopoietin (TSLP) is a potent immunomodulatory cytokine involved in Th2- and Th17-mediated immune responses in different autoimmune diseases. TSLP expression in relation to disease activity was studied in salivary glands of primary Sjögren's syndrome (pSS) patients as compared to non-SS sicca (nSS) controls.

Methods. Tissue sections of minor salivary glands from pSS and nSS patients were stained with monoclonal antibodies against human TSLP, CD3, CD19 and cytokeratin high molecular weight (CK HMW) or stained for Alcian blue to detect mucus production. The number of TSLP-expressing cells was quantified and expression was correlated to local and systemic disease parameters.

Results. The number of TSLP-expressing cells was significantly lower in pSS patients than in nSS controls and correlated with a range of disease markers. In pSS patients, TSLP was expressed outside of lymphocytic infiltrates at sections that also encompassed high numbers of intact acinar cells. This difference was independent of tissue destruction.

Conclusions. Reduced TSLP expression in pSS patients is associated with increased local and systemic inflammatory markers. Loss of TSLP expression may contribute to Th1/Th17-associated immunopathology in pSS, in line with previous studies demonstrating that TSLP promotes a protective Th2 milieu at mucosal sites.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of the salivary and lachrymal exocrine glands that leads to dryness of mouth and eyes. Infiltrates mainly consist of activated T- and B lymphocytes in conjunction with a range of antigen presenting cells, including monocytes, macrophages and dendritic cells (DCs). The infiltrating leukocytes and activated local epithelial cells produce a range of proinflammatory mediators including cytokines and proteases that interfere with glandular cell-function and drive tissue destruction, resulting in dryness symptoms[1]. $CD4^+$ T cells are thought to be critical for disease, as they are essential for efficient activation of autoantibody-producing B cells and production of proinflammatory cytokines. $CD4^+$ T cell subsets T-helper (Th)1 and Th17 cells producing the proinflammatory cytokines interferon (IFN)-γ and interleukin (IL)-17 respectively, are both associated with tissue damage in pSS[2].

The IL-7-related cytokine thymic stromal lymphopoietin (TSLP) is often described as a factor in Th2-mediated atopic diseases, but it also induces homeostatic T-cell expansion[3]. TSLP is produced by fibroblasts, epithelial cells and keratinocytes and mainly acts on classical dendritic cells (cDCs, also referred to as myeloid dendritic cells/mDCs), which strongly activate $CD4^+$ T-cells upon TSLP stimulation[4]. TSLP is a pro-inflammatory mediator in murine arthritis models and *in vitro* experiments with rheumatoid arthritis (RA) patient material[5,6]. Considering the presence of cDCs and factors that can induce TSLP production in the salivary glands[7,8], we investigated TSLP production in the salivary glands of patients with pSS in relation to parameters of disease activity.

Materials and Methods

Patients

Labial salivary gland (LSG) specimens of 39 pSS patients were used for immunohistochemistry (IHC) and LSG specimens of 6 pSS patients were used for mRNA analyses. All patients were diagnosed according to American-European consensus group criteria[9]. 26 sicca syndrome patients not fulfilling these criteria were classified as patients with non-Sjögren's sicca (nSS) and were included as control group for IHC (n=18) and mRNA analysis (n=8). Patient characteristics are shown in Table 1. EULAR Sjögren's Syndrome disease Activity

Index (ESSDAI) was calculated as previously described[10]. None of the patients received immunosuppressant drugs. The institutional review board (TcBio) of the UMC Utrecht approved this study without the additional need for written informed consent of the donors provided.

Histology and Immunohistochemistry

Frozen tissue sections were prepared and processed as previously described[11]. Serial tissue sections were stained with various monoclonal antibodies or Alcian blue. Tissue slides were incubated overnight with mouse monoclonal antibody against human TSLP (IgG1κ, a kind gift from Mike Comeau, Amgen, Thousand Oaks, CA, USA), cytokeratin HMW (34βE12, DAKO, Glostrup, Denmark), CD3 (BD Bioscience, San Jose, CA, USA) or CD19 (BD). We confirmed specificity of the TSLP antibody on western blot using TSLP produced by HEK293 cells. In addition, when used for staining of skin biopsies from atopic dermatitis patients the antibody identified TSLP-expressing keratinocytes. Control stainings were performed omitting the primary antibody or replacing it for an isotype control (M1, IgG1κ, Amgen). Antigen-antibody complexes were visualized with powervision-peroxidase-3,3'-diaminobenzidine (Leica Microsystems, Wetzlar, Germany) according to standard protocols. Scoring of stained cells was performed by investigators blinded to patient characteristics. Finally, mucopolysaccharides were stained with 1 percent Alcian blue (Sigma-Aldrich, St. Louis, MO, USA) in citrate buffer at pH 2.5 for 15 minutes at room temperature.

	nSS	pSS
N (M/F)	26 (2/24)	45 (5/40)
Age	50 ± 14	48 ± 14
ESR	18 ± 17	36 ± 24
sIgG	11 ± 2.6	20 ± 8.0
LFS	0.0 ± 0.0	3.1 ± 1.8
%IgA+	77 ± 14	46 ± 20
ESSDAI	-	2.4 ± 2.7
Anti-SSA (pos/neg)	1/25	33/12
Anti-SSB (pos/neg)	1/25	23/22

Table 1. Patients' characteristics. Characteristics of all patients used for immunohistochemistry and quantitative PCR analyses. ESR: erythrocyte sedimentation rate; sIgG: serum immunoglobulin G; LFS: lymphocyte focus score; %IgA+: percentage of IgA-expressing plasma cells in labial biopsy; ESSDAI: EULAR Sjögren's syndrome disease activity index. Data shown are mean ± standard deviation unless stated otherwise.

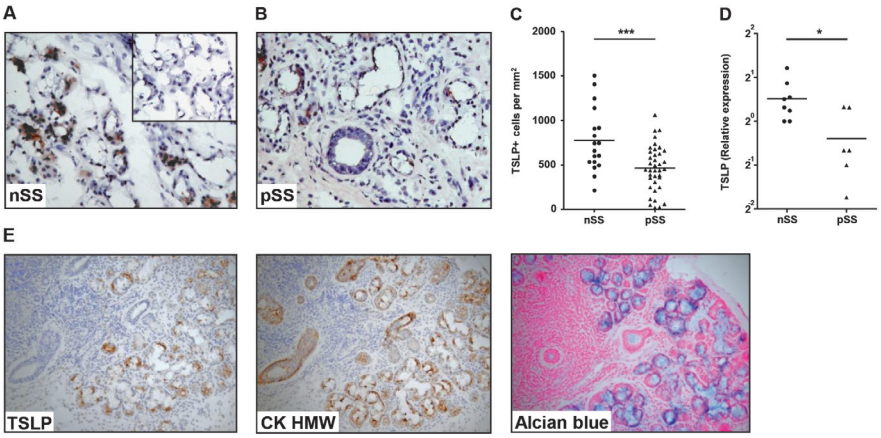

Figure 1. TSLP expression is decreased in labial salivary glands of pSS patients as compared to nSS patients. Labial salivary gland biopsy cryo-sections of primary Sjögren's syndrome (pSS) patients and non-Sjögren's sicca (nSS) controls and were stained for TSLP, Cytokeratin HMW or an isotype control. pSS patients show a lower number of TSLP positive cells per mm2 as compared to nSS. Representative photographs of TSLP staining in a nSS control (A, isotype control inset) and a pSS patient with LFS 2 (B). Significantly lower amounts of TSLP-producing cells per mm2 were quantified in pSS patients as compared to nSS (C). Lower TSLP expression in pSS salivary glands was confirmed on mRNA level with qPCR (D). Serial slides from the salivary glands of a pSS patient with LFS 2 show that TSLP expression is located in structures that are positive for Cytokeratin HMW and Alcian Blue, indicative of intact epithelial acinar cell structures that produce mucus (E). Original magnification x400 for A and B, x100x for E. Inset shows a higher magnification. * and *** indicate statistical differences of $p<0.05$ and $p<0.005$ respectively.

mRNA analysis

Material of 6 pSS patients and 8 nSS patients with characteristics conform the avarages depicted in Table 1 was used for mRNA analysis. Total RNA was isolated from LSG biopsy samples using RNeasy microkit (Qiagen, Valencia, CA, USA). TSLP gene expression was determined by quantative real-time PCR using the following primers: forward, AGTGGGACCAAAAGTACCGAGTT; reverse, GGATTGAAGGTTAGGCTCTGG. Results were normalized by comparison with expression of GAPDH. Relative expression was calculated by using the ΔCT method and dividing by the ΔCT of the lowest sample in the control group, which was set at 1.

Statistics

Mann-Whitney U test was used to evaluate differences between pSS and nSS patients. Spearman correlation was used to evaluate correlations between TSLP expression and T-cell numbers or disease parameters. Values of $p \leq 0.05$ were considered statistically significant.

Results

TSLP expression is decreased in salivary glands of pSS patients and localized in the epithelial cells with acinar cell structure

TSLP was abundantly expressed in the salivary glands of nSS controls (Figure 1a) and to a lesser extent in pSS patients (Figure 1b), while control stainings were negative (Figure 1a inset). The number of TSLP-expressing cells per mm2 was significantly decreased in pSS patients as compared to nSS controls (Figure 1c). We confirmed the IHC data by determining TSLP mRNA content in LSG tissue. Messenger RNA data correspondingly showed significantly decreased TSLP levels in pSS patients when compared to nSS controls (Figure 1d). In both groups TSLP was almost exclusively expressed by epithelial cells with acinar cell structures, which expressed epithelial cell marker CK HMW and were stained with Alcian blue, indicative of mucin production (Figure 1e).

The decrease in TSLP expression in pSS patients is not due to a general decrease in salivary epithelial cells

TSLP seems to be mainly produced by epithelial cells in the salivary gland and these cells are often disarranged and destroyed in pSS patients. To exclude the possibility that the observed reduction in TSLP-expression in pSS patients is solely due to a loss of epithelial cells in the salivary glands, we quantified the amount of TSLP-expressing cells in parts of the salivary gland tissue with intact epithelial cell structures, as determined by CK-HMW staining, and away from lymphocytic aggregates, as determined by CD3- and CD19 staining. In-line with the data per mm^2, the amount of TSLP-expressing cells as a percentage of the total cell number was significantly lower in the pSS patients as compared to the nSS controls (Figure 2a). The percentage of TSLP-expressing cells negatively correlated with the number of diffusely infiltrating CD3-expressing T-cells (Figure 2b). In addition, there were no differences between pSS patients and nSS controls in the amount of CK-HMW-expressing epithelial cells present in the counted sections (Mean ± SEM; 3498 ± 283 cells/mm^2 vs. 3784 ± 200 cells/mm^2, p=0.21).

Decreased TSLP expression correlates with local and systemic disease parameters

The number of TSLP-expressing cells per mm^2 in LSG tissue of pSS patients negatively correlated with ESSDAI (r= -0.321, p=0.016). The number of TSLP-expressing cells per mm^2 in LSG tissue of pSS and nSS sicca patients inversely correlated with Lymphocyte Focus Score (LFS) (Figure 3a), serum IgG levels (Figure 3b) and Erythrocyte Sedimentation Rate (ESR) (Figure 3c) and correlated positively with the percentage of local IgA producing plasma cells (Figure 3d).

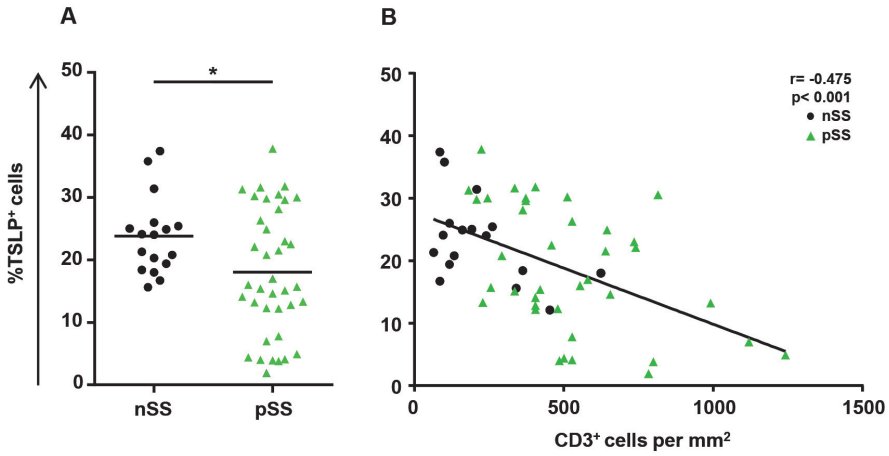

Figure 2. The percentage of TSLP-expressing cells in intact salivary gland tissue is decreased in pSS patients and correlates with T-cell infiltration. In sections of salivary gland tissue away from lymphocytic aggregates and with intact epithelial tissue as determined by CK-HMW, CD3 and CD19 staining, the percentage of cells that express TSLP is decreased in pSS patients as compared to nSS controls (A). This is correlated with numbers of diffusely infiltrating T-cells at these sites (B). * shows a statistical difference of p<0.05. Spearman's correlation coefficient (r) and p-values are shown.

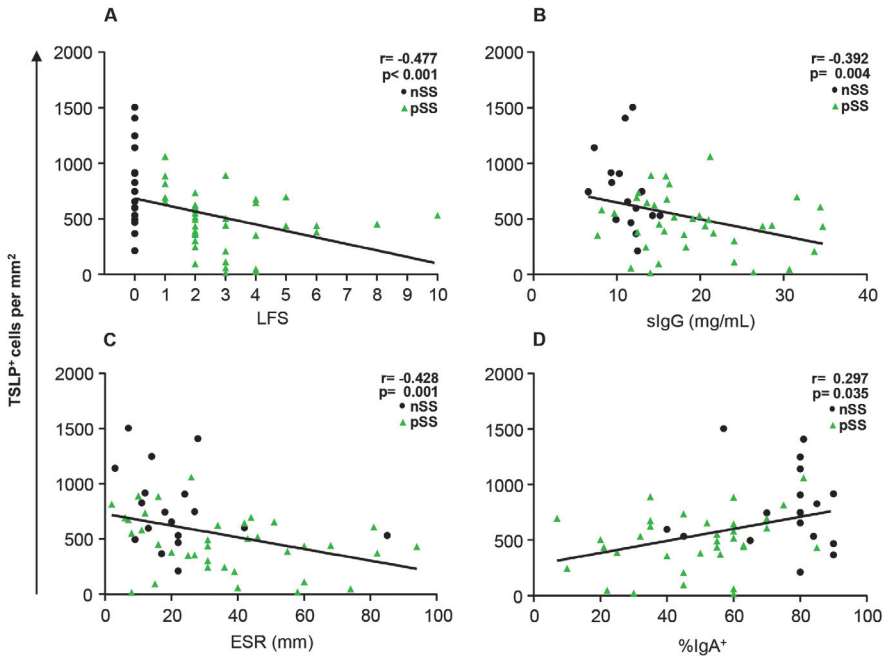

Figure 3. TSLP expression correlates with local and systemic disease parameters in sicca patients. The numbers of TSLP positive cells per mm2 in nSS and pSS patients were significantly correlated with Lymphocyte Focus Score (LFS) (A), serum IgG (sIgG) levels (B), erythrocyte sedimentation rate (ESR) (C) and percentage of IgA+ plasma cells (D). Spearman's correlation coefficients (r) and p-values are shown.

Discussion

Here we show that the number of TSLP-producing cells and TSLP mRNA content in the minor salivary glands is decreased in pSS patients as compared to nSS controls, and that the amount of TSLP-expressing cells correlates with local and systemic disease markers. This decrease in expression is not merely a consequency of tissue destruction as the percentage of TSLP-producing cells outside of lymphocytic foci, at sites with intact epithelial cells is also significantly decreased in pSS patients.

As TSLP is a pro-inflammatory mediator in a range of rheumatic diseases including RA[6] and systemic sclerosis (SSc)[12], we did not expect to find decreased expression in the LSG of pSS patients. However, differences in

immunological microenvironment between pSS and RA or SSc may explain this discrepancy. A decrease in TSLP expression has also been shown in patients with Crohn's disease[13]. Epithelial cells from the gut constitutively express TSLP, promoting DC-driven Th2-activity and protecting against Th1-mediated immunopathology[14]. Decreased TSLP production by the gut epithelial cells in Crohn's disease patients enhances inflammation by reducing Th2-mediated protection against inflammation. We hypothesize that TSLP produced by epithelial cells is similarly regulated in the mucosal tissue of the salivary glands and mediates tolerance by promoting Th2-activity and thereby preventing Th1- and Th-17-driven inflammatory activity. This is supported by the correlation of TSLP expression with local and systemic disease parameters.

In this study the Th1/Th2 balance in relation to TSLP was not investigated. However, we have previously shown highly increased local numbers of IFNγ-producing cells and a strong shift towards Th1-activity in pSS but not nSS patients[15]. Here we show an inverse correlation between percentage of TSLP-expressing cells and the number of diffuse infiltrating T-cells. As there is a strong shift towards Th1 activity in pSS patients versus nSS patients while Th2-activity is equal between these groups[15,16], it is anticipated that these infiltrating T-cells largely represent Th1 cells. However, the proposed induction of Th2-activity by TSLP can also be pro-inflammatory, supporting germinal center reaction. In the salivary glands, Th2- cells are increased in germinal centers and likely to represent T-follicular helper cells (Tfh) that co-express IL-4, IL-21 and Bcl-6[16, 17]. As germinal centers are related to disease severity, IL-4-producing Tfh cells may contribute to ongoing inflammation.

Furthermore, TSLP has recently been suggested to have direct anti-microbial activities. Therefore, reduced TSLP expression may contribute to loss of barrier function and subsequently to enhanced sialadenitis due bacterial- or viral-triggered inflammation[18,19], but this requires further validation.

In conclusion our data show decreased production of TSLP in LSG of patients with pSS that is associated with local and systemic markers of inflammation. Future studies should reveal whether this lower TSLP expression is the cause or the effect of the inflammatory tissue damage in the LSG of pSS patients.

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5

Dendritic cells, T-cells and epithelial cells: a crucial interplay in immunopathology of primary Sjögren's syndrome

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Summary

Sjögren's syndrome (pSS) is a chronic autoimmune disease that is characterized by mononuclear cell infiltration of exocrine glands. T-cells have been shown to play a central role in tissue destruction and regulation of B-cell activity and the production of autoantibodies typifying pSS. Despite the fact that dendritic cells (DCs) are candidate key players in the activation of T- and B-cells in pSS, their contribution has been under evaluated. This manuscript reviews current insights in DC biology and examines literature on the role of DCs in the immunopathology of primary Sjögren's syndrome, focusing on the interplay between dendritic cells, epithelial cells and T-cells.

Primary Sjögren's syndrome

Sjögren's syndrome (pSS) is a chronic autoimmune disease characterized by mononuclear cell infiltration of exocrine glands. The clinical manifestations include dry eyes (keratoconjunctivitis sicca), dry mouth (xerostomia) and systemic features including invalidating fatigue, myalgia and arthralgia. Depending on further organ involvement, extraglandular manifestations may occur[1]. In addition, patients are at increased risk of developing B cell lymphoma[2]. The immunopathology of mononu-clear cell infiltration and subsequent destruction of glands in Sjögren's syndrome still remains largely unknown, despite years of extensive research. T-cells have been shown to play a detrimental role in the regulation of B-cell activity and the production of autoantibodies typifying pSS. Despite the fact that dendritic cells (DCs) are candidate key players in the activation of T- and B-cells in pSS, their contribution has been under evaluated. This is partly due to the low frequency of these cells in the circulation and inflamed tissue. This manuscript reviews current insights in DC biology and examines literature on the role of DCs in the immunopathology of primary Sjögren's syndrome.

Blood dendritic cells

Several types of dendritic cells are described in blood, characterized by the absence of lineage markers CD3, CD19, CD14, CD20, CD56 and glycophorin A (Lin-) and high expression of MHC class II. Peripheral blood DCs are further divided into plasmacytoid dendritic cells (pDCs) and two types of classical dendritic cells (cDCs), though several other subsets have been proposed[3,4]. Currently, the blood-DC antigen (BDCA)-molecules are the most important markers to distinguish blood DCs from each other. BDCA-1, -2, -3 and -4 have been described so far and within the Lin- population all four are specific for DCs (see Table 1). Other than BDCA-2 and -4, which are both expressed on pDCs, the BDCA-markers are non-overlapping and can thus be used with great accuracy[5,6].

Classical DCs, also often referred to as myeloid DCs (mDCs), are highly specialized cells that are found in lymphoid and non-lymphoid tissues. They capture environmental- and cell-associated antigens and migrate via the lymphatic system to the T-cell zone of secondary lymphoid organs. cDCs present the captured antigens to prime naive T-cells and an adaptive immune response is initiated or inhibited[4,7]. The classical DCs are often characterized as $Lin^{-}MHC–II^{+}CD11c^{+}$, though CD11c is not a marker exclusively for cDCs in both humans and mice. It is also expressed on most human monocytes and

macrophages[4] and on a large proportion of murine monocytes[3]. The cDCs are more accurately identified and subdivided by expression of CD1c (BDCA-1)$^+$ and CD141 (BDCA-3)$^+$, distinguishing type-1 and type-2 cDCs respectively[5]. BDCA-1$^+$ cDC1s are potent attractors and activators of CD4$^+$ T-cells and important for peripheral tolerance. BDCA-3$^+$ cDC2s are potent cross-presenting cells capable of inducing CD8$^+$ T-cell activation with antigens taken up from the environment[4]. In mice, the BDCA-1$^+$ DC subset is characterized as CD4$^+$/CD11b$^+$ cDCs and the BDCA-3$^+$ DC subset is characterized as CD8α$^+$/CD103$^+$ cDCs[3].

Plasmacytoid DCs are characterized as Lin$^-$MHC-II$^+$BDCA-2$^+$BDCA-4$^+$ and are known for their ability to secrete large amounts of type 1 interferon (IFN) after viral challenge[4,6]. In mice, pDCs have been characterized by high CD45 and low CD11c expression. Activation of pDCs not only induces IFN production, but it also renders them more apt at antigen- presentation. However, pDCs are not phagocytic and express low levels of MHC class II molecules with a high turnover, which makes them inefficient at antigen-presentation compared to cDCs. Instead, pDCs induce early activation of NK cells and mediate T cell survival via IFN production, making pDCs essential in controlling chronic viral infections[8].

6-Sulfo LacNac (slan)-DCs represent a major celltype close to the DC subset, though they are often considered to be closer to monocytes primarily due to their expression of low levels of CD14 and high levels of CD16. However, slan-DCs display a phenotype markedly different from CD16$^+$ monocytes, with expression of Lin$^-$MHC–II$^+$CD16$^+$CD11c$^-$CD123^{low}BDCA-2$^-$BDCA-4$^-$[9,10]. If classified as a separate DC subset, slanDCs form the largest population of human blood DCs composing 0.6%-2% of peripheral blood mononuclear cells. In blood, slan-DCs appear to remain immature, possibly due to inhibitory signals. However, in the absence of inhibitory signals or in the presence of IFN-γ, slan-DCs are capable of rapid maturation. This suggests that slan-DCs are activated once they migrate into tissue[11,12]. Indeed, slan-DCs have been identified as inflammatory cells in psoriatic skin lesions, capable of inducing strong Th1 and Th17 responses[13]. However, slan-DCs were also shown to be present as resident cells in healthy skin[14], indicating they play a role in steady-state processes.

Tissue dendritic cells

Outside of the blood, DCs have mainly been described in lymphoid tissues, but also in a large range of non-lymphoid tissues. When migrating into tissues,

DC subset	CD14	HLA-DR	CD86	CD11c	BDCA-1	BDCA-2	BDCA-3	BDCA-4	CD4+ T-cell activation	CD8+ T-cell activation
cDC1	-	+++	+++	++	++	-	+	-	+++	+
cDC2	-	+++	+++	++	-	-	+++	-	+	+++
pDC	-	++	++	-	-	++	+	++	+	+
slanDC	+	+	+	-	-	-	-	-	-	-

Table 1. Human peripheral blood dendritic cell characteristics. Characteristics of the major DC subsets found in human peripheral blood. DC subsets described are cDCs, pDCs and slan-DCs. Depicted are expression of CD14, HLA-DR, CD86, BDCA-1, -2, -3 and -4 and potential of the DC to induce activation of CD4+ and CD8+ T-cells. Expression is subdivided in four categories: negative (-), low (+), medium (++) and high (+++) expression. Abbreviations; cDC: classical DC; pDC: plasmacytoid DC; slan-DC: 6-Sulfo LacNac-expressing DC; BDCA: blood DC antigen.

DCs change their phenotype and display different surface markers compared to blood DCs. DCs in the tissue make up only a small percentage of present cells. Moreover, in addition to migratory DCs, resident DCs exist in most tissues. Consequently, delineating the DC population is very difficult in humans.

Follicular dendritic cells (fDCs) are located in primary and secondary lymphoid follicles where they form networks. fDCs are marked by expression of a large amount of Fc- and complement-receptors, including, most notably CD11b, CD14, CD16, CD32 and CD35. Unlike other DC subtypes, fDCs do not express CD45 and are not bone-marrow-derived[15]. Due to expression of complement- and Fc receptors and consistent recycling of immune-complexes, DCs are capable of presenting antibody-antigen complexes for extended periods of time[16]. In fDC networks, B cells may recognize the antigens presented by the fDCs. Once a B cell recognizes antigen it processes and presents the antigen to T cells. B cell-T cell interaction ensures survival of the antigen-specific B cell while T cells stimulate the B cell to undergo clonal expansion, which may result in the formation of germinal centers[17]. In many chronic immune diseases including rheumatoid arthritis and Sjögren's syndrome, ectopic germinal centers are formed in which fDCs are present. This process is often associated with very strong immune activation and tissue damage[18,19]. In pSS, formation of ectopic germinal centers is a possible predicting factor for lymphoma development[20].

Langerhans cells (LCs) are dendritic cells that are found in stratified squamous epithelia, such as the skin. LCs are characterized by the expression of CD1a, CD45, MHC class II, E-cadherin, the C-type lectin Langerin (CD207), Lag-antigen, and typically contain Birbeck granules[21,22]. Epidermal LCs and dermal DCs migrate to dermal lymph nodes where they can activate antigen-specific T cells. As mentioned, slan-DCs have also been identified as inflammatory cells in skin lesions, capable of inducing strong Th1 and Th17 responses[13]. It remains unclear whether slanDCs can migrate into other tissue sites.

Dendritic cells and their role in the immunopathology of primary Sjögren's syndrome

Only a few studies have looked explicitly at the changes in dendritic cell populations in primary Sjögren's syndrome (pSS) patients and in those studies, different markers are used to identify DC subsets. Though this makes it difficult to interpret available data, several lines of research suggest an important role for DCs in Sjögren's syndrome.

Changes in circulating dendritic cells in peripheral blood

Multiple studies report a decrease in peripheral blood DCs, but different markers have been used to discriminate DC subsets. A decrease in numbers was shown for $CD11c^{+}$[23], $CD11c^{+}CD1a^{+}$[24] and BDCA-3^{+}[25] cDCs in pSS patients, while BDCA-1^{+} cDC numbers are unchanged[25] (Table 2). BDCA-2^{+} pDC numbers are reduced in peripheral blood[25,26], though this is not true for $HLA\text{-}DR^{+}$ $CD11c^{-}$ cells[23,24]. As BDCA-2 is a specific marker for the pDC population and the Lin- $HLA\text{-}DR^{+}$ subset contains cell types other than cDCs and pDCs[5,6] (Table 2), the data using BDCA-2 as a marker are more convincing. The decrease in peripheral blood DC numbers of pSS patients sparks the idea that DCs migrate from peripheral blood towards the exocrine glands to initiate and perpetuate an autoimmune response[23]. This hypothesis is supported by data from a MLR/lpr mouse model in which DCs form the first infiltrating population in the salivary gland, followed by infiltration of lymphoid cells and immunopathology, though this temporal effect was not seen in NOD mice in the same study, indicating that DCs play a role in different phases of the disease in these models[27]. This indicates that different cell types might initiate sialidenitis in different pSS patients and that DC activation may initiate or boost inflammation. In addition, data showing a positive correlation between disease duration and absolute $CD11c^{+}$ cDC in the peripheral blood have suggested that cDCs play a more prominent role early in disease. However, the study is cross-sectional and the data are not supported by paired assessments of local cDC numbers, making it difficult to interpret the results[23].

Article	DC subset	Markers used	Location	Main findings
Ozaki et al. 2001 [24]	cDCs	CD11c$^+$, CD1a$^+$	PB	Lower percentage, no functional differences
	pDCs	CD11c$^-$	PB	No differences
	cDCs	CD11c$^+$, Fascin$^+$	SG	Present in pSS LSG
Gottenberg et al. 2006 [33]	pDCs	CD123$^+$	SG	Increased numbers
Manoussakis et al. 2007 [30]	iDCs, LCs	S-100$^+$	SG	Increased numbers
Wildenberg et al. 2008 [26]	pDCs	BDCA-2$^+$	PB	Decreased percentage, increased activation
Ozaki et al. 2010 [23]	cDCs	CD11c$^+$	PB	Decreased numbers, normalizes during disease
	pDCs	CD11c$^-$	PB	No differences
	cDCs	CD11c$^+$, Fascin$^+$	SG	Present in pSS LSG, numbers decrease during disease
Vogelsang et al. 2010 [25]	cDC1s	BDCA-1$^+$	PB	No differences
	cDC2s	BDCA-3$^+$	PB	Lower percentage and decreased numbers
	pDCs	BDCA-2$^+$	PB	Lower percentage and decreased numbers
	pDCs	BDCA-2$^+$	SG	Present in pSS LSG, no correlation with numbers in PB

Table 2. Studies on DCs in pSS patients. The main papers that present data on DCs from pSS patients are described with an overview of the main findings reported therein. Findings were categorized on DC subset described, divided into cDCs, pDCs, iDCs and LCs. Markers used to identify DCs are stated. For PB data, all papers use a preselection on lineage$^-$ HLA-DR$^+$ cells. Location of the DCs studied is depicted, either PB or SG. Main findings are stated, changes indicated are all compared to healthy controls. Abbreviations; cDCs: classical DCs; pDCs: plasmacytoid DCs; iDCs: interdigitating DCs, LCs: Langerhans cells; PB: peripheral blood; SG: salivary glands; BDCA: blood DC antigen; LSG: labial SG.

Dendritic cell numbers in salivary glands

In healthy human salivary glands HLA$^-$DR$^+$ DCs comprise 5-10% of the striated- and excretory duct walls. DCs are located mainly between epithelial cells, often with processes extending basally along the duct wall, or apically with processes extending towards the lumen. Furthermore, DCs can be found in intercalated ducts, in the intercellular spaces of the acini, and throughout interstitial tissues. CD11c$^+$ DCs show a similar distribution[28].

In salivary glands of pSS patients, T- and B cells form the large majority of infiltrating mononuclear cells (MNCs). Other infiltrating MNCs include macrophages and DCs. Fascin$^+$ fDC have been described to form aggregates around vessels, usually at the periphery of the infiltrates. In severe lesions and when germinal centers (GCs) are present according to histopathologic criteria, the fDCs form into fDC/T-cell/B-cell networks[29]. Furthermore, S100+ interdigitating DC (iDC) and CD11c$^+$ fascin$^+$ cDC infiltration have been described in pSS salivary glands[23,29,30]. Though Fascin and S100 are not DC-specific markers[31], the latter results are supported by providing additional data on other DC properties such as morphology and HLA-DR expression[23,30]. The preferential location for DC infiltration seems to be ductal epithelial structures[23,29,30]. Intraepithelial infiltration by DCs is significantly more frequent in pSS patients when compared to healthy donors and secondary Sjögren's syndrome (sSS) patients, which have Sjögren's syndrome characteristics overlapping with another autoimmune disease[30]. Around epithelial structures, CD1a- and DC-LAMP-expressing cDCs are mainly located within the lymphoid aggregates[32]. pDC infiltration in pSS salivary glands has also been reported, using BDCA-2 as a pDC marker,[25,33], while no pDCs were detected in healthy human salivary glands or glands from non-Sjögren's sicca (nSS) patients which experience dryness symptoms without the presence of autoimmunity[28,33].

Interestingly, one of the studies describes that the increase in cDC numbers in pSS salivary glands normalizes after several years after diagnosis, showing that the number of infiltrating DCs correlates with disease duration[23]. This suggests a role for DCs mainly in initiation of inflammation in the salivary glands. However, this study uses Fascin expression as a marker for cDCs, which is not strictly cDC-specific[31]. In addition, the percentage rather than absolute number of DCs is used for the correlation and as such the described correlation can also be the result of increased numbers of infiltrating lymphocytes. Moreover, the correlation between time after diagnosis and number of DCs present is not studied over time but cross-sectionally, making interpretation difficult[23]. Still, these data form an interesting lead for future research. For pDCs, a correlation between blood and tissue levels could not be confirmed[25], but as is indicated above, it might be possible that the length of time from disease onset until measurement of DC numbers is important for the measurement outcome, as initial changes might resolve too in the pDC subset.

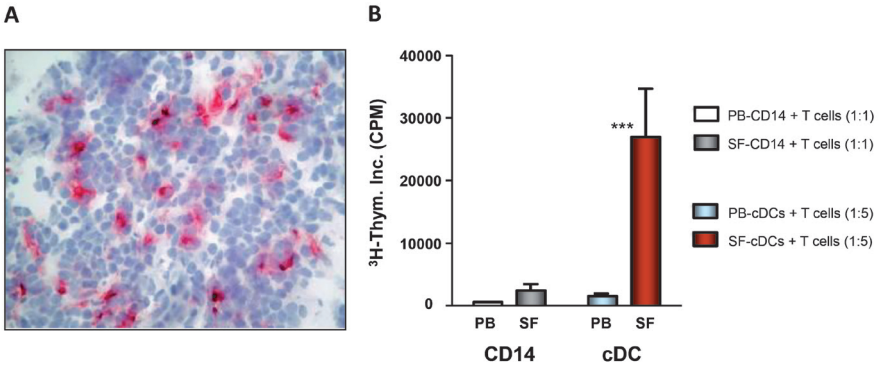

Figure 1. Mature inflammatory cDCs are present in salivary gland tissue of primary Sjögren's syndrome patients and are very potent T cell activators. Immunohistochemical staining of CD208/DC-LAMP in cryo-section of pSS patient (**A**). CD14-expressing monocytes and BDCA-1-expressing myeloid/classical DCs (mDCs/cDCs) were isolated from paired peripheral blood (PB) and synovial fluid (SF) samples of rheumatoid arthritis patients, activated and co-cultured with $5x10^4$ autologous $CD4^+$ T cells. Co-culture for 6 days in a cell ratio of 1:1 for monocytes and 1:5 ratio for cDCs ($1x10^4$ cells). Proliferation of T cells measured by tritium incorporation for 18 hours in counts per minute (CPM) (**B**).

Plasmacytoid DCs and immunopathology of pSS

Of the different DC subsets, the role of pDCs has been most thoroughly studied. pSS patients have an activated type-1 IFN system and genes activated by the IFN produced are detectable in a range of cell types in these patients, including monocytes and epithelial cells[26,33-35]. Up regulation of these genes is seen in around half of all pSS patients and this activation is referred to as an IFN signature. Presence of IFN signature in pSS monocytes was shown to be correlated with disease activity and production of autoantibodies[36]. The effects of type-1 IFN in disease are partly mediated by B-cell activating factor (BAFF), a cytokine involved in activation of B-cells. BAFF levels in pSS patient serum are correlated with a range of disease processes in human and mouse studies[37-39]. BAFF expression was shown to be induced by IFN-α stimulation on pSS monocytes and salivary gland epithelial cells (SGECs)[33,36].

pDCs are extremely potent IFN-α producing cells and are thought to be responsible for the majority of IFN-α produced in these patients[8,33]. A collection

of studies on pDC levels in peripheral blood and in salivary glands support the hypothesis that pDCs migrate from the blood into the glands[25,26,33], especially since pDCs are absent in control biopsies[28]. However, increased serum levels in pSS patients have not been reported and measurements have not been consistent[34,40]. pDCs in pSS peripheral blood appear to be activated and their activation status correlates to the presence of an IFN signature, suggesting a link between activation of the pDCs in blood and type-1 IFN production[26]. In addition, it has been demonstrated that apoptotic or necrotic material combined with Ro/SSA or La/SSB autoantibody-containing pSS serum can activate pDCs to produce IFN-α[34]. The significance of pDCs in pSS development is further supported by data from the ID3 -/- knockout mouse model. ID3 is an inhibitory transcription factor that might play a role in inhibition of pDC development, but its exact role remains to be determined[41-43]. ID3 deficient mice were shown to develop pSS like symptoms[44]. Thus, pDCs seem important in production of IFN-α and subsequent activation of several pro-inflammatory pathways, though the exact implications of this system need further investigation. Especially the initial activation of pDCs in pSS patients is an interesting topic for future research. Viruses have been implicated to play an initial role in pSS pathogenesis as they stimulate TLRs and are very good inducers of IFN-α production[34,45].

In addition to IFN-α, IFN-γ was also shown to be capable of inducing expression of genes included in the IFN signature. IFN genes that were upregulated in salivary gland biopsy specimen of pSS patients were induced by IFN-γ[33]. Several lines of evidence suggest that the interplay between cDCs, epithelial cells and T-cells plays a critical role in the development of Th1 (IFN-γ) responses and immunopathology.

Interplay of cDCs, epithelial cells and T-cells in pSS

A number of studies have reported on expression of maturation markers by the DCs present in the salivary glands. Expression of HLA-DR, CD1a, CD83, DC-SIGN and DC-LAMP on DCs was shown in pSS salivary glands[27,32,46]. DC lysosome-associated membrane glycoprotein (DC-LAMP, i.e. CD208) is involved in antigen presentation and expression of MHC class-II. It is described as a marker for mature DCs[47]. DC-specific ICAM-3 grabbing non-integrin (DC-SIGN, i.e. CD209), a C-type lectin receptor, is expressed at high levels by immature cDCs in non-lymphoid tissues and mature cDCs in lymphoid tissues[48,49]. It is involved in a range of processes including DC migration, recognition of pathogens by DCs and activation of T-cells[50]. CD1a is involved in presentation of lipids to T-cells and is used as marker for immature

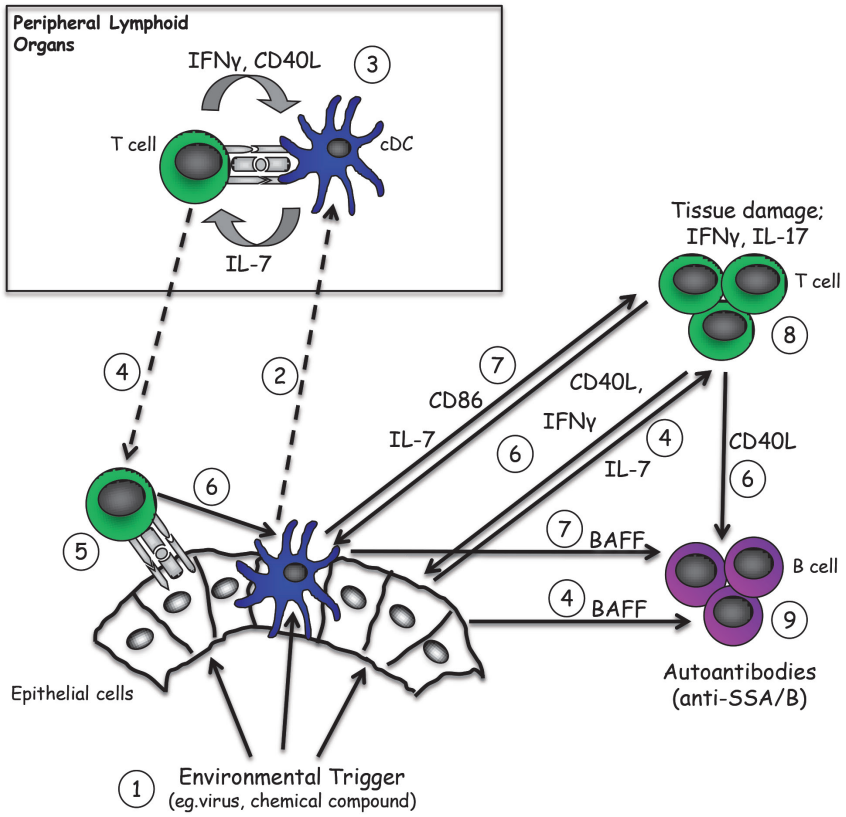

Figure 2. Interplay between cDCs, T-cells and epithelial cells leads to tissue damage and auto-antibody production in primary Sjögren's syndrome. Epithelial cells and classical dendritic cells (cDCs) present in the salivary glands (SG) can be triggered by environmental factors, such as viral infections or chemical compounds (**1**). cDCs activated in the SG travel to the peripheral lymphoid organs to encounter T-cells, leading to T-cell activation (**2**). cDCs induce T-cell activation via antigen presentation and cytokine production (eg. IL-7). T-cells induce maturation of immature cDCs via CD40L and proinflammatory cytokines (eg. IFNγ) (**3**). Triggered epithelial cells attract cDCs and T-cells to the SG by secreting chemokines. In addition, they produce BAFF to activate B-cells and IL-7 to activate T-cells (**4**). Alternatively, surveying T-cells may recognize an antigen present in the SG and become activated by antigen presenting cDCs or epithelial cells (**5**). T-cells subsequently activate the cDCs, epithelial cells and B-cells via CD40L and proinflammatory cytokines (**6**). cDCs can cause diversification of the immune response by activating T-cells via IL-7 and B-cells via BAFF (**7**). Activated T-cells induce tissue damage via production of proinflammatory cytokines (**8**). B-cells are activated by BAFF from the epithelial cells and CD40L by the T-cells. They produce auto-antibodies, involved in extraglandular manifestations (**9**). Arrows depict activation, broken arrows depict chemotaxis .

DCs[51]. In addition, CD83, CD86 and HLA-DR are often used as markers for DC maturation. In pSS, DC-SIGN$^+$ cells were found dispersed throughout the tissue and the infiltrate, whereas DC-LAMP$^+$ and CD1a$^+$ cells were confined to lymphocytic infiltrates[46]. Expression of DC-SIGN, DC-LAMP and CD1a correlated with expression of the IL-7 receptor, which in turn is a marker for T-cell infiltration[32]. Thus, both immature and mature DCs are present in the salivary glands and all three markers are indirectly related to T-cell activity.

Migration and differentiation of monocytes may be one of the mechanisms contributing to increased DC numbers in the salivary glands. Increased levels of non-classical CD14^{low}CD16$^+$ monocytes are present in pSS patients compared to healthy controls and these cells may be a source of DCs that infiltrate the salivary glands[46]. In an *in vitro* reverse transmigration assay performed to study the fate of human monocytes that migrate out of the blood, both classical CD14$^+$CD16$^-$ and non-classical CD14^{low}CD16$^+$ monocyte subsets were shown to partly develop into DCs. Monocytes migrating in the assay start expressing HLA-DR, CD83, CD1a, DC-SIGN and DC-LAMP, indicating maturation into DCs. The non-classical monocytes primarily differentiate into DC-LAMP$^+$ CD83$^+$ mature DCs. This differentiation was confirmed in a NOD mouse model, where non-classical monocytes were shown to migrate to the salivary glands and preferentially develop into tissue DCs that are CD11c positive. The number of non-classical monocytes was increased in pSS patients, indicating that their migration and differentiation may contribute to increased cDC infiltration in the salivary glands of these patients[46]. However, the process of monocyte differentiation into DCs is still controversial in humans, making these data difficult to interpret at this time.

Although cDCs are present in the pSS salivary glands and there are clear signs that they are mature and capable of potent lymphocyte activation, their exact function remains unclear. Data from human *in vitro* and mouse studies suggest a pivotal role of these DCs in pSS.

Classical DCs as drivers of pSS-like disease

Several studies on blood DC levels and immunohistochemical analyses of pSS salivary glands have suggested trafficking from blood DCs towards the salivary glands early in disease[23,33,46]. Although not shown for cDCs from the inflammatory site of pSS patients, the capacity of inflammatory DCs to vigorously activate T-cells and induce Th1 and Th17 responses was recently shown in rheumatoid arthritis (RA) patients[52]. In the absence of exogenously

added antigen, BDCA-1-expressing cDCs from the synovial fluid of RA patients induced a 15-fold higher $CD4^+$ T-cell proliferation at a cell-ratio of 1 DC to 5 T-cells (Figure 1). Robust T-cell activation was already found at DC/T-cell ratio of 1:50. Although this remains to be demonstrated for pSS patients, it illustrates the potential of a small number of DCs to induce robust T-cell activation.

Also in mice changes in DC populations and their potential have been observed. In the NOD mouse, it has been demonstrated that DC infiltration precedes lymphocyte infiltration[27], possibly accompanied by a phenotype switch[46]. A proposed mecha-nism of disease describes the trafficking of DCs loaded with autoantigens from the salivary glands to lymph nodes, activating antigen-specific T cells (Figure 2). This leads to influx of autoreactive T cells in the salivary gland[27]. The dendritic cell immunoreceptor (Dcir) knockout mouse model further supports a central role of DCs in the pathogenesis of pSS. Dcir is a C-type lectin receptor containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) that is essential in maintaining appropriate DC levels, which has been suggested to be important in self-tolerance preservation. Dcir mRNA is expressed highest in DCs in lymphatic organs. In the absence of Dcir, mice spontaneous develop sialoadenitis, with lymphocytic infiltration of the salivary glands and glandular destruction as seen in pSS patients. At a later stage, the mice develop significantly more anti- Ro/SSA and anti La/SSB antibodies, together with an increase in numbers of activated T-cells in the peripheral blood and $CD11c^+$ DCs in the lymph nodes compared to control mice[53].

cDC driving inflammation in response to epithelial cell activation

Epithelial cells are not part of the immune system, but they are known to have a strong effect on immune activation in tissues. Especially in the salivary gland where epithelial cells make up the majority of the tissue, they are critical in modulating the immune response. The factors driving salivary epithelial activation are not clear, but are thought to include environmental factors such as chemical compounds, viruses infecting the salivary glands and TLR ligands present in the saliva[1,54].

In human salivary glands, DCs accumulate in- and around ductal epithelia[23,29,30]. However, the mechanism initiating MNC infiltration in pSS remains unexplained. One proposed mechanism is the epithelial expression of chemokines attracting DCs (Figure 2). DCs express a large range of chemokine receptors on their surface. Some of these are required for migration to lymphoid structures and are generally up regulated upon activation and maturation of

the DC. Others serve for attraction into tissues and are expressed mainly on immature DCs while being down regulated upon maturation[55]. mRNA of the chemokines BCA-1 (CXCL13), TARC (CCL17), ELC (CCL19), SLC (CCL21) and MDC (CCL22) was found in ductal epithelial cells in the salivary glands of pSS patients[56,57]. mRNA of these chemokines was present in total salivary gland lysate of a larger proportion of pSS patients compared to nSS patients or patients with an unrelated autoimmune disease[56]. In addition, expression of TARC and MDC mRNA was shown to correlate with lymphocyte focus score[57]. All of the described chemokines ligate receptors that are expressed by DCs, though some are only expressed on mature DCs[55].

Further evidence for activation of SGECs in pSS patients comes from the observed CD40 expression on SGEC cell lines derived from SS patients[58,59]. As a large proportion of lymphocytes that infiltrate the salivary gland tissue are CD40L-expressing T-cells, it is plausible that CD40-CD40L interaction between epithelial cells and infiltrating T-cells induces activation of the epithelial cells[58]. CD40 expression on SGECs is up regulated by IFN-γ and IL-1β exposure. IFN-γ is present in the salivary glands in pSS patients, it is produced by infiltrating T cells in SS and is involved in destruction of epithelia[58,60,61]. Significantly increased numbers of IFN-γ producing $CD4^+$ T cells are present in salivary glands of pSS patients compared to healthy donors[24,60]. In an allogenic co-culture system with naive $CD4^+$ T cells and $CD1a^+CD11c^+$ cDCs, an increased percentage of T-cells from pSS patients starts producing IFN-γ compared to T-cells from healthy donors, suggesting the T-cells of pSS patients are more prone to produce IFN-γ[23]. Additionally, CD40 is essential in promoting Fas-dependent apoptosis in cultured SGECs[59]. SGECs cell lines from SS patients show increased expression of Fas and FasL, which could be mediated by IFN-γ and TNF-α, leading to increased apoptotic death[61].

In addition, SGECs seem to be capable of inducing T-cell activation. They were shown to produce the T-cell activating cytokine IL-7 upon activation[62] and have been reported to express co-stimulatory molecules CD80 and CD86, the adhesion molecules ICAM-1 (intercellular adhesion molecule 1) and VCAM (vascular cell adhesion molecule), E-selectin, HLA-DR[63] and TLRs[64]. Expression of these molecules could be upregulated by either IFN-γ or TNF-α. These findings suggest a role for SGECs as non-professional APCs[64].

Furthermore, epithelial cells may be involved in B-cell activation. BAFF mRNA is significantly increased in SGECs of pSS patients compared to controls after

stimulation with interferons. BAFF expression is increased by IFN-γ (and IFN-α) stimulation in both pSS patients and controls[65]. BAFF is essential in promoting B lymphocyte activation and survival and might be critical in autoimmune B cell activation[66]. Thus, epithelial cells in pSS salivary glands play an important role in activating B cells under the influence of Th1 activity. Alternatively, Th1 activation induces activation of myeloid cells such as macrophages and cDCs to produce BAFF, enhancing B-cell activation.

T-cells as driving force for cDC activation and immunopathology

An alternative route of DC activation and immunopathology involves T-cells, in particular Th1 cells associated with IFN-γ production (Figure 2). As described, T-cells that express the ligand for CD40 are abundant in the pSS salivary gland[58]. In addition to epithelial cells, these T-cells are also able to induce maturation of DCs. Several cytokines are involved in this process, including IFN-γ and IL-12[67]. Especially IFN-γ is abundantly present in the salivary glands of pSS patients and is known to mediate tissue damage[58,61]. Thus, T-cells that enter the salivary gland can be activated by antigen-primed cDCs to initiate T-cell activation. As cDC-T-cell interactions can induce highly efficient T-cell proliferation and cytokine secretion, this interaction seems to be an important part of the local inflammation and tissue destruction.

IL-7 is one of the factors involved in cDC-T-cell interactions and is indicated to play a role in the induction of autoimmunity in the salivary glands[68]. It is mainly produced by stromal cells in primary lymphoid organs and plays a pivotal role in T-cell homeostasis. IL-7 mainly acts on T-cells expressing the high affinity IL-7 receptor (IL-7R) and can induce T-cell-dependent activation of macrophages, DCs and B-cells in pSS patients[69]. IL-7 can also be produced by cDCs upon stimulation by T-cells and T-cells are more efficiently activated by IL-7 in the presence of cDCs or other APCs[70,71]. T-cells that express the IL-7R are abundantly present in the pSS salivary glands and their numbers correlate with the presence of DCs expressing CD1a and DC-LAMP and enhanced numbers of B-cells and plasma cells. In addition, IL-7 expression in LSG of pSS patients is increased as compared to nSS controls and correlates with increased inflammation and IL-7R expression. Furthermore, IL-7R-expressing T-cells respond very strongly to IL-7 stimulation, with efficient proliferation and robust production of IFN-γ and IL-17[32]. There is also recent data indicating that IL-7 plays a critical role in a Th1-cell-driven experimental model of Sjögren's syndrome[72]. pSS-like disease in B6.NOD-Aec mice was shown to be associated with increased levels of IL-7

in the salivary glands and serum, in-line with observations in pSS patients[73]. Gain- and loss-of-function experiments showed that IL-7 plays a critical role in the development and onset of experimental pSS by enhancing Th1 responses and IFN-γ-dependent expression of Th1 cell-attracting CXCR3 ligands by glandular epithelial cells. Endogenous IL-7 production by salivary gland epithelial cells that was induced by injecting Poly I:C, a TLR3 ligand that mimics viral RNA, had similar effects in this model[62]. IL-7-driven immune activation was associated with disease phenomena characteristic of pSS, including increased apoptosis of epithelial cells, increased lymphocytic infiltration, production of anti-nuclear antibodies and reduced salivary flow[72]. These mouse data confirm that viral RNA can induce IL-7 production by epithelial cells, indicating that this cytokine is involved early in disease. IFN-α and IFN-γ, at the early disease phase likely produced by epithelial cells and NK-cells respectively, are critical for this induction. At a later disease stage, the effects of epithelial cell-derived IL-7 and T-cell derived IFN-γ reinforce one another creating a milieu where Th1 cells are very effectively attracted and activated [73]. IFN-α produced by pDCs as well as cDC-derived IL-7 may further potentiate this system.

Conclusions

Taken together, a considerable body of evidence supports a role for both plasmacytoid and myeloid/classical dendritic cells in pSS justifying careful evaluation of the exact phenotypic and functional properties of these cells. In line with this, the first initiatives to study genetic and epigenetic regulation of cDCs and pDCs in healthy controls and pSS patients are ongoing. In this regard, pSS patients with a high risk to develop extraglandular manifestations and lymphoma are of particular interest.

pSS research the last decade has put a lot of focus on trials with efficacious treatments approved in other generalized auto-immune disease such as rheumatoid arthritis, while only subgroups of pSS patients might benefit from these treatments. Focusing on cell-types other than lymphocytes may be required to develop new treatment options for pSS patients in the future. Given the potential interplay of DCs and epithelial cells and the role of DCs in T- and B-cell activation, delineating molecular pathways that are associated with DC activation may prove critical in understanding the disease and thus finding novel biomarkers to monitor disease and new and better therapeutic targets.

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6

Differential expression of miRNAs in type-1 classical DCs from patients with primary Sjögren's syndrome suggests dysregulated signalling pathways

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Abstract

Objectives. Type-1 classical dendritic cells (cDCs) are very potent antigen presenting cells known to induce strong T-cell activation. Despite the fact that cDC1s are candidate key players in the activation of local T and B-cells in primary Sjögren's syndrome (pSS), they have rarely been studied in this disease. To better understand the role of cDC1s in pSS we investigated the expression of micro RNAs (miRNA) in these cells in pSS patients.

Methods. Two independent cohorts (discovery and validation) were established including a total of 29 pSS patients, 16 non-Sjögren's sicca (nSS) patients – defined as patients with dryness complaints but not clinically considered pSS and not fulfilling the classification criteria for pSS - and 17 healthy controls (HC). BDCA-1-expressing cDC1s were isolated and miRNA profiling was performed for 758 targets. miRNAs found to be differentially expressed in the discovery cohort were assessed in the independent validation cohort. Finally, pathway enrichment was performed with the experimentally supported targets of the validated miRNAs.

Results. A total of 24 miRNAs were downregulated in pSS patients versus HC in the discovery cohort, 16 of those were selected for validation. Two miRNAs were subsequently validated. Pathway enrichment indicated that these miRNAs are involved in various pathways. Intriguingly, expression of these miRNAs was remarkably similar in patients with pSS as well as nSS.

Conclusions. We here for the first time show differential expression of two miRNAs in isolated type-1 classical dendritic cells of pSS patients. As these miRNAs are involved in multiple signalling pathways, they might be directly involved in steering the immune response in these patients, a conceptual framework that deserves further investigation.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltration of the salivary and lachrymal exocrine glands that leads to dryness of mouth and eyes. T-cells play a central role in tissue destruction and regulate the activity of B-cells, which produce the autoantibodies typifying pSS [1]. Classical dendritic cells (cDCs, also referred to as myeloid DCs/mDCs) are very potent antigen presenting cells known to induce strong T-cell proliferation and cytokine production. Two subsets of cDCs can be distinguished in the peripheral blood based on the expression of blood dendritic cell antigens (BDCA), type-1 cDC expressing BDCA-1 (CD1c) and type-2 cDCs which express BDCA-3 (CD141). cDC1 are potent attractors and activators of CD4+ T-cells, while cDC2 are strong cross-presenting cells capable of activating CD8+ T-cells [2]. In pSS patients, total cDC numbers as measured by expression of CD11c are lower in the peripheral blood and higher in the salivary glands, suggesting migration of these cells towards the exocrine glands [3,4]. The decrease in peripheral blood cDC numbers seems to be due to cDC2 numbers, with unchanged numbers of cDC1s [5]. While the subsets have not been studied separately in the salivary glands, local expression of markers for both mature and immature cDCs, including CD1a, CD208 and CD209, have been shown, correlating with markers of inflammation [6]. *Ex vivo* analysis of cDC1s showed induction of vigorous T-cell activation in rheumatoid arthritis [7].

Micro RNA (miRNA) are small noncoding RNA molecules that play a key role in regulating the expression of protein-coding genes at the post-transcriptional level by inducing degradation of messenger RNA (mRNA) or interfering with translation of target mRNA. Typically, miRNA have multiple targets and are involved in complex signalling pathways. miRNA regulate inflammatory pathways and are frequently dysregulated in cells of the immune system in patients with autoimmune disorders. Specific miRNAs are differentially expressed in several autoimmune diseases, including systemic sclerosis and systemic lupus erythematosus [8,9]. In pSS, dysregulation of miR-146a, miR-155, miR-181a and miR-223 have been described in peripheral blood mononuclear cells (PBMCs) [10-12]. In addition, miR-181a is differentially expressed in pSS salivary glands [10] and salivary gland expression levels of miR-574 and miR-768-3p allow discrimination between patients with low and high focus scores [13].

Despite the fact that cDC1s are potential central players in the activation of local T and B-cells in pSS [14], they have rarely been studied in pSS. This is partly

due to the low relative frequency of these cells in the circulation and inflamed tissue. Considering the crucial role that dysregulation of cDC1s in pSS patients could play in local autoimmune activity and the important role of miRNAs in regulation of cellular processes, we investigated the expression of >750 miRNAs in isolated cDC1s of pSS patients.

Methods

Patients and controls

Two separate cohorts of patients (discovery and validation), under treatment in the University Medical Center Utrecht, and controls were established. The was no overlap in donors between the two cohorts. The characteristics of discovery and validation cohorts are depicted in Table 1 and Table 2, respectively. pSS patients (n=29) were classified according to American-European consensus group criteria [15]. Of the pSS patients, 3 individuals were clinically diagnosed as pSS but did not fulfil the classification criteria (Supplementary Table 1). These patients did not differ from the other pSS patients in any analysis. 16 individuals who were clinically not considered to be pSS patients and did not fulfill the classification criteria for pSS were defined as non-Sjögren's sicca (nSS) patients and included in the two cohorts. 17 healthy donors were included as control and divided over both cohorts. There were no significant differences in sex and gender between any of the groups in either cohort. This research was performed in accordance with the medical ethical regulations of the University Medical Center Utrecht and all patients gave their written informed consent.

Dendritic cell isolation

Mononuclear cells (MCs) were isolated from heparinized peripheral blood by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). $CD19^-$ $CD1c^+$ cDCs were isolated from PBMCs by magnetic-activated cell sorting (MACS) using CD1c $(BDCA\text{-}1)^+$ isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. As B-cells can express CD1c, $CD19^+$ cells were depleted from PBMCs. To confirm consistent purity of isolated cDCs, cells were stained with CD45-PerCP (Biolegend), CD1c-PE (Biolegend) and CD19-FITC (BD Bioscience, San Jose, USA) and the proportion of $CD19^-$ $CD1c^+$ cells within isolated fraction was measured using fluorescence-activated cell sorting (FACS) and a FACSCanto II flow cytometer (BD Bioscience, San Jose, USA). Gating strategy is shown in Figure 1. Mean purity of all samples was 90%. There were no significant differences in purity between any of the groups in either cohort. Cells were lysed in RLTPlus buffer (Qiagen, Venlo, Netherlands) supplemented with beta-mercaptoethanol.

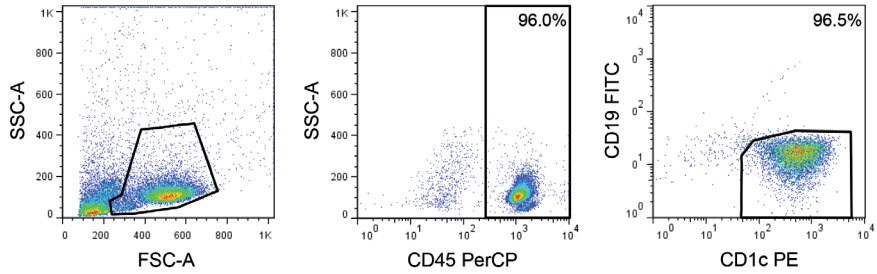

Figure 1. Representative gating strategy to calculate purity of isolated CD19$^-$ BDCA-1$^+$ type-1 classical dendritic cells. Isolated cell-fraction was stained with anti-CD45-PerCP, anti-CD19-FITC and anti-BDCA-1-PE. Live cells were gated based on forward scatter (FSC) and side-scatter (SSC) and leukocytes were gated based on CD45 expression within the live gate. cDC1 purity was calculated based on the percentage of CD19$^-$ CD1c$^+$ cells within the CD45$^+$ population.

RNA isolation

Total RNA containing the small-RNA fraction was purified from lysates of isolated cells using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were assessed with Qubit RNA Kit (Life Technologies, Carlsbad, CA, USA) and the Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) respectively.

miRNA profiling and analysis

miRNA profiling was performed by TaqMan RT-qPCR on the OpenArray platform (LifeTechnologies) following manufacturer's instructions. This method allows for the simultaneous analysis of 758 miRNAs split into two equal pools (A and B). 10ng of total RNA were reverse-transcribed by using the miRNA multiplex RT primers pools, either v2.1 for pool A or v3.0 for pool B, and the TaqMan miRNA reverse transcription kit (LifeTechnologies). RT products were pre-amplified using the Megaplex PreAmp Primers pools A and B in the presence of the TaqMan PreAmp Master mix (Life Technologies), by using the following thermal cycler conditions: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min and 16 cycles of 95°C for 15 sec and 60°C for 4 min and one single cycle of 96°C for 10 minutes. The miRNA OpenArray profiling was performed on the amplified cDNA, diluted in a 1:40 ratio using Taqman OpenArray master mix on the QuantStudio 12k flex Real-Time PCR system (LifeTechnologies). miRNA profiling data were analyzed using ExpressionSuite software (LifeTechnlogies). Data were normalized using the global normalization approach [16]. The analysis

was conducted on data points detected at crt lower than 27, by rejecting samples with an amplification score lower than 1.24. miRNAs that showed a significant difference at $p<0.05$ with relative fold change (FC) of ≥ 2 or ≤ 0.5 in pSS compared to HC were considered differentially expressed.

To validate the differential expression of selected miRNAs, cDNA was synthesized from 6.6 ng of total RNA by using a pool of 20 RNA-specific RT primers contained in the TaqMan Human miRNA assays (each diluted to 0.02x) in the presence of 10 U/µl MultiScribe RT enzyme, 0.25 U/µl RNase Inhibitor and 2mM dNTPs (LifeTechnologies). The following thermal cycler conditions were used: 10 min at 4°C; 30 min at 16°C; 20 min at 42°C; 5 min at 85°C. The RT product was pre-amplified as described above with a pool of the correspondent miRNA TaqMan assays diluted to 0.03x. The quantification of each miRNA was performed on the OpenArray platform by using a custom panel of 18 TaqMan assays (indicated in Table 3) and 2 for small-nucleolar RNAs. Data were analyzed according to the comparative threshold cycle method [17], after normalization by the mean Ct of 2 miRNAs (miR-17 and miR-191) and 2 small nucleolar RNA (RNU48 and U6) which showed good abundance and stable expression between patients and controls. The miRNA expression of each donor was depicted as FC compared to one healthy control, which was set at 1.

Pathway enrichment

A list of genes predicted to be target of the differentially expressed miRNAs and validated by *in vitro* experiments or supported by indirect experimental data (from here on referred to as experimentally supported targets) was retrieved from three databases: StarBase [18], mirTarBase [19] and TarBase [20]. From StarBase, only those miRNA-target pairs that were predicted by at least 3 software programs and supported by two separate pieces of evidence were selected. For mirTarBase, all experimentally supported targets were included. For TarBase, all experimentally supported downregulated targets of the miRNAs were selected. Pathway enrichment was performed using Toppgene Suite [21] on the union of experimentally-supported targets retrieved from abovementioned databases. Relevant enriched pathways were selected using the B&H FDR corrected p-value with a threshold of $p<0.05$, excluding pathways directly related to a specific type of cancer and duplicated pathways. Pathways were grouped based on overlap of targets in the pathways and literature.

Integration of cohort data

To compare expression of differentially expressed miRNAs between nSS and pSS

	HC	pSS	nSS
N (M/F)	6 (0/6)	15 (2/14)	9 (0/9)
Age (yr.)	56 [54 – 67]	53 [29-77]	43 [25- 68]
LFS (foci/4 mm^2)	-	1.7 [1.0 – 4.0]	0.0 [0.0 – 1.0]
ESSDAI	-	2.0 [0.0 – 19]	-
ESSPRI	-	3.3 [1.8 – 8.8]	-
Schirmer (mm/5 mins)	-	4.5 [0.5 – 25]	3.3 [0.0 – 24]
ANA (no. positive/% of group)	-	13 /87%	3 /33%
SSA (no. positive/% of group)	-	8 /53%	1 /11%
SSB (no. positive/% of group)	-	3 /20%	0 /0%
Rheumatoid factor (no. positive/% of group)	-	4 /27%	0 /0%
Serum IgG (g/L)	-	14 [8.3 – 30]	12 [6.8 – 17]
ESR (mm/hour)	-	9 [5.0 – 30]	11 [4.0 – 17]
CRP (mg/L)	-	1.0 [0.0 – 8.0]	0.7 [0.0 – 4.0]
C3 (g/L)	-	1.0 [0.8 – 1.3]	1.2 [0.6 – 1.7]
C4 (g/L)	-	0.3 [0.1 – 0.3]	0.3 [0.2 – 0.4]

Table 1. Discovery Cohort Characteristics. HC: Healthy control; nSS: non-Sjögren's Sicca; pSS: primary Sjögren's syndrome; LFS: Lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: Anti-nuclear antibodies; SSA: Anti-SSA/Ro; SSB: Anti-SSB/La; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein. Values are median [range] unless stated otherwise.

patients and for correlations with disease parameters, relative miRNA expression levels of both cohorts were integrated. Geometric mean (GM) of expression in HC groups of both cohorts was calculated using Graphpad software version 6.02 (Graphpad, Lo Jolla, CA, USA). FC of all samples in validation cohort was corrected for the ratio between GM in HC of discovery cohort and GM of HC in the validation cohort.

Statistics

Differences in miRNA expression between pSS patients and HC in discovery cohort were analyzed using ExpressionSuite software (Lifetechnologies). For validation, statistical analysis was performed with GraphPad Prism software version 6.02 (GraphPad) using the Mann-Whitney U test (two-sided). For correlations with disease parameters, Spearman's rho was used. For comparison

of HC, nSS and pSS miRNA expression, Kruskall-Wallis test was performed with post-hoc Mann-Whitney U test. Differences and correlations were considered statistically significant at $p<0.05$.

Results

24 miRNAs were differentially expressed in pSS patients as compared to healthy controls in screening array

RNA was extracted from isolated cDC1 populations of patients and controls. To screen for differentially expressed miRNAs in pSS patients we performed OpenArray-based screening of 758 miRNAs on RNA isolated from pSS patients and healthy controls (Table 1). miRNAs were considered to be differentially expressed when the expression in the pSS patients relative to that of the HC group was greater than 2 or lower than 0.5, with a p-value of $p<0.05$. Using this discovery approach, we detected 24 miRNAs that were differentially expressed in pSS patients (Table 3).

Significant downregulation of miR-708 and miR-130a was confirmed in validation cohort

Of the 24 miRNAs that were differentially expressed in the discovery cohort, we

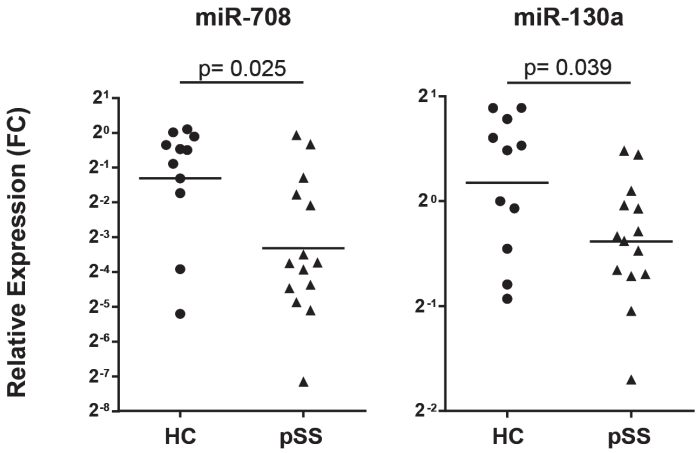

Figure 2. miR-708 and miR-130a are downregulated in cDC1s of pSS patients. A selection of miRNAs differentially expressed in pSS patients as compared to healthy donors (HC) in the discovery cohort were measured in an independent validation cohort of patients and controls. Expression was calculated relative to 4 housekeeping RNAs and depicted compared to a HC sample set at 1. Geometric means and Mann-Whitney U test p-values are shown.

	HC	pSS	nSS
N (M/F)	11 (1/9)	14 (1/13)	7 (0/7)
Age (yr.)	50 [29 – 64]	54 [26 – 69]	47 [37 – 69]
LFS (foci/4 mm^2)	-	2.0 [1.0 – 4.0]	0.0 [0.0 – 0.7]
ESSDAI	-	5.0 [0.0 – 13]	-
ESSPRI	-	5.3 [1.0 – 8.0]	-
Schirmer (mm/5 min)	-	15 [0.5 – 30]	5.8 [1.5 – 32]
ANA (no. positive/% of group)	-	12 /86%	6 /86%
SSA (no. positive/% of group)	-	10 /72%	3 /43%
SSB (no. positive/% of group)	-	9 /64%	0 /0%
Rheumatoid factor (no. positive/% of group)	-	5 /45%	1 /20%
Serum IgG (g/L)	-	17 [9 – 33]	13 [11 – 14]
ESR (mm/hour)	-	17 [4.0 – 38]	10 [2.0 – 29]
CRP (mg/L)	-	1.3 [0.0 – 3.1]	1.3 [0.0 – 5.2]
C3 (g/L)	-	1.0 [0.8 – 1.3]	1.1 [0.8 – 1.3]
C4 (g/L)	-	0.2 [0.1 – 0.3]	0.3 [0.2 – 0.4]

Table 2. Validation Cohort Characteristics. HC: Healthy control; nSS: non-Sjögren's Sicca; pSS: primary Sjögren's syndrome; LFS: Lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: Anti-nuclear antibodies; SSA: Anti-SSA/Ro; SSB: Anti-SSB/La; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein. Values are median [range] unless stated otherwise.

measured the expression of 16 miRNAs in a separate cohort of pSS patients and healthy controls in a separate experiment (Table 2). Selection was based on the level of expression and quality of amplification. Of these 16 miRNAs, two were confirmed to be downregulated in pSS patients as compared to HC with p-value <0.05 (Figure 2).

To investigate whether the differences in miRNA expression between pSS patients and HC were associated with disease parameters, we integrated the data of both cohorts and performed correlation analyses within the pSS patient group. No significant correlations between expression of miR-708 or miR-130a and LFS, ESSDAI, ESSPRI, Schirmer test, serum IgG, ESR, CRP, C3 or C4 were found. In addition, there were no significant differences between pSS patients with auto-antibodies and those without, including anti-nuclear antibodies, SSA or SSB (data not shown).

Target	Fold Change pSS vs HC	p-value	Measured in Validation
miR-29a#	0.158	0.020	x
miR-708	0.159	0.013	x
miR-126#	0.293	0.020	x
miR-30e-3p	0.308	0.029	x
miR-30d	0.328	0.031	x
miR-29b	0.362	0.029	x
miR-340	0.387	0.008	
miR-21	0.403	0.027	x
Let-7g	0.407	0.002	x
miR-223	0.413	0.005	x
miR-25	0.413	0.001	x
miR-213	0.413	0.042	x
miR-345	0.434	0.001	
miR-150	0.441	0.002	x
miR-26a	0.449	0.002	
miR-324-5p	0.449	0.024	
miR-9	0.451	0.005	x
miR-532	0.452	0.004	x
miR-16	0.456	0.001	
miR-221	0.457	0.003	
Let-7f	0.463	0.001	x
miR-374-5p	0.468	0.004	
miR-103	0.490	0.007	
miR-130a	0.498	0.013	x

Table 3. List of differentially expressed miRNAs in pSS patients in discovery cohort. 758 miRNAs were measured on OpenArray. All miRNAs differentially expressed in pSS patients as compared to healthy donors with a relative fold-change of ≤0.5 or ≥2.0 and a p-value of ≤0.05 are listed. miRNAs selected to be measured in the validation cohort are marked in the rightmost column.

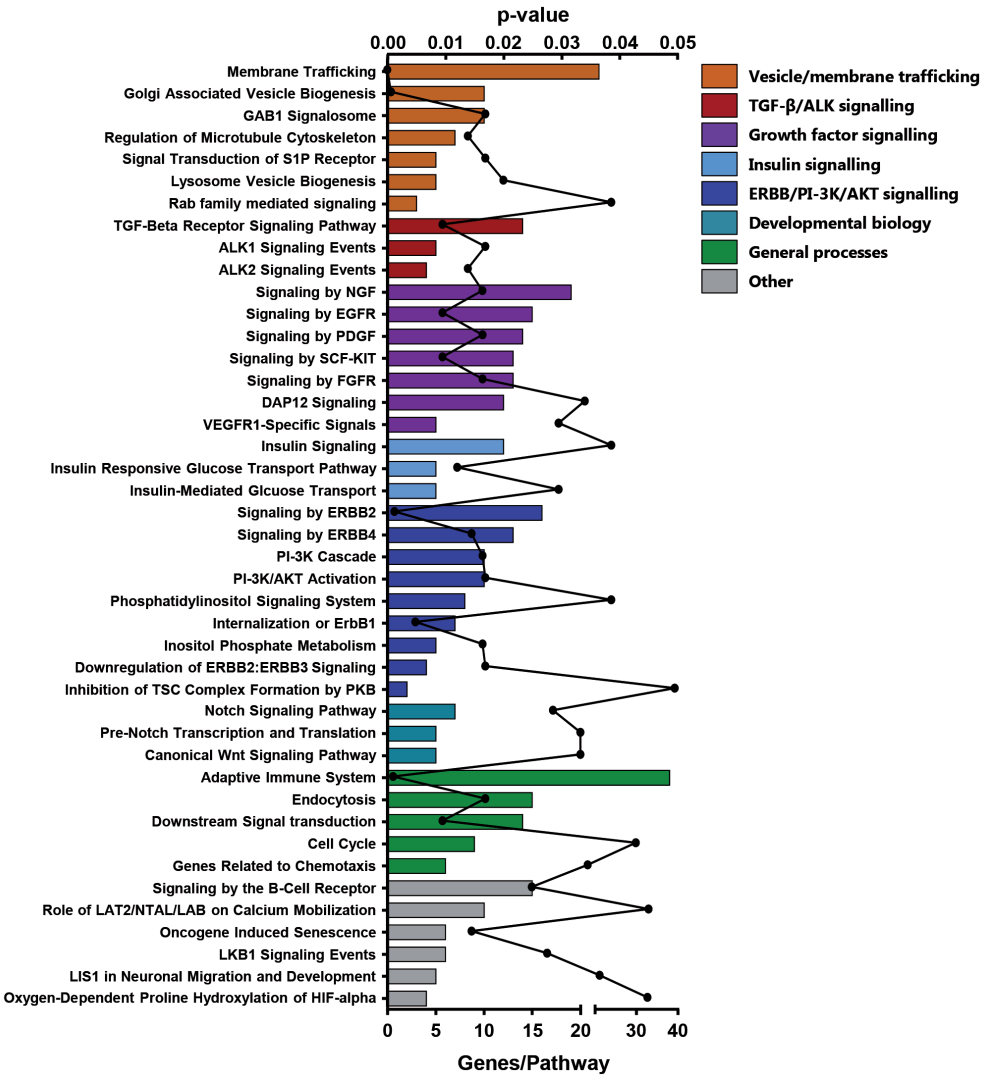

Figure 3. Pathway enrichment of experimentally supported miRNA targets. Experimentally supported targets of miR-708 and miR-130a were retrieved from three separate databases. The targets of both miRNAs were pooled and pathway enrichment was performed. Pathways significant at $p<0.05$ with B&H FDR correction were considered significantly enriched and are depicted here. Pathways directly related to a specific form of cancer and duplicate pathways were excluded. Bars show the number of miRNA target genes involved in each pathway, lines indicate B&H FDR corrected p-values. Pathways were divided into categories based on the miRNA target genes therein and biological function.

miR-708 and miR-130a are involved in numerous signalling pathways and membrane trafficking

In order to identify the potential role of miR-708 and miR-130a in the regulation of cDC1, we performed pathway enrichment analysis on the union of their experimentally supported targets retrieved from three independent databases. 43 pathways were significantly enriched, including various growth factor signalling pathways and pathways involved in membrane trafficking (Figure 3).

Expression of miR-708 and miR-130a in cDC1s of nSS patients is similar to pSS patients

To compare the specificity of miRNA expression levels in pSS patients, a group of nSS patients was included in both cohorts. The relative expression of miR-708 and miR-130a from both cohorts was integrated and we compared the expression in nSS patients with pSS patients. No significant differences between pSS and nSS patients in expression of these miRNAs were observed (Figure 4). This did not seem to be related to autoimmune activity, as the patients with a LFS of >0.0 and/or with presence of anti-SSA antibodies were similar to those patients without.

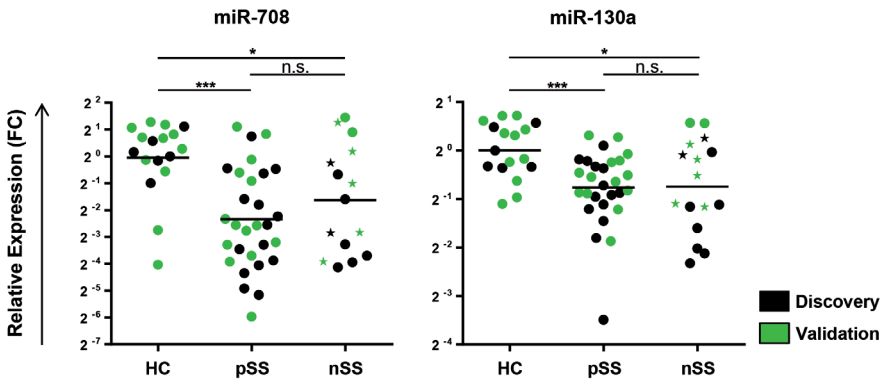

Figure 4. Down-regulated expression of miR-708 and miR-130a is characteristic of both nSS patients and pSS patients. Relative expression of miR-708 and miR-130a in discovery cohort and validation cohort was pooled by correcting for the difference in relative expression between the HC groups. nSS patients with LFS >0.0 and/or SSA+ shown as a star. Geometric means are shown. * and *** indicate statistical differences of $p<0.05$ and $p<0.001$ respectively.

Discussion

We here for the first time show differentially expressed miRNAs in an isolated dendritic cell subset of pSS patients. Two miRNAs are downregulated in type-1 cDCs of pSS patients compared to healthy donors in two independent cohorts, providing the first evidence that type-1 cDCs have dysregulated molecular pathways in pSS. miR-708 and miR-130a are involved in regulation of numerous signalling pathways and membrane trafficking. Furthermore, we show that dysregulated expression of these miRNAs is a characteristic of sicca patients, as nSS patients and pSS patients show similar down-regulation.

We chose to perform target validation in a separate cohort of patients and controls to distinguish true differentially expressed targets from false positives, instead of correcting p-values for statistical analyses. Even though the 16 targets measured in the validation cohort were selected based on a strict relative expression threshold and quality control, only 2 targets were validated, indicating that biological validation as performed here is a critical step in large miRNA screening experiments. This is especially true for studies in low-frequency isolated cell populations as performed here, where experiments with small sample size using large-scale screening tools are attractive due to limiting amounts of RNA and high costs per sample.

The present study did not confirm dysregulated expression of any miRNAs previously described to be differentially expressed in pSS PBMCs [10-12]. Similarly, we did not find any evidence for differences in expression of miR-181a, miR-574 and miR-768-3p, previously reported to be differentially expressed in the pSS salivary glands [10,13]. However, as cDC1s make up only 0.5% of the PBMCs and constitute a low number of cells at the site of inflammation it is to be expected that differences found in these DCs are not reflected in total PBMCs or salivary gland tissue.

Though we based the discovery and validation of differentially expressed miRNAs purely on the difference in expression between pSS and HC, we included nSS patients in both cohorts to compare their miRNA expression to that in pSS patients. Strikingly, no differences between pSS and nSS patients were observed in expression of miR-708 and miR-130a. It must be noted that individual nSS patients in both cohorts show some signs of autoimmunity or inflammation in the salivary gland. In both cohorts combined, three patients

have a LFS between 0.4 and 1.0, three patients are positive for anti-SSA and one patient has a LFS of 0.1 and is SSA-positive. In none of those patients a clinical diagnosis of pSS is made. However, clearly the pSS patients in both cohorts have increased autoimmunity, with much higher parameters of local and systemic immune activation and proportion of individuals with presence of antibodies against SSA and/or SSB. Though the lack of difference in miRNA expression between pSS and nSS was unexpected, we can not explain this by any limitations of our study. There are no relevant differences in sex or age between any of the groups in both cohorts. Material from controls and patients was treated in exactly the same manner and there were no temporal differences in sample collection between the groups. Furthermore, patient and control samples were randomly divided over all arrays and analyses.

The absence of clear differences in expression of miR-708 and miR-130a between nSS and pSS is in line with the absence of any correlations of miRNA expression levels with disease parameters. As there are clear differences in immune activity parameters between pSS and nSS patients, also in both of our cohorts, the differences in miRNA expression are not directly linked to disease activity. This striking observation is however difficult to interpret. As nSS patients have markedly less inflammation in salivary glands compared to pSS patients [6], our data suggest a disconnect between immunopathology in the tissue and the peripheral blood in nSS patients. Perhaps peripheral cDC1s are similarly dysregulated in both groups, while the local tissue determines whether marked inflammatory activity is induced upon a certain trigger. In-line with this, we could not find any differences in expression of activation markers on circulating cDC1s (CD86, HLA-DR) between nSS and pSS patients (M.R. Hillen, unpublished data). In addition, as peripheral blood cDCs are considered less mature cells than their counterparts in tissues [22], maturation at the site of inflammation could give rise to more significant differences between the patient groups.

Thus, we here show the first evidence for dysregulation of isolated circulating cDC1c from patients with primary Sjögren's syndrome, indicating defective signalling in these cells. Though cDC1s are very potent activators of CD4 T-cells, research into this cell subset in pSS pathogenesis is virtually non-existent. It is not clear what causes dysregulation in cDCs from the peripheral blood and to what extent they play a role in disease at this time. Multiple hits may be involved in pathogenesis, including dysregulated miRNA regulation with at least some of the hits in the inflamed tissue. Dysregulation in cDC1s may

contribute to local immunopathology when these cells migrate from the blood and receive additional signals, which could differ between nSS and pSS patients. Further investigation on the role of cDC1s in pSS patients, in particular those from the site of inflammation, should yield additional insights on their role in pathogenesis.

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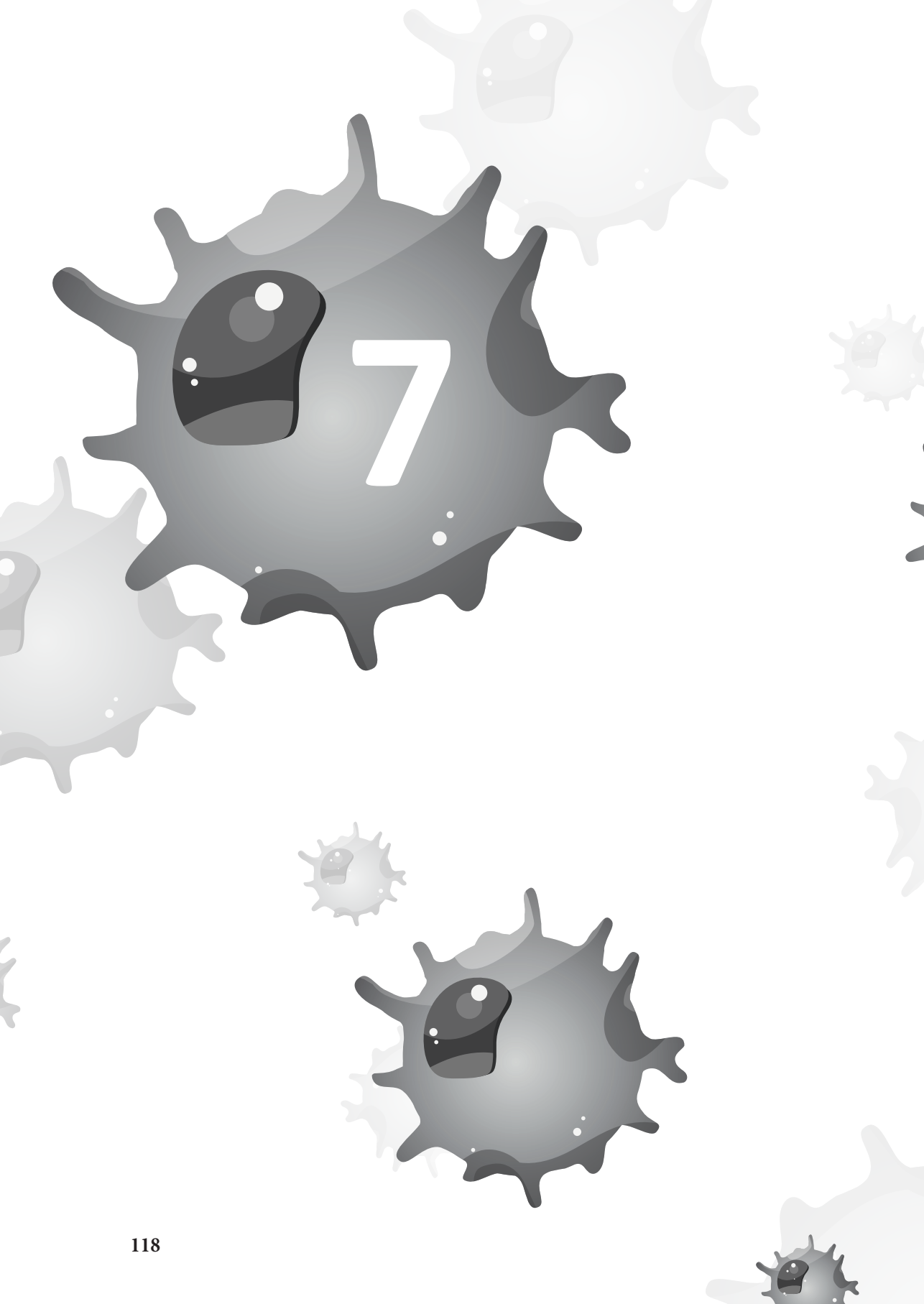
Patient age/sex	LFS	Autoantibodies	Description
57 yr./Female	>3	ANA+	Oral dryness, 50% IgA+ plasmacells in SLSG, neuropathy nerve VIII
60 yr./Female	4	ANA+	Ocular, oral and vaginal dryness, <70% IgA+ plasmacells in SLSG, Schirmer OD not performed due to congenital absence of iris, enucleated OD due to persistent corneal ulcer
65 yr./Female	3	ANA+	Ocular and oral dryness, Schirmer ODS 20 mm/5 min, 50% IgA+ plasmacells in SLSG, auto-immune hepatitis

Supplementary Table 1. pSS patients included in study that did not meet AECG classification criteria. LFS: Lymphocyte focus score; ANA: anti-nuclear antibodies; SLSG: Sublabial salivary gland specimen; OD: Oculus dexter; OS: Oculus sinister.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A multitude of down-regulated miRNAs in plasmacytoid DCs of primary Sjögren's syndrome patients indicates dysregulated signalling

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Abstract

Objectives. Plasmacytoid dendritic cells (pDCs) are key players in the pathogenesis of pSS and important producers of type-1 interferon (IFN), which is associated with disease. To better understand the role of pDCs in pSS, we investigated the expression of micro RNAs (miRNA) in these cells in pSS patients.

Methods. Two independent cohorts (discovery and validation) were established including a total of 30 pSS patients, 16 non-Sjögren's sicca (nSS) patients – defined as patients with dryness complaints but not clinically considered pSS and not fulfilling the classification criteria for pSS - and 15 healthy controls (HC). BDCA-4-expressing pDCs were isolated from peripheral blood and profiling of 758 miRNAs was performed. miRNAs found to be differentially expressed in the discovery cohort were subsequently measured in an independent validation cohort. Experimentally supported targets of validated miRNAs were used to perform pathway enrichment.

Results. A total of 24 miRNAs were downregulated in pSS patients versus HC in the discovery cohort. 15 miRNAs were selected to be measured in the validation cohort and ten of these were subsequently validated. Pathway enrichment indicated that these miRNAs are involved in the regulation of numerous signalling pathways. Interestingly, the expression of the validated miRNAs was not different between patients with pSS and those with nSS.

Conclusions. We here for the first time show differential expression of ten miRNAs in isolated pDCs of pSS patients. As these miRNAs are involved in regulation of multiple signalling pathways, they might be directly involved in the enhanced production of type-1 IFN in these patients.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by mononuclear infiltration of the exocrine glands. Clinical manifestations include dryness of mouth and eyes, fatigue, myalgia and arthralgia. [1]. Plasmacytoid dendritic cells (pDCs) comprise a rare cell subset human peripheral blood and are characterized by expression of high levels of HLA-DR in conjunction with blood dendritic cell antigens (BDCA)-2 and -4 [2]. pDCs are considered to be the main producers of type-1 interferons (IFN), which is thought to be an important process in immunity against viruses [3,4].

pDCs are thought to be key players in the pathogenesis of pSS, at least in part due to their type-1 IFN production which is reflected in the IFN-signature that is present in the majority of these patients [4]. Genes enhanced or induced by type-1 IFN are detectable in the majority of pSS patients in a range of cells, including monocytes and epithelial cells. Presence of IFN signature in monocytes is correlated with disease activity and presence of autoantibodies [4]. In addition, type-1 IFN enhance production of B-cell activating factor (BAFF), which is an important mediator in pSS involved in induction of B-cell activation and enhanced production of auto-antibodies [4-6]. Furthermore, deficiency for the type-1 IFN receptor in mice prevents murine Sjögren's syndrome [7]. pDCs numbers in the pSS salivary glands are increased [8,9] while they are decreased in the blood [9,10], suggesting migration towards the site of inflammation. While the activity of pDCs in pSS has been studied to some extent [8], the mechanisms that lead to or are associated with derailed activity of pDCs have not been examined in detail. This is partly due to the low frequency of these cells in the blood and the inflamed tissue.

MicroRNAs (miRNA) are small noncoding RNA molecules that play a key role in regulating the expression of protein-coding genes at the post-transcriptional level. By base-pairing to a target messenger RNA (mRNA), miRNAs mediate the binding of the RNA-induced silencing complex (RISC) to the mRNA, which results in inhibition of translation or even degradation of the target mRNA. miRNAs are crucial players in both physiological and pathological processes and specific miRNAs are differentially expressed in several autoimmune diseases, including other type-1 IFN signature diseases, in which pDCs are considered to play an important role [11-13]. In pSS, dysregulation of several miRNAs in peripheral blood mononuclear cells (PBMCs) and salivary glands have been described [14-16]. In addition, salivary gland expression levels of miR-

574 and miR-768-3p allow discrimination between patients with low and high focus scores [17].

Though pDCs are considered to be key players in the pathogenesis of pSS, the underlying mechanisms driving pDC derailment have not been extensively studied. Considering the crucial role of miRNAs in regulation of cellular processes, we investigated the expression of around 750 miRNAs in pDCs isolated from pSS patients.

Methods

Patients and controls

Two separate cohorts of patients (discovery and validation), under treatment in the University Medical Center Utrecht, and controls were established. There was no overlap in donors between the two cohorts. The characteristics of discovery and validation cohorts are depicted in Table 1 and Table 2, respectively. pSS patients (n=30) were classified according to American-European consensus group criteria [18]. Of the pSS patients, 2 individuals were clinically diagnosed as pSS but did not fulfill the classification criteria (Supplementary Table 1). These patients did not differ from the rest of the patients in any analysis. 16 individuals who were clinically not considered to be pSS patients and did not fulfill the classification criteria for pSS were defined as non-Sjögren's sicca (nSS) patients and included in the two cohorts. 15 healthy donors were included as control and divided over both cohorts. There were no significant differences in sex and gender between any of the groups in either cohort. This research was performed in accordance with the medical ethical regulations of the University Medical Center Utrecht and all patients gave their written informed consent.

Dendritic cell isolation

Mononuclear cells (MCs) were isolated from heparinized peripheral blood by density centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). BDCA-4$^+$ pDCs were isolated from PBMCs by magnetic-activated cell sorting (MACS) using BDCA-4$^+$ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. To confirm consistent purity of isolated pDCs, cells were stained with CD45-PerCP (Biolegend, San Diego, CA, USA), CD123-PE (BD Biosciences, San Jose, CA, USA), BDCA-2-FITC (BD) and BDCA-4-APC (BD) and the proportion of BDCA-2$^+$ CD123$^+$ cells within isolated fraction was measured using fluorescence-activated cell sorting (FACS) and a FACSCanto II flow cytometer (BD Bioscience, San Jose,

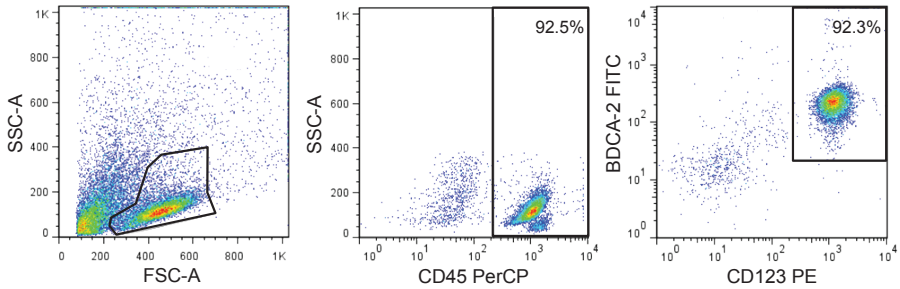

Figure 1. Gating strategy for purity check of isolated BDCA-4$^+$ plasmacytoid dendritic cells. Isolated cell-fraction was stained with anti-CD45-PerCP, anti-BDCA-2-FITC, anti-BDCA-4-APC and anti-CD123-PE. Live cells were gated based on forward scatter (FSC) and side-scatter (SSC) and leukocytes were gated based on CD45 expression within the live gate. pDC purity was calculated based on the percentage of CD123$^+$ BDCA-2$^+$ cells within total CD45$^+$ leucocytes.

	HC	pSS	nSS
N (M/F)	5 (0/5)	14 (1/13)	8 (0/8)
Age (yr.)	58 [54 – 67]	56 [29 – 70]	43 [25 – 68]
LFS (foci/4 mm^2)	-	2.1 [1.0 - 5.0)	0.0 [0.0 – 1.0]
ESSDAI	-	2.0 [0.0 – 19]	-
ESSPRI	-	5.0 [2.0 – 8.8]	-
Schirmer (mm/5 mins)	-	5.0 [0.0 – 25]	3.0 (0.0 – 24)
ANA (no. positive/% of group)	-	11 /79%	2 /25%
SSA (no. positive/% of group)	-	8 /57%	2 /25%
SSB (no. positive/% of group)	-	4 /29%	0 /0%
Rheumatoid factor (no. positive/% of group)	-	5 /42%	0 /0%
Serum IgG (g/L)	-	14 [8.3 – 30]	10 [6.8 – 17]
ESR (mm/hour)	-	11 [5.0 – 36]	12 [4.0 – 17]
CRP (mg/L)	-	1.0 [0.0 – 7.0]	1.9 [0.0 – 4.0]
C3 (g/L)	-	1.1 [0.7 – 1.3]	1.2 [0.6 – 1.7]
C4 (g/L)	-	0.3 [0.0 – 0.4]	0.3 [0.2 – 0.4]

Table 1. Discovery Cohort Characteristics. HC: Healthy control; nSS: non-Sjögren's Sicca; pSS: primary Sjögren's syndrome; LFS: Lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: Anti-nuclear antibodies; SSA: Anti-SSA/Ro; SSB: Anti-SSB/La; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein. Values are median [range] unless stated otherwise.

USA). The used gating strategy is shown in Figure 1. Mean purity of all samples was 90%, there were no significant differences in purity between any of the groups in either cohort. Cells were lysed in RLTPlus buffer (Qiagen, Venlo, Netherlands) supplemented with beta-mercaptoethanol.

RNA isolation

Total RNA containing the small-RNA fraction was purified from lysates of isolated cells using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity were assessed with Qubit RNA Kit (Life Technologies, Carlsbad, CA, USA) and the Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA) respectively.

miRNA profiling and analysis

miRNA profiling was performed by TaqMan RT-qPCR on the OpenArray platform (LifeTechnologies) following manufacturer's instructions. This method allows for the simultaneous analysis of 758 miRNAs split into two equal pools (A and B). 10ng of total RNA were reverse-transcribed by using the miRNA multiplex RT primers pools, either v2.1 for pool A or v3.0 for pool B, and the TaqMan miRNA reverse transcription kit (LifeTechnologies). RT products were pre-amplified using the Megaplex PreAmp Primers pools A and B in the presence of the TaqMan PreAmp Master mix (Life Technologies), by using the following thermal cycler conditions: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min and 16 cycles of 95°C for 15 sec and 60°C for 4 min and one single cycle of 96°C for 10 minutes. The miRNA OpenArray profiling was performed on the amplified cDNA, diluted in a 1:40 ratio using Taqman OpenArray master mix on the QuantStudio 12k flex Real-Time PCR system (LifeTechnologies). miRNA profiling data were analysed using ExpressionSuite software (LifeTechnlogies). Data were normalized using the global normalization approach [19]. The analysis was conducted on data points detected at crt lower than 27, by rejecting samples with an amplification score lower than 1.24 or including them as crt =27. miRNAs that showed a significant difference at $p<0.05$ with relative fold change (FC) of ≥ 2 or ≤ 0.5 in pSS compared to HC were considered differentially expressed.

To validate the differential expression of selected miRNAs, cDNA was synthesized from 6.6 ng of total RNA by using a pool of 19 miRNA-specific RT primers contained in the TaqMan Human miRNA assays (each diluted to 0.02x) in the presence of 10 U/µl MultiScribe RT enzyme, 0.25 U/µl RNase Inhibitor and 2mM dNTPs (LifeTechnologies). The following thermal cycler conditions

	HC	pSS	nSS
N (M/F)	10 (1/9)	16 (0/16)	8 (0/8)
Age (yr.)	53 [29 – 64]	52 [26 – 70]	47 [34 – 69]
LFS (foci/4 mm^2)	-	2.0 [1.0 – 4.0]	0.0 [0.0 – 0.7]
ESSDAI	-	5.0 [0.0 – 13]	-
ESSPRI	-	5.3 [1.0 – 8.0]	-
Schirmer (mm/5 min)	-	6.5 [0.0 – 30]	5.5 [1.5 – 32]
ANA (no. positive/% of group)	-	14 /93%	6 /75%
SSA (no. positive/% of group)	-	13 /82%	3 /38%
SSB (no. positive/% of group)	-	10 /63%	0 /0%
Rheumatoid factor (no. positive/% of group)	-	7 /54%	1 /17%)
Serum IgG (g/L)	-	18 [9 – 33]	13 [11 – 14]
ESR (mm/hour)	-	16 [3.0 – 38]	13 [2.0 – 29]
CRP (mg/L)	-	1.3 [0.0 – 5.0]	1.0 [0.0 – 5.2]
C3 (g/L)	-	1.1 [0.7 – 1.3]	1.2 [0.6 – 1.7]
C4 (g/L)	-	0.2 [0.1 – 0.4]	0.3 [0.2 – 0.4]

Table 2. Validation cohort characteristics HC: Healthy control; nSS: non-Sjögren's Sicca; pSS: primary Sjögren's syndrome; LFS: Lymphocyte focus score; ESSDAI: EULAR Sjögren's syndrome disease activity index; ESSPRI: EULAR Sjögren's syndrome patient reported index; ANA: Anti-nuclear antibodies; SSA: Anti-SSA/Ro; SSB: Anti-SSB/La; RF: Rheumatoid Factor; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein. Values are median [range] unless stated otherwise.

were used: 10 min at 4°C; 30 min at 16°C; 20 min at 42°C; 5 min at 85°C. The RT product was pre-amplified as described above with a pool of the correspondent miRNA TaqMan assays diluted to 0.03x. The quantification of each miRNA was performed on the OpenArray platform by using a custom panel of 17 TaqMan assays (indicated in Table 3) and 2 small-nucleolar RNAs. Data were analyzed according to the comparative threshold cycle method [20], after normalization by the mean Ct of 2 miRNAs (miR-17 and miR-191) and 2 small nucleolar RNA (RNU48 and U6) which showed good abundance and stable expression between patients and controls. The miRNA expression of each donor was depicted as FC compared to one healthy-control, which was set at 1.

Pathway enrichment

A list of genes predicted to be target of the differentially expressed miRNAs and validated by in vitro experiments or supported by indirect experimental data (from here on referred to as experimentally supported targets) was retrieved

from three databases: StarBase [21], mirTarBase [22] and TarBase [23]. From StarBase, only those miRNA-target pairs that were predicted by at least 3 software programs and supported by two separate pieces of evidence were selected. For mirTarBase, all experimentally supported targets were included. For TarBase, all experimentally supported downregulated targets of the miRNAs were selected. Pathway enrichment was performed on the retrieved targets of each separate miRNA using Toppgene Suite [24]. Subsequently, pathways that were significantly enriched using the B&H FDR corrected p-value with a threshold of $p<0.05$ for 3 or more miRNAs were considered relevant, excluding pathways directly related to a specific type of cancer and duplicated pathways. Pathways were grouped based on overlap of targets in the pathways and literature.

Integration of cohort data

To compare expression of differentially expressed miRNAs between nSS and pSS patients and for correlations with disease parameters, relative miRNA expression levels of both cohorts were integrated. Geometric mean (GM) of expression in HC groups of both cohorts was calculated using Graphpad software version 6.02 (Graphpad, Lo Jolla, CA, USA). FC of all samples in validation cohort was corrected for the ratio between GM in HC of discovery cohort and GM of HC in validation cohort.

Statistics

Differences in miRNA expression between pSS patients and HC in discovery cohort were analyzed using ExpressionSuite software (Lifetechnologies). For validation, statistical analysis was performed with GraphPad Prism software version 6.02 (GraphPad) using the Mann-Whitney U test (two-sided). For correlations with disease parameters, Spearman's rho was used. For comparisons of HC, nSS and pSS miRNA expression, Kruskall-Wallis test was performed with post-hoc Mann-Whitney U test. Differences and correlations were considered statistically significant at $p<0.05$.

Results

24 miRNAs are differentially expressed in pSS patients as compared to healthy controls in the discovery cohort

Expression of 758 miRNAs was assessed in pDCs obtained from pSS patients and healthy donors from the discovery cohort (Table 1) using an OpenArray-based screening. miRNAs were considered to be differentially expressed when their detected levels in patients compared to controls was at least two-fold

higher or two-fold lower with a p-value of <0.05. We observed 24 miRNAs that were differentially expressed in pSS patients as compared to the healthy controls (Table 3).

Target	Fold Change pSS vs HC.	p-value	Measured in Validation
miR-30e-3p	0.098	0.008	x
miR-16	0.187	0.006	
miR-223#	0.222	0.016	
miR-103	0.239	0.001	
miR-362-3p	0.258	0.018	
miR-28	0.311	0.019	x
miR-29a	0.325	0.037	x
miR-29c	0.343	0.025	x
miR-30d#	0.345	0.020	x
miR-502-3p	0.360	0.016	
miR-532	0.371	0.021	x
miR-25	0.386	0.026	x
Let-7g	0.404	0.041	x
miR-186	0.405	0.044	x
miR-340	0.417	0.035	
miR-532-3p	0.432	0.001	
miR-195	0.447	0.001	x
miR-130b	0.453	0.003	
miR-128a	0.468	0.048	x
miR-361	0.471	0.021	x
miR-181c	0.477	0.047	x
miR-130a	0.479	0.003	x
miR-20a#	0.499	0.043	
miR-126#	2.060	0.030	x

Table 3. List of miRNAs differentially expressed in pSS patients compared to healthy donors in discovery cohort. 758 miRNAs were measured on OpenArray. All miRNAs differentially expressed in pSS patients as compared to healthy donors with a relative fold-change of ≤0.5 or ≥2.0 and a p-value of p<0.05 are listed. miRNAs selected to be measured in the validation cohort are marked in the rightmost column.

Ten differentially expressed miRNAs were validated in an independent validation cohort

Of the 24 miRNAs differentially expressed in the discovery cohort, we measured the expression of 15 miRNAs in an independent validation cohort of pSS patients and healthy donors (Table 2). These targets were selected based on the level of expression and the quality of amplification. Of these 15 miRNAs, 10 miRNAs were validated to be downregulated in pDCs of pSS patients compared to HC with $p<0.05$ (Figure 2).

Downregulated miRNAs do not correlate with disease activity or inflammatory parameters

To investigate whether the differences in miRNA expression between pDCs of pSS patients and HC were associated with disease parameters, we integrated the data of both cohorts and performed correlation analyses within the pSS patient group. No significant correlations between expression of the 10 miRNAs

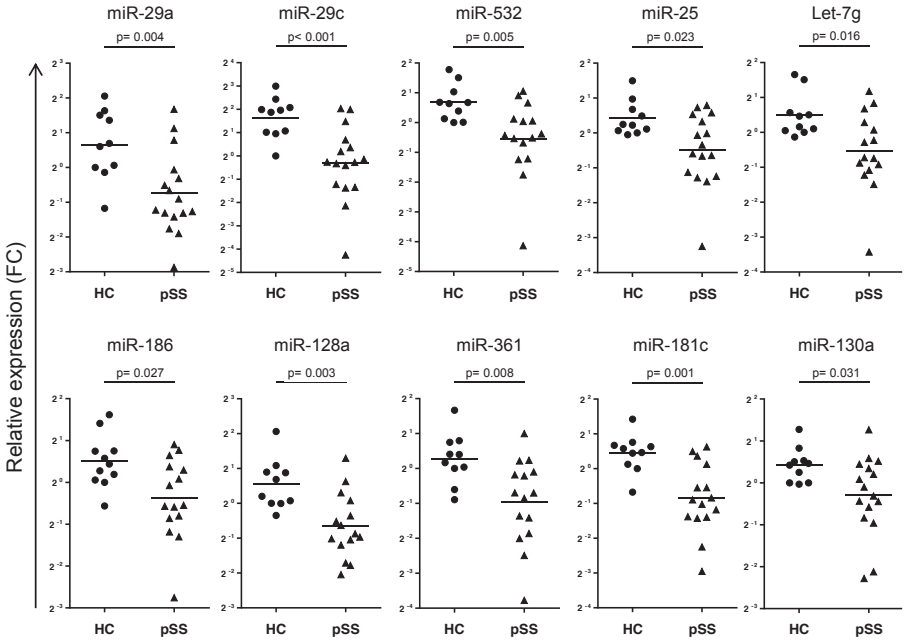

Figure 2. miRNAs confirmed to be differentially expressed in pSS patients in the validation cohort. 10 miRNAs that were differentially expressed in the discovery cohort were validated to be downregulated in pSS compared to HC in the validation cohort. Depicted are Geometric mean and p-value of Mann-Whitney U tests.

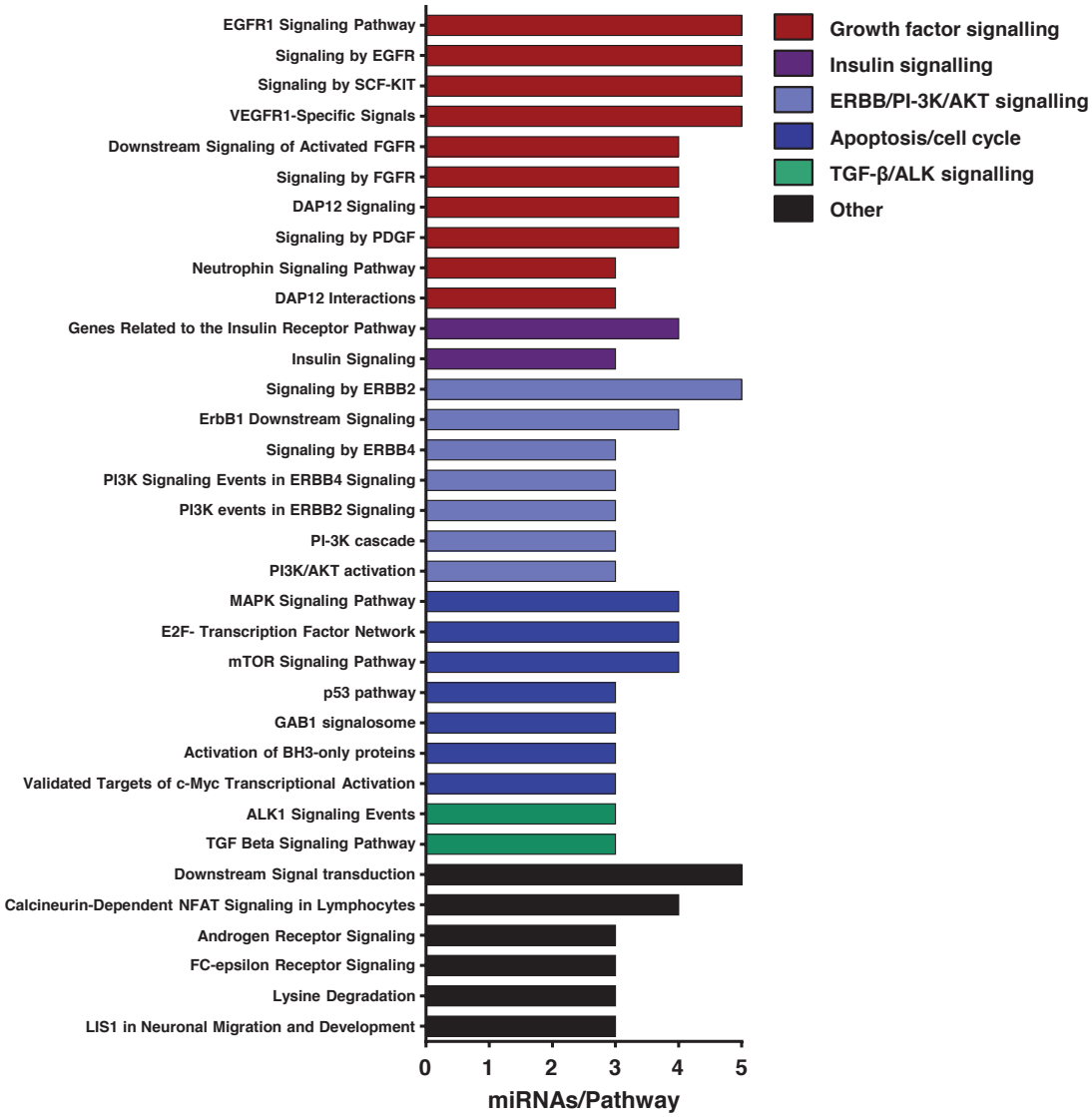

Figure 3. Intersect of pathway enrichment of validated miRNAs targets. Experimentally supported targets of the differentially expressed miRNAs were retrieved from three separate databases. Pathway enrichment was performed for each miRNA separately. Pathways significant at $p<0.05$ with B&H FDR correction were considered significantly enriched. Pathways present in pathway enrichment for at least 3 miRNAs are depicted. Pathways directly related to a specific form of cancer and duplicate pathways were excluded. Bars show the number of miRNAs for which the pathway was enriched. Pathways were divided into categories based on the miRNA target genes therein and biological function.

and LFS, ESSDAI, ESSPRI, Schirmer test, serum IgG, ESR, CRP, C3 or C4 were found. In addition, there were no significant differences between pSS patients with auto-antibodies and those without, including anti-nuclear antibodies, SSA or SSB (data not shown).

Downregulated miRNAs are involved in a multitude of signalling pathways, including cell cycle regulation

In order to identify the potential roles of the 10 miRNAs found to be downregulated in pSS pDCs, we performed pathway enrichment analysis on the experimentally supported targets retrieved from three independent databases. Separate pathway enrichment analyses were performed for the targets of each miRNA, followed by a selection of pathways that were significantly represented in the analysis of 3 or more miRNAs. 34 pathways were significantly enriched for at least 3 miRNAs, with a large amount of them (n=28) involved in signalling pathways. (Figure 3).

Expression of downregulated miRNAs in pDCs of pSS patients is similar to nSS patients

To investigate the specificity of miRNA expression levels in pSS patients, a group of nSS patients was included in both cohorts. No significant differences between pSS and nSS patients in expression of any of the 10 miRNAs were observed (Figure 4). This did not seem to be related to the presence of signs of autoimmunity, as the patients with a LFS of >0.0 and/or with presence of anti-SSA antibodies were similar to those without.

Discussion

We here for the first time show differentially expressed miRNAs in isolated pDCs of pSS patients. Ten miRNAs were downregulated in pDCs of pSS patients compared to healthy donors in two independent cohorts. Furthermore, our data indicate that dysregulated expression of these miRNAs is characteristic of sicca patients, as we observed similar expression patterns between nSS and pSS patients.

Using our robust discovery and validation approach, only significantly downregulated miRNAs were found in pDCs of sicca patients. Pathway enrichment analysis predicts that the miRNA-regulated signalling pathways in derailed pDCs of pSS and nSS patients are associated with enhanced

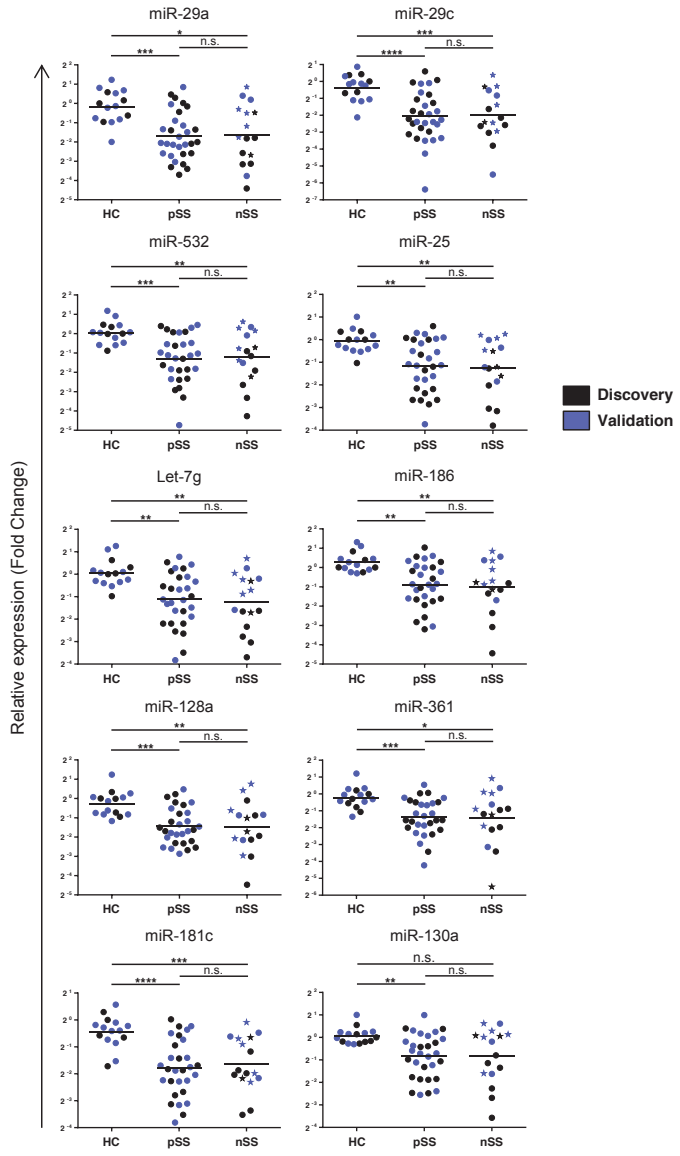

Figure 4. Expression of all differentially expressed miRNAs is similar in nSS patients and pSS patients. Relative expression of the validated miRNAs from discovery cohort and validation cohort was integrated by correcting for the difference in relative expression between the HC groups. nSS patients with LFS >0.0 and/or SSA+ shown as a star. Geometric means are shown. *, **, *** and **** indicate statistical differences of $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$ respectively.

activation of these cells. As such, knock-in and knock-out experiments by using miRNA micis or anti-miRs respectively should reveal the potential of the identified miRNAs to regulate the activity and phenotype of pDCs.

We could not confirm dysregulation of any miRNAs previously described to be differentially expressed in pSS PBMCs or salivary gland tissue [14-17]. However, as pDCs make up only 0.1% of the PBMCs it is to be expected that differences found in PBMCs are not reflected in isolated pDCs. Similarly, with regards to findings in salivary gland miRNA expression, relatively low numbers of pDCs at the site of inflammation is likely the reason that we did not find any previously described miRNAs to be differentially expressed.

Though we based the discovery and validation of differentially expressed miRNAs purely on the difference in expression between pSS and HC, we included nSS patients in both cohorts to compare their miRNA expression to that found in pSS patients. Upon integration of the data from both cohorts, no differences between pSS patients and nSS patients were observed in expression of any of the miRNAs. It must be noted that individual nSS patients in both cohorts may show some signs of autoimmunity or inflammation. In both cohorts combined, three patients have a LFS between 0.4 and 1.0, four patients are positive for anti-SSA and one patient has a LFS of 0.1 and is SSA-positive. In none of these patients a clinical diagnosis of pSS is made. Furthermore, the pSS patients in both cohorts clearly have increased levels of autoimmune activity, with higher parameters of local and systemic immune activation. Though the lack of difference in miRNA expression between pSS and nSS was unexpected, we can not explain this by any limitations of our study. There are no relevant differences in sex or age between any of the groups in both cohorts and no differences in purity of isolated cells between the groups. Material from healthy donors and patients was treated in exactly the same manner and there were no temporal differences in sample collection between the groups. Furthermore, patient and control samples were randomly divided over all arrays and analyses.

The similarities in expression of these miRNAs between nSS and pSS is in line with the absence of any correlations of miRNA expression levels with disease parameters. As nSS and pSS patients clearly differ in their immune activity parameters, also in both of our cohorts, the alterations in miRNA expression are not directly linked to disease activity. Though dysregulation of pDCs has been described in pSS patients [8,10], blood and salivary gland pDCs have not before been studied in patients with nSS. nSS patients have markedly less

inflammation in the salivary glands compared to pSS patients based on the general absence of foci and less expression of proinflammatory cytokines [25,26]. Thus, our data suggest a disconnect between immunopathology in the salivary gland tissue and the peripheral blood in nSS patients. The local inflammatory response associated with production of type-1 IFN observed in pSS patients may be induced by increased migration of these dysregulated pDCs observed in pSS [9], though pDC numbers in nSS patients have not yet been studied. In addition, divergence in local inflammatory signals that drive pDC maturation, prolonged retention in the tissue or enhanced production of type-1 IFN, such as TLR ligands, may cause the differences in the salivary glands observed between pSS and nSS patients.

Thus, we here show the first evidence for dysregulation of miRNAs in circulating pDCs of Sjögren's syndrome patients and their possible implications in derailed mechanisms characterizing these cells in pSS. We did not observe differences in expression of the validated miRNAs between pSS and nSS patients, indicating that similar mechanisms might lead to miRNA alterations both patient groups. However, as a type-1 IFN signature is present in a large part of pSS but not nSS patients, these results suggest that other signals might be required for enhanced IFN production by pDCs. Further research should help to dissect the factors involved in pDC activation and IFN production as well as the associated autoimmune response and tissue damage in pSS.

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Patient age/sex	LFS	Autoantibodies	Description
60 yr./Female	4	ANA+	Ocular, oral and vaginal dryness, <70% IgA+ plasmacells in SLSG, Schirmer OD not performed due to congenital absence of iris, enucleated OD due to persistent corneal ulcer
65 yr./Female	3	ANA+	Ocular and oral dryness, Schirmer ODS 20 mm/5 min, 50% IgA+ plasmacells in SLSG, auto-immune hepatitis

Supplementary Table 1. pSS patients included in study that did not meet AECG classification criteria. LFS: Lymphocyte focus score; ANA: anti-nuclear antibodies; SLSG: Sublabial salivary gland specimen; OD: Oculus dexter; OS: Oculus sinister. .


8

Summary & Discussion

Parts of this chapter have been published in:
M.R. Hillen, T.R.D.J. Radstake, C.E. Hack, J.A.G. van Roon. Thymic stromal lymphopoietin (TSLP) as a novel mediator amplifying immunopathology in rheumatic disease. Rheumatology (Oxford). 2015; doi:10.1093/rheumatology/kev241

The first chapters of this thesis focused on the role of thymic stromal lymphopoietin (TSLP), in conjunction with interleukin (IL)-7 and thymus and activation regulated chemokine (TARC), in rheumatoid arthritis (RA) and the role of TSLP in primary Sjögren's syndrome (pSS). As described in the introduction (**Chapter 1**), TSLP is a cytokine with important roles in lymphocyte development. In addition, it is an important factor in homeostasis of T-lymphocytes. TSLP is extensively described as a mediator in atopic diseases but increasing evidence suggests that it plays a role in many different conditions including cancer and autoimmunity.

Dendritic cells, TSLP and TARC in rheumatoid arthritis

Previous data show that intra-articular concentrations of TSLP are increased in the majority of RA patients [1,2]. Fibroblasts from RA patients were shown to express TSLP *ex vivo* and TSLP secretion was induced *in vitro* upon stimulation with proinflammatory cytokines IL-1β and TNF-α and ligands for TLR3 and TLR4 [3], which are present in RA synovium and are implicated in immunopathology [4]. In addition, TSLP was shown to be produced by a monocyte cell-line upon stimulation with IL-32, associated with differentiation to macrophages, indicating that monocytes and macrophages may contribute to the TSLP levels in the RA joint [5].

Blood dendritic cell antigen (BDCA-1/CD1c)-expressing type-1 classical DCs (cDC1s), were found to be increased in the synovial fluid (SF) of RA patients compared to the peripheral blood. This subset of DCs displays higher expression of BDCA-1, MHC class-II, CD80, CD86, CD40 and TSLP receptor (TSLPR) in RA [6]. When stimulated with TSLP, cDC1s induce T-cell proliferation and secretion of Th1-, Th2- and Th17-associated cytokines IFN-γ and IL-17 by T-cells. cDC1s from the RA SF were able to induce more T-cell proliferation

compared to paired cDC1s from the peripheral blood [1]. In addition, these cells produce high levels of T-cell attracting chemokines including TARC [7] and macrophage inflammatory protein (MIP)-1α [1].

Several mouse models demonstrate that TSLP increases inflammation in experimental arthritis. Administration of neutralizing antibodies against TSLP ameliorates disease in a TNF-α- and immune complex-dependent collagen type II antibody-induced arthritis (CAIA) model [2]. In the collagen-induced arthritis model (CIA), which is dependent on both B- and T-cells, systemic administration of recombinant TSLP caused more severe disease and tissue destruction as measured by clinical arthritis score and histology. Furthermore, TSLPR-deficient (TSLPR-/-) mice have less severe proteoglycan-induced arthritis (PGIA) when compared to wild-type mice, associated with a decrease in Th17- and Th2 activity and osteoclast activity [8].

In **Chapter 2**, we observed almost complete prevention of immunopathology in a PGIA model in TSLPR-/- mice treated with anti-IL-7R. This was associated with marked decreases in T-cell attracting- and co-stimulatory factors and levels of cytokines associated with Th17 activity. We further observed robust additive effects of IL-7 and TSLP on T-cell proliferation and Th17 activation in human DC/T-cell *in vitro* co-cultures, suggesting these data are relevant for the human setting. As TSLP and IL-7 share the IL-7Rα chain for signalling, targeting this subunit to prevent signalling of both cytokines presents a potentially very beneficial treatment option for RA patients.

Upon stimulation of cDC1s with TSLP, TARC is produced in high concentrations [7]. *In vitro*, TSLP concentrations of 20 ng/mL are required for optimal induction of T-cell proliferation by DCs, but concentrations of 1 ng/mL are enough for optimal TARC production by these cells (M.R. Hillen, unpublished data). TARC is a chemokine that attracts cells expressing C-C chemokine receptor (CCR) 4, which is present on CD4 T-cells with Th2, Th17, Th22 and Treg phenotypes [9-12], NKT-cells [13] and fibroblasts [14]. In RA patients, increased numbers of peripheral blood CD4 T-cells express CCR4 [15]. Moreover, the majority of IL-17-producing cells in the RA synovium express CCR4 [16], suggesting that ligation of this receptor is involved in migration towards or retention in the synovial compartment.

In **Chapter 3**, we show increased TARC levels in the RA SF as compared to OA SF and increased production of TARC by RA SF mononuclear cells (MCs)

and cDC1s. The correlation of TARC concentrations with numbers of cDC1s in the synovial fluid suggested an important contribution of these cells to TARC production. Indeed, depletion of cDCs from MC fractions strongly prevented spontaneous TARC production by MCs from the SF of RA patients. Furthermore, blocking TARC in a transwell migration assay not only prevented T-cell migration and the production of Th1- and Th17 cytokines but also largely inhibited TNF-α production. Thus, TARC production by TSLP-activated cDC1s may mediate immunopathology by attracting cell-types that produce cytokine involved in RA immunopathology. These data indicate that targeting the cDC1s as the main source of TARC might be a successful method. Effective type-1 cDC-depletion using an antibody would be an efficacious way to prevent TARC production as shown in our DC-depletion experiments. Moreover, this will prevent production of a multitude of other pro-inflammatory mediators that these cells produce, such as TNF-α, MIP-1α, and will inhibit local T-cell proliferation induced by cDCs.

TSLP in primary Sjögren's syndrome

As described in **Chapter 4**, we observed reduced TSLP-expression in labial salivary gland biopsy samples of pSS patients as compared to non-Sjögren's sicca (nSS) controls, which experience dryness symptoms without signs of autoimmunity or presence of lymphocytic foci. In contrast to what is described for other rheumatic diseases, TSLP expression was lower in pSS patients compared to nSS patients, correlating with a decrease in disease parameters. In addition, reduced TSLP was associated with increased numbers of lymphocytic foci, disease activity index and numbers of infiltrating T-cells.

The functional consequences of this reduced TSLP expression in pSS are unknown. However, regulation of the immune system in the mucosal-associated tissue of the salivary gland is very different from that of the joint or skin and is more similar to that of other mucosal tissues such as the gut [17]. In the gut, TSLP expression is decreased in patients with Crohn's disease compared with healthy donors [18] and TSLPR-deficiency induces Th1- and Th17 activity in an experimental colitis model [19]. Furthermore, in mice that are unable to form micro RNAs (miR) in the gut, it was shown that miR-375 is needed for efficient TSLP production. miR-375 levels are much lower in the colon of patients with inflammatory bowel disease (IBD) and lowered TSLP levels could play an important role here [20]. Moreover, cDCs conditioned by TSLP derived from gut epithelial cells are strongly polarized towards induction of Th2 activity in co-culture with $CD4^+$ T-cells [21]. Based on these data the concept has been put

forward that TSLP is constitutively expressed in healthy gut epithelial cells and prevents inflammation by skewing the milieu towards Th2 activity which protects against Th1/Th17-mediated immunopathology [21].

Hence, it can be speculated that the expression of TSLP in the salivary glands resembles what is shown in Crohn's disease and that TSLP plays an immune-regulatory role in the salivary glands. Consequently, decreased expression of TSLP in these glands as occurs in pSS may favor Th1/Th17 activity, which promotes tissue destruction and exocrine gland dysfunction. Although this concept is plausible, recent findings on TSLP isoforms have indicated there is increased complexity. Epithelial cells at mucosal sites including salivary glands appear to produce high levels of a shorter isoform of TSLP that does not bind to the TSLPR but instead has strong anti-microbial properties. This raises the possibility that lowered expression of this shorter isoform leads to a reduced barrier function by diminished anti-microbial activity and subsequently enhanced immune activation [22]. Future studies will have to demonstrate which isoforms are expressed in the salivary glands of pSS patients.

TSLP effects are dependent on immunological micro-environment

Thus, TSLP plays a role in several rheumatic diseases and processes involved therein. The activity that TSLP promotes varies per condition and seems to be critically dependent on the immunological milieu at the site of inflammation and the major antigens against which a response is mounted. This results mostly in Th2/Th17-driven responses in the skin and Th1/Th17 responses in the joint.

TSLP production in the joint is most likely triggered by TLR-ligands or inflammatory cytokines [3]. When stimulated with TSLP, cDCs from the joint of RA patients promote production of Th1, Th2 and Th17 mediators by autologous CD4 T-cells [1]. As these cultures are not dependent on presence of allogeneic antigens and required cell-cell contact for effective T-cell activation, these cDCs seem to present auto-antigens and induce homeostatic T-cell expansion without skewing Th-responses, as was previously described [23]. Local mediators in the synovium likely mediate skewing of these expanded T-cells towards Th1- and Th17 activity, which results in the pro-inflammatory effects of the cytokine as observed in mouse experiments (Figure 1). IL-12 and TLR3L were shown to skew TSLP-cDC-activated T-cells towards Th1- and Th17-cells, even in the presence of Th2 mediators including IL-4 and OX40L [24-26].

However, the role of TSLP in barrier-associated tissues seems to be different. In the skin, TSLP mediates Th2-skewing as has been extensively studied in atopic dermatitis (AD) and is also true for systemic sclerosis [27,28]. Upon insult to the tissue, TSLP is produced as defense measure against helminths or other microbial pathogens by epithelial cells and creates an environment that is highly permissive for Th2 activity to clear the infection (Figure 1) [29]. Similar to the skin in AD patients, TSLP is a very strong Th2-skewing factor in patients with SSc. Here, TSLP induces Th2 activity which seems to be a critical component of the pro-fibrotic effects as shown by the disease amelioration in mice with defective IL-13/IL-4 signalling [27].

In mucosal tissues, which also have an important barrier function, TSLP seems to be more critical for maintenance of tolerance by inducing Th2 activity and diminishing Th1/Th17 activity responses [21]. This is in contrast to the skin where it seems to be primarily produced to prevent insults to underlying tissues by initiating an inflammatory response after initial penetration of the barrier [29]. Lower expression in salivary glands and possibly other exocrine glands makes TSLP an unattractive target for therapy in pSS. However, future studies should shed light on the isoforms that are expressed at mucosal sites and what isoforms are targeted by therapy. Importantly, in trials using anti-TSLP treatment in asthma patients no specific adverse events in the digestive tract were reported [30].

Blockade of TSLP signalling has shown promising results in RA and SSc mouse models warranting studies to target this cytokine in these diseases. In this respect, a recent clinical study with anti-TSLP shows promising effects on prevention of T-cell driven immunity in asthma without significant adverse effects [30], setting the stage for TSLP as a promising target in chronic inflammatory diseases.

Dendritic cells in primary Sjögren's syndrome

The second part of this thesis focuses on the role of dendritic cells in pSS. As reviewed in **Chapter 5**, numbers of subsets of dendritic cells are decreased in peripheral blood of pSS patients and locally increased numbers are present. We used a non-hypothesis driven approach to study potential dysregulation in signalling pathways in cDC1s and pDCs in these patients. To this end, we examined the expression levels of micro RNAs (miRNA) in these cells. miRNA are small noncoding RNA molecules that play a key role in regulating the expression of protein-coding genes at the post-transcriptional level by degradation of messenger RNA (mRNA) or interfering with translation of target mRNA.

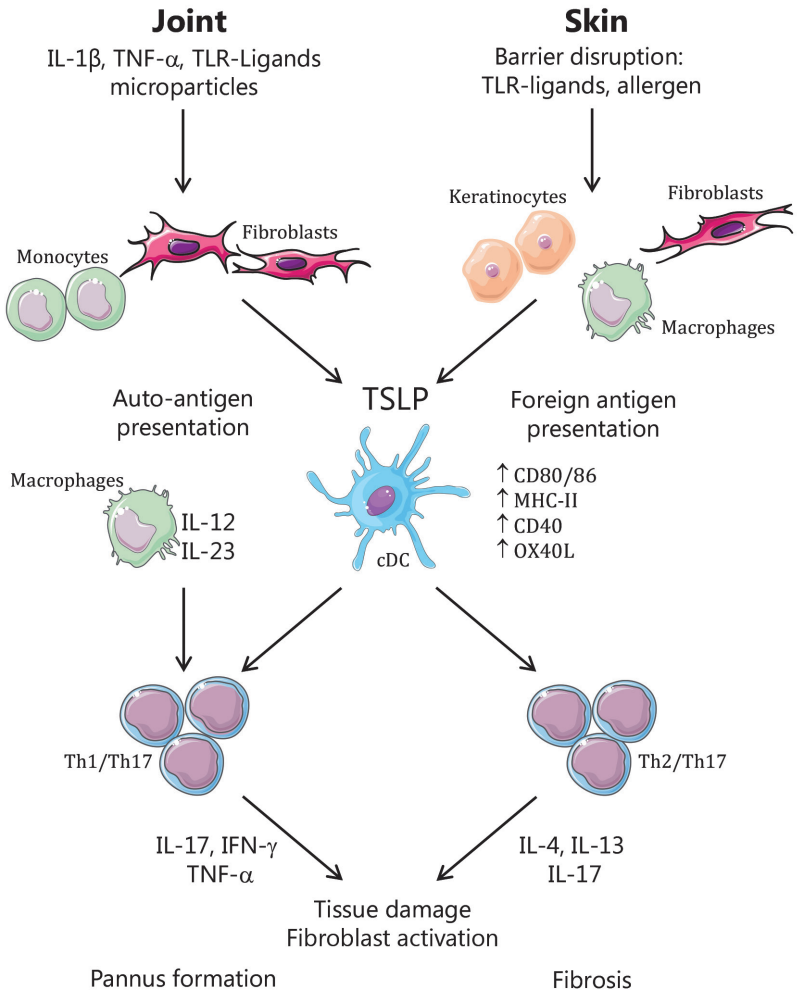

Figure 1. Effects of TSLP are dependent on the tissue and immunological environment. In the joint, TSLP-stimulated cDCs induce homeostatic proliferation of T-cells without skewing the T-cell response. The microenvironment in the joint will induce production of Th1/Th17 cytokines. In the skin, barrier disruption allows entry of TLR-ligands, which enhance TSLP production by macrophages and keratinocytes. Activation of the cDCs induces strong Th2/Th17 activity by the CD4 T-cells.

The functional capacities and molecular characteristics of Type-1 cDCs have not previously been studied in pSS, but these cells may be important mediators in the disease. They are very potent activators of CD4 T-cells and are also involved in B-cell activation [31], both critical mediators in pSS immunopathology and tissue damage. Previous work indicates that there are mature cDCs in the pSS salivary glands and a decrease in cDC numbers in peripheral blood of these patients. In **Chapter 6**, we show the first data on differentially expressed miRNAs in an isolated immune cell subset of pSS patients and the first data on isolated cDC1s in this disease. In two independent cohorts, two miRNAs were downregulated in type-1 cDCs of pSS patients compared to healthy donors. miR-708 and miR-130a are involved in a range of downstream signalling pathways, including growth factor signalling as well as vesicle trafficking. Furthermore, we show that dysregulated expression of these miRNAs is a characteristic of sicca patients, as we observed no differences between nSS patients and pSS patients.

Plasmacytoid dendritic cells (pDCs) are known for their capacity to secrete large amounts of type-1 interferons (IFN), which is thought to be an important process in immunity against viruses [31]. Genes enhanced or induced by type-1 IFN are detectable in the majority of pSS patients in a range of cells, including monocytes and epithelial cells, which is referred to as the IFN signature. Presence of IFN signature in monocytes is correlated with increased disease activity and presence of autoantibodies [32]. In addition, type-1 IFN enhance production of B-cell activating factor (BAFF), which is an important mediator in pSS inducing B-cell activation and production of auto-antibodies [32,33].[34]. Numbers of pDCs in the pSS salivary glands are increased [35,36] while they are decreased in the blood [36,37], suggesting migration towards the site of inflammation or lymphoid organs. In **Chapter 7**, we for show differentially expressed miRNAs in isolated pDCs of pSS patients. Ten miRNAs were downregulated in pDCs of pSS patients compared to healthy donors in two independent cohorts, providing the first evidence that pDCs are dysregulated at the miRNA level in pSS. These miRNAs are involved in a multitude of signalling pathways and regulation of the cell cycle. Furthermore, as observed for cDC1s, dysregulated expression of these miRNAs seemed to be a characteristic of sicca patients as we observed similar expression patterns between nSS patients and pSS patients.

pSS research in the last years has put a lot of focus on trials with efficacious treatments approved in other generalized autoimmune diseases such as RA, while only subgroups of pSS patients benefit from these treatments. Given the prominent B-cell hyperactivation in pSS, strategies have largely been focused

on inhibition of B-cell activity. Multiple strategies that target these cells directly (anti-CD-20/Rituximab; anti-BAFF/Belimumab) or indirectly via inhibition of T-cell activity (CTLA-4-Ig/Abatacept) hold some promise, especially for treatment of systemic manifestations [38-40]. Though these studies are clearly worthwhile, focusing on cell types crucial for initiation and perpetuation of lymphocyte activation may yield new treatment options for pSS patients in the future. The miRNA data presented in this thesis are the first evidence of dysregulation in pSS cDC1s and shed further light on the processes that are different in pSS pDCs, as well as indicate the pathways that are dysregulated in these cells. Many of the pathways have not been previously described to be involved or dysregulated in pSS, allowing for new insight in the function of these cells in disease.

Interestingly, the lack of differences between pSS and nSS patients suggests that these patient groups overlap in peripheral DC dysregulation, at least at the miRNA level. As nSS and pSS patients are clearly different with regards to local immune activation, this suggests a disconnect between the local immune response and systemic activity of DCs. Local immune activity may require additional triggering at the site of inflammation or possibly in lymphoid organs. Alternatively, initial triggering of the DCs may occur during differentiation in the bone marrow due to hereditary defects or imprinting in this compartment as a reaction to for example viral infection [41,42].

It is possible that the group of nSS patients is comprised of patients with pre-clinical SS as there is some evidence that autoantibody presence precedes pSS symptom onset by several years [43], though there is little evidence for progression of nSS patients towards pSS. However, as the group of nSS patients is very heterogeneous, it is difficult to interpret the miRNA data. As we have collected the material studied in chapters 6 and 7 during standard patient care, we did not select for subsets of patients. Hence, the group of nSS patients under study has a relatively high prevalence of signs of autoimmunity including autoantibodies and presence of small numbers of lymphocytic aggregates. In addition, the pSS patients included in this research have relatively low markers of systemic inflammation compared to those studied in Chapter 4 or previous research from our group [44]. However, as no correlations of miRNA expression with clinical parameters were observed it is unclear what the influence of this is. For follow-up research, we will include a group of patients with only oral and/or ocular symptoms for comparison, as these patients may represent a distinct subset of patients. Furthermore, additional measures of dryness will be assessed,

in particular objective measures of oral dryness including unstimulated whole saliva.

Because we look at the peripheral DC populations as a whole, a range of factors might influence the differences we find between groups. These include hereditary defects, activation/maturation status, DC migration into tissue and lymphoid organs and possibly recirculation of cells from the inflamed tissue [36,45-47]. As there is very little research into DCs in pSS, the roles of many of these processes are unknown. To ensure proper data interpretation, current and future research of our group aims to elucidate the role of these processes. We are currently investigating DC numbers in the blood of the three groups under study in relation to expression of miRNAs. Furthermore, we will investigate the dynamics of DC migration and potential recirculation in an animal model for Sjögren's syndrome.

Though only one layer of data is described in this thesis in the form of miRNA expression, we are currently pursuing full characterization of these cells on multiple levels for both pSS and nSS patients. Integration of miRNA expression data with RNA sequencing data of the DCs and disease parameters will provide a complete picture of pathways and processes involved in disease. Initial analysis of RNAseq data reveals that various pathways enriched in the miRNA predicted targets analysis are also dysregulated on the mRNA level. In addition, we have observed inverse correlations of a range of miRNAs with predicted targets indicating that these targets are indeed regulated by the miRNAs. Ultimately we hope to identify the main pathways to target in the patient population, and, if possible, identify which type of treatment fits which individual patient. These efforts require taking a step back and focusing on unbiased analyses of data. Though this approach requires major investments, the potential to discover previously unrecognized processes and pathways involved in disease can be a game changer for pSS research and, more importantly, patients. In addition, therapeutic options that target DCs or administer tolerogenic-DCs have gained a lot of interest in several other autoimmune diseases [48-50] and may be options in pSS as wel

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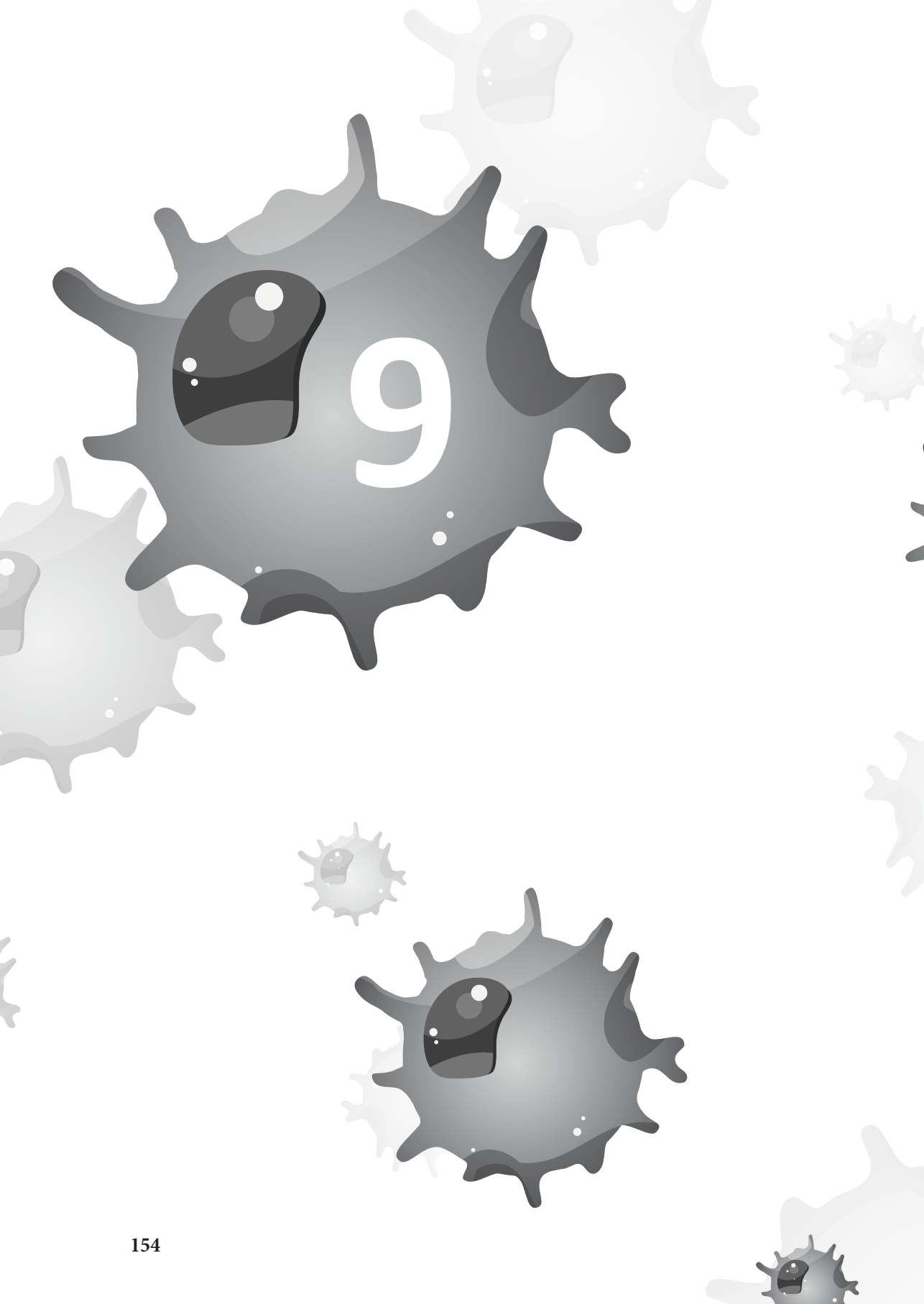
9

Appendix

Nederlandstalige samenvatting

Het afweersysteem heeft de taak het lichaam te beschermen tegen indringers. Het is daarom ook bekend als het immuunsysteem, afgeleid van het Latijnse woord *immunitas*, wat vrij zijn van betekent. Bacteriën, schimmels en virussen zouden zonder ingrijpen van het afweersysteem ongeremd kunnen delen en schade toebrengen aan het lichaam. Het afweersysteem bestaat voornamelijk uit witte bloedcellen, of immuuncellen, die in een groot aantal verschillende celtypen zijn in te delen. Elk van deze cellen heeft een specifieke taak en de verschillende celtypen moeten goed samenwerken om het lichaam te kunnen beschermen. Voor optimale samenwerking moeten de immuuncellen communiceren, hiervoor gebruiken ze een scala aan verschillende ontstekingseiwitten, die we cytokines noemen. Wanneer ergens in het lichaam een ziekteverwekker wordt gesignaleerd zullen de immuuncellen ter plaatse cytokines maken om andere cellen aan te trekken en te activeren. De grote hoeveelheid geactiveerde immuuncellen zullen de ziekteverwekker opruimen. Zo'n reactie van het afweersysteem, of immuunrespons, vormt de basis van een ontsteking.

Dendritische cellen

Een van de typen witte bloedcellen is de dendritische cel (DC). Deze cellen zijn zeer zeldzaam, minder dan een procent van de immuuncellen in het bloed bestaat uit DCs. Ze zijn echter wel zeer potent. DCs zijn aanwezig in bijna alle weefsels van het lichaam en houden hier het milieu scherp in de gaten, op zoek naar mogelijke ziekteverwekkers of andere lichaamsvreemde moleculen. Wanneer ze iets verdachts tegenkomen nemen ze het in zich op en maken er kleine stukjes van, hierna reizen ze naar de lymfeknoop waar een breed scala aan verschillende typen immuuncellen aanwezig is. De DCs brengen de kleine stukjes van het opgenomen molecuul, nu antigeen genoemd, naar buiten en binden ze op hun buitenkant zodat andere immuuncellen het kunnen binden. Dit proces heet antigeen presentatie. Als het antigeen door andere cellen van het immuunsysteem ook als lichaamsvreemd wordt gezien ontstaat er in de lymfeknoop activatie van een deel van de witte bloedcellen. Deze zullen naar de plaats reizen waar het antigeen door de DC is opgenomen en een ontsteking teweeg brengen. DCs zijn de beste antigeen presenterende cellen in het lichaam en ze zijn vooral heel goed in het activeren van T-cellen. Deze T-cellen, genoemd naar de thymus (zwezerik) waar ze uitrijpen, vormen een belangrijk onderdeel van het afweersysteem. Als ze zijn geactiveerd kunnen ze heel efficiënt vermenidgvuldigen en heel erg gericht reageren op het stukje antigeen dat aan

ze is gepresenteerd, daarnaast activeren ze op hun beurt weer andere witte bloedcellen door productie van cytokines. Antigeen presentatie door de DC is dus het begin van een kettingreactie die leidt tot een sterk leger immuuncellen dat de bedreiging opruimt.

Auto-immuunziekten

Het afweersysteem is enorm sterk, de wereld om ons heen zit vol ziekteverwekkers maar toch worden we haast nooit ziek. Deze kracht kan echter ook gevaarlijk zijn. Geactiveerde immuuncellen maken allerlei stoffen die niet alleen schadelijk zijn voor ziekteverwekkers, maar ook voor het eigen lichaamsweefsel. Daarom zijn er een aantal processen die het immuunsysteem moeten afremmen in werking. Dit is enerzijds om te voorkomen dat voor een enkele bacterie een enorme immuunrespons optreedt, wat schade aan het weefsel kan geven. Anderzijds is dit belangrijk tegen het ontstaan van een immuunrespons tegen lichaamseigen cellen. Een ontsteking ontstaat in de gezonde situatie alleen wanneer er indringers of lichaamsvreemde moleculen worden gesignaleerd. Er zijn een groot aantal processen die ervoor zorgen dat er geen ontsteking ontstaat tegen lichaamseigen cellen, deze processen noemen we zelf-tolerantie. Soms gaat er echter iets verkeerd in de herkenning van lichaamseigen cellen en wordt de zelf-tolerantie doorbroken, dit is wanneer auto-immuunziekten ontstaan. Bij patiënten met auto-immuunziekten worden lichaamseigen cellen door het afweersysteem gezien als lichaamsvreemd en ontstaat een ontsteking. Dit betekent dus dat een aantal van de processen die een afweerreactie tegen lichaamseigen cellen moeten voorkomen niet goed werken. Dit is onder andere het geval bij de reumatische ziekten reumatoïde artritis en het primair syndroom van Sjögren. In dit proefschrift is onderzocht hoe DCs een rol spelen in deze ziektebeelden.

De rol van DCs en TSLP in reumatoïde artritis

Reumatoïde artritis (RA) is één van de meest voorkomende auto-immuunziekten in de wereld en komt voor in ongeveer een procent van de bevolking. Bij patiënten met RA wordt een onderdeel van het gewricht door het afweersysteem als lichaamsvreemd gezien en ontstaat een ontsteking, wat zorgt voor pijnlijke, gezwollen gewrichten en stijfheid. De ontsteking in gewrichten van RA patiënten is chronisch en blijft dus gedurende lange tijd aanwezig. Door de langdurige pijn en vermoeidheid is het voor RA patiënten moeilijk om deel te nemen aan dagelijkse activiteiten als werken en sporten. T-cellen spelen een belangrijke rol in dit proces omdat ze bepaalde eiwitten maken die betrokken zijn bij weefselschade en omdat ze verantwoordelijk zijn voor het activeren van andere

immuuncellen die op hun beurt ook bij de immuunrespons betrokken zijn. We weten bijvoorbeeld dat T-cellen de cytokines tumornecrosefactor-alfa (TNF-α), interferon-gamma (IFN-γ) en interleukine-17 (IL-17) kunnen maken, die op verschillende manieren bijdragen aan het ziekteproces.

Voorafgaand onderzoek

Ondanks het feit dat DCs zo goed zijn in het activeren van deze T-cellen is er over de rol van DCs in RA nog niet veel bekend. Eerder onderzoek van onze onderzoeksgroep heeft laten zien dat er een sterk toegenomen aantal DCs voorkomt in de gewrichtsvloeistof, of synoviaal vocht (SF), van RA patiënten. Deze vloeistof ontstaat in het gewricht tijdens de ontsteking en bevat een groot aantal immuuncellen en ontstekingsstoffen, waaronder veel cytokines. Daarnaast hebben we laten zien dat deze DCs goed in staat zijn om T-cellen te activeren en te zorgen dat ze onder andere de hierboven genoemde cytokines IFN-γ, TNF-α en IL-17 gaan maken. Ook hebben we onderzoek gedaan naar de rol van het cytokine thymic stromal lymphopoietin (TSLP). TSLP is een cytokine dat heel erg goed is in het activeren van DCs. In het SF van RA patiënten zien we een grote aanwezigheid van dit cytokine. Als je in een kweekplaat (aangeduid met het Latijnse in vitro, oftewel in glas) TSLP bij DCs voegt, zie je dat de DCs extra eiwitten gaan maken die betrokken zijn bij de antigeen presentatie. Als je deze aan TSLP blootgestelde DCs vervolgens (nog steeds *in vitro*) bij T-cellen brengt gaan de T-cellen heel erg snel en vaak delen en grote hoeveelheden IFN-γ, TNF-α en IL-17 produceren.

Verder heeft onze groep onderzoek gedaan in muizen, waarbij geprobeerd wordt om RA na te bootsen in een levend dier (aangeduid met het Latijnse *in vivo*, oftewel in het leven). Muizen worden extra ziek als je ze injecteert met TSLP. Verder hebben we gezien dat als je het genetisch materiaal van een muis zo aanpast dat ze niet meer op TSLP kunnen reageren (dit noemen we een TSLPR-/- muis), de muizen juist minder ziek worden van de nagebootste RA.

Dit proefschrift

Hoofdstuk 2 beschrijft onderzoek naar de rol van TSLP in combinatie met het cytokine IL-7. Dit cytokine lijkt op TSLP, maar geeft een directe activatie aan de T-cellen in plaats van aan de DCs zoals TSLP doet. TSLP en IL-7 beïnvloeden dus de DC/T-cel interactie van twee verschillende kanten. Daarnaast gebruiken TSLP en IL-7 hetzelfde eiwit om hun signaal af te geven aan cellen. Dit betekent dat als je dit ene eiwit kunt aanpakken, je de functie van beide cytokines kunt blokkeren. Om te onderzoeken of dit een goed aangrijpingspunt is hebben we muizen

gebruikt waarin we RA hebben nagebootst. Daarnaast hebben we deze muizen aangepast zodat ze niet op TSLP kunnen reageren (TSLPR-/- muizen). Vervolgens hebben we de muizen geïnjecteerd met een stof (anti-IL-7Rα) die ervoor zorgt dat ze ook niet op IL-7 kunnen reageren. Wat we zagen is dat de muizen die niet op TSLP én IL-7 kunnen reageren duidelijk minder ziek worden dan de muizen die niet zijn aangepast of behandeld (WT muizen). Ook worden de muizen die niet op TSLP én IL-7 kunnen reageren minder ziek dan de muizen die alleen niet op TSLP óf IL-7 kunnen reageren. Het lijkt dus extra zin te hebben om beide cytokines aan te pakken ten opzichte van maar één van de twee. Verder zien we dat het voorkomen van reageren op TSLP en IL-7 ook zorgt voor minder productie van vooral IL-17, één van de belangrijke cytokines in RA.

Als laatste hebben we ook in een kweekplaat (*in vitro*) gekeken naar de effecten van TSLP en IL-7 samen. We hebben DCs en T-cellen uit het bloed gehaald en gekeken wat er gebeurt als je TSLP, IL-7 of allebei toevoegt. We zien dat zowel TSLP als IL-7 veel deling van T-cellen geeft. Daarnaast zorgen beide cytokines voor productie van cytokines. Maar, zowel T-cel deling als productie van cytokines is nog hoger als je zowel TSLP als IL-7 toevoegt. Hierbij is, net als in de muizenproef, vooral IL-17 sterk beïnvloed door beide cytokines. Het lijkt er dus op dat het aanpakken van zowel TSLP als IL-7 een mogelijk aangrijpingspunt is voor therapie in RA. Omdat beide cytokines ook in andere reumatische aandoeningen een rol spelen zou dit ook perspectief kunnen bieden voor andere ziekten.

In Hoofdstuk 3 hebben we gekeken naar een eiwit dat heel veel wordt gemaakt door DCs wanneer ze aan TSLP worden blootgesteld. Thymus and activation regulated chemokine (TARC) zorgt voor aantrekking van immuuncellen, waaronder T-cellen, naar de plaats waar het geproduceerd wordt. TARC is een chemokine, wat betekent dat het een cytokine is dat voor migratie van immuuncellen zorgt naar de plaats van ontsteking.

We hebben hier laten zien dat grote hoeveelheden TARC aanwezig zijn in het gewrichtsvloeistof (SF) van RA patiënten. TARC lijkt te zijn betrokken bij het aantrekken van immuuncellen naar het gewricht, wat zorgt voor extra ontsteking. We zagen dat de immuuncellen in het gewricht verantwoordelijk zijn voor de TARC productie, dit leek voornamelijk van de DCs afkomstig. Vervolgens hebben we een deel van de immuuncellen (MCs) uit het SF gehaald. Als je uit deze MCs de DCs verwijdert zie je dat bijna alle TARC productie verdwijnt. De DCs zijn dus essentieel voor de TARC productie. Ook onderzochten we het type T-cel dat TARC kan aantrekken. Hiervoor namen we DCs die we stimuleerden

met TSLP om TARC te gaan maken. Deze cellen deden we onderin een plastic kweekplaat, daarboven zetten we een klein gaasje met gaatjes erin. Bovenop dit gaasje deden we T-cellen. De gaatjes in het gaasje zijn net te klein voor de T-cellen om doorheen te gaan, maar als ze worden geactiveerd door een chemokine zoals TARC zullen ze zich er toch doorheen kunnen wurmen. We gaven de T-cellen de tijd om door het gaasje te bewegen en daarna vingen we de T-cellen op en keken we welke cytokines ze konden maken. TARC bleek een van de meest belangrijke eiwitten te zijn voor de aantrekking van T-cellen door DCs die aan TSLP zijn blootgesteld. Verder zagen we dat de door TARC aangetrokken T-cellen vooral TNF-α en in mindere mate IFN-γ en IL-17, konden maken, alle drie belangrijke cytokines die ontsteking bevorderen in RA.

Dus, TARC is aanwezig in het RA gewricht, lijkt hier vooral door DCs te worden geproduceerd en kan T-cellen aantrekken, welke op hun beurt cytokines kunnen uitscheiden die een rol spelen bij de schade in het gewricht. Omdat TARC zo sterk wordt gemaakt onder invloed van TSLP geeft deze informatie ook meer inzicht over de rol van TSLP in het RA gewricht.

De rol van DCs en TSLP in het primair syndroom van Sjögren

Het primair syndroom van Sjögren (pSS) is na RA de tweede meest voorkomende reumatische ziekte in Nederland. In plaats van de gewrichten worden in pSS onderdelen van de speekselklieren en traanklieren door het afweersysteem als lichaamsvreemd gezien. Hierdoor ontstaat er een ontsteking in deze klieren, wat er voor kan zorgen dat pSS patiënten last hebben van een droge mond en droge ogen. Daarnaast zijn veel pSS patiënten heel erg moe, wat een grote impact heeft op het dagelijks leven. We weten dat een aantal processen die in RA belangrijk zijn ook een rol spelen bij het ontstaan van ontsteking in de klieren van pSS patiënten. Zo spelen T-cellen die naar de klieren bewegen een belangrijke rol in het ontstaan van schade.

Voorafgaand onderzoek

Ook in pSS is nog maar weinig onderzoek gedaan naar de rol van DCs. In Hoofdstuk 5 is een samenvatting gegeven van wat er op dit moment bekend is over de rol van DCs in pSS. Er is vooral veel onderzoek gedaan naar DCs in bloed en we weten maar heel erg weinig over de DCs die in de klieren zitten op de plaats van de ontsteking. Dit komt vooral omdat het heel erg moeilijk is om deze cellen te onderzoeken. De gewrichtsvloeistof in RA patiënten is een mooie

bron voor het onderzoeken van de plaats van ontsteking. Omdat de ontsteking in pSS patiënten in de klieren zit is hier heel erg moeilijk bij te komen en de klieren zijn erg klein, waardoor het onderzoeken van zo'n weinig voorkomend celtype als DCs nog moeilijker is. Uit onderzoek dat eerder gedaan is blijkt dat de DCs samen met de andere immuuncellen naar de klieren van pSS patiënten toe gaan. In bloed zien we dat er een vermindering is van het aantal DCs in vergelijking met gezonde individuen, wat erop lijkt te duiden dat de DCs het bloed verlaten en migreren naar de plek van ontsteking, de speeksel en traanklieren.

Dit proefschrift

In Hoofdstuk 4 hebben we onderzocht of er ook TSLP aanwezig is in de speekselklieren van pSS patiënten en of we verschillen zien met speekselklieren van mensen die geen auto-immuunziekte hebben maar wel last hebben van droogte in mond en ogen (nSS patiënten). Wat we hier zien is heel erg anders dan wat we in RA patiënten zien. In pSS patiënten is juist minder TSLP aanwezig in de speekselklieren dan in de nSS patiënten en we zien ook dat de patiënten die minder TSLP hebben ook minder ziek lijken te zijn. Dit is dus volledig anders dan in RA, waar patiënten juist méér TSLP op de plaats van ontsteking hebben en TSLP lijkt bij te dragen aan de ontsteking.

Er zijn een tweetal mogelijke verklaringen voor deze uitkomst, maar er zal verder onderzoek gedaan moeten worden om zeker te weten wat er precies aan de hand is. De eerste verklaring is dat TSLP een ander soort rol speelt in pSS dan het doet in RA. Dit heeft te maken met de speciale eigenschappen van het weefsel dat deel uitmaakt van ons spijsverteringsstelsel. Deze weefsels komen constant dingen van buiten het lichaam tegen, waaronder de dingen die je eet en de goede bacteriën die je bij je draagt in je lichaam. Het is belangrijk dat tegen deze niet gevaarlijke bacteriën en onderdelen van etenswaren geen ontsteking wordt gericht, omdat anders het verteringsproces niet goed kan werken en je zelfs schade zou kunnen krijgen aan je darmen. In de darmen is bekend dat TSLP kan bijdragen aan het voorkomen van dit soort ontsteking en wellicht speelt het ook zo'n rol in de speekselklieren. Een andere mogelijkheid is dat TSLP hier in een andere vorm voorkomt, waarin het kan werken als een antibacterieel eiwit zoals onlangs is aangetoond. De vermindering van TSLP in patiënten zou dan kunnen bijdragen aan de extra problemen die zij ondervinden met tandbederf.
In Hoofdstukken 6 en 7 hebben we gekeken naar de verschillen in twee subtypen van DCs tussen pSS patiënten en gezonde individuen. We hebben deze DCs uit het bloed gehaald en met de nieuwste technieken gekeken of we verschillen in de cel konden vinden. We hebben ons hierbij gericht op verschillen in micro RNAs

(miRNA), die betrokken zijn bij het controleren van het maken van eiwitten in de cel.

Alle eiwitten in een cel worden gemaakt aan de hand van een specifieke code die zich op het DNA bevindt. Wanneer een cel een eiwit wil maken, zal het op het DNA zoeken naar de code voor dit eiwit en met een complex proces zorgen dat deze code wordt overgeschreven in RNA. Dit RNA is een soort overtrekvel waarop de code die op het DNA staat wordt gekopieerd. Bijna alle RNA in een cel wordt gebruikt om eiwitten te maken, de overgeschreven code bevat de blauwdruk van het te maken eiwit. Het RNA wordt daarvoor naar een specifiek mechanisme in de cel gebracht en daar wordt het eiwit in elkaar gezet. Hoe meer RNA er van het DNA wordt overgetrokken hoe meer eiwit er gemaakt kan worden. De hoeveelheid van een eiwit die gemaakt wordt is echter een heel erg precies werkje en is essentieel voor het gezond blijven van een cel. Daarom zijn er verschillende mechanismen om te regelen dat precies de goede hoeveelheid eiwit wordt gemaakt, miRNA zijn er hier één van. miRNA zijn kleine stukjes RNA (vandaar de naam) die niet een blauwdruk voor een eiwit vormen maar zorgen dat bepaalde blauwdruk RNAs worden afgebroken. Elk miRNA kan een aantal specifieke blauwdruk RNAs herkennen en afbreken, waardoor ze niet in eiwit kunnen worden omgezet. Hoe meer er van een miRNA aanwezig is in een cel, hoe meer er van de blauwdruk RNAs worden afgebroken en hoe minder eiwit er dus gemaakt kan worden. Dit betekent dat de hoeveelheid van een bepaald miRNA in een cel iets zegt over hoe sterk het maken van een groep eiwitten aan banden wordt gelegd.

Tijdens het bestuderen van de aanwezigheid van deze miRNA in DCs van pSS patiënten zagen we verschillen met gezonde individuen. Een aantal miRNAs leken minder voor te komen in de DCs van de patiënten. Door te kijken welke blauwdruk RNAs kunnen worden afgebroken door de miRNAs waar we verschillen in vonden, vinden we eiwitten waarvan de aanwezigheid wel eens anders zou kunnen zijn in patiënten. Dit kan dan weer duiden op processen in de cel die in patiënten anders zijn dan in gezonde individuen en wellicht betrokken kunnen zijn bij het ziekteproces. Met deze methode kunnen we nog volledig onbekende processen die een rol spelen bij het ziekteproces ontdekken. Wel is het lastig om op basis van alleen miRNAs iets te zeggen over wat er in de cel gebeurt. Deze gegevens gebruiken we vooral als aanknopingspunt voor volgend onderzoek. Zo zijn we op dit moment aan het kijken naar de aanwezigheid van de blauwdruk RNAs in deze cellen. Daarnaast gaan we kijken wat er gebeurt wanneer je één van deze miRNAs uitschakelt in gezonde cellen.

Conclusie

Het onderzoek in deze thesis geeft een aantal aangrijpingspunten voor verder onderzoek. We laten zien dat het eiwit dat zowel TSLP als IL-7 gebruikt om op cellen te werken mogelijk interessant is om medicijnen op te richten. Verder beschrijven we dat TARC kan bijdragen aan ontsteking in RA, wat meer duidelijkheid geeft over de rol van TSLP en DCs in RA. In het tweede deel van de thesis hebben we de fundering gelegd voor zeer gedetailleerd onderzoek naar DCs in pSS. We hebben het eerste bewijs gevonden dat een bepaald subtype DCs anders is in patiënten dan in gezonde individuen. In een ander subtype DCs hebben we nieuwe veranderingen vastgesteld, die meer duidelijkheid kunnen geven over hoe pSS precies werkt. Door deze cellen verder te onderzoeken kunnen we in de toekomst nieuwe inzichten over de werking van de ziekte krijgen en misschien zelfs nieuwe medicijnen ontwikkelen.

Verklarende woordenlijst en gebruikte afkortingen

Term	Betekenis
RA (reumatoïde artritis)	Auto-immuunziekte in gewrichten
pSS (primair syndroom van Sjögren)	Auto-immuunziekte in (speeksel)klieren
DC (dendritische cel)	Zeldzame cel die T-cellen sterk activeert
Immuuncel	Witte bloedcel, cel uit het afweersysteem
T-cel	Cel die betrokken is bij schade in RA en pSS
Cytokine	Ontstekingseiwit
Antigeen	Door DCs opgenomen en afgebroken molecuul, wordt gepresenteerd aan andere immuuncellen
TNF-α	Een ontstekingseiwit dat wordt gemaakt in RA en pSS
IFN-γ	Een ontstekingseiwit dat wordt gemaakt in RA en pSS
IL	Een familie van ontstekingseiwitten, elk eiwit heeft een eigen nummer
TSLP	Een ontstekingseiwit dat DCs activeert
TSLPR-/-	Muis die niet op TSLP kan reageren
WT	Normale, niet aangepaste muis
Anti-IL-7Rα	Stof waardoor muizen niet op IL-7 kunnen reageren
TARC	Eiwit dat immuuncellen aantrekt naar ontsteking
SF	Gewrichtsvloeistof
MC	Deel van de immuuncellen dat oa. de DCs en T-cellen bevat
DNA	Genetisch materiaal, bevat de blauwdruk voor eiwitten
RNA	Overgetrokken DNA, bevat meestal de blauwdruk voor een eiwit. Nodig om elk eiwit te kunnen maken.
miRNA (micro RNA)	RNA molecuul dat geen blauwdruk voor een eiwit bevat. Breekt RNA af en reguleert zo eiwit aanmaak

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Beste Aike, als expert op het gebied van pSS ben jij van enorme waarde voor mijn onderzoek geweest. Niet alleen was je hofleverancier van patiënten samples, je hebt ook veel tijd geïnvesteerd in het bijbrengen van de subtiele nuances van de classificatie van Sjögren patiënten aan deze biomedisch wetenschapper. Ik bewonder de manier waarop jij altijd correct, weloverwogen en genuanceerd kunt spreken. Dit staat soms in schril contrast met mijn wat meer bruuske benadering, toch begrepen we elkaar uiteindelijk altijd tijdens een discussie of overleg. Dank voor al je input en inspanningen!

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Alle medewerkers van het researchlab van de reumatologie, bedankt voor alle input, interesse en gezelligheid. Ook al hoorde ik maar half bij de groep, ik was altijd volledig welkom. Kim, dank je wel voor alle tijd die je stak in het uitvoeren en uitleggen van allerhande experimenten en analyses. We hebben samen talloze experimenten ingezet en isolaties gedaan. Je was er altijd voor raad en daad en we hebben ons nooit verveeld tijdens de lange uren. Luid vertellend over muziek, je man en je kinderen vloog de tijd om (en de samples soms ook). Arno, jij stond altijd klaar om dingen over te nemen of te regelen en me tot blauwe plekken toe op mijn arm te stompen. Je bent ongelooflijk precies en flexibel en dat zijn hele goede kwaliteiten in een collega. Daarnaast spande je ook altijd zeer enthousiast samen met Astrid om mij liefst dagelijks naar de sportschool te krijgen, waar ik je zeer ondankbaar voor ben. Marion, ik heb veel van je geleerd over immuunhistochemie en over het stimuleren van zelf nadenken (wat denk je zelf?). Ook gaf je een volstrekt nieuwe betekenis aan een bekend merk voor verzorgingsproducten, wat zowel hilarisch als nuttig was. Dank je wel! Katja, de lab Brabo, ik kon je meestal niet verstaan maar ik denk dat je best wel vriendelijk bent. Je was altijd bereid even in te springen om bloed op te werken en andere dingen te regelen. Daarnaast werd ik zeer geïnspireerd

door jouw bureaubladachtergronden en facebookposts, iets met brandingen en wolkenluchten enzo. Best wel diep.

Alle (oud) AIOs en OIOs bij de reumatologie, bedankt voor de discussies, voor de AIO etentjes en de congresreisjes, voor de lol die we hebben gehad tijdens het maken van filmpjes/dansjes/liedjes voor promoties, voor het meezingen met de Backstreetboys, voor de labuitjes en de koffiemomentjes. Fréderique, Freakie, wat hebben we gelachen om facial tissues, jouw beperkte vocabulair aan eufemismen, skateboarden door het lab en flauwe grappen. Daarnaast heb ik wetenschappelijk ook veel aan je gehad, jouw data waren de basis voor één van mijn hoofdstukken en je was altijd bereid wat voor me na te zoeken of op te sturen uit je candystore. Bedankt! Angela, jouw schoenen waren niet de minsten om op te vullen (alleen al qua hoogte van de hak), maar dat lag zeker niet aan jou. Je hebt enorm veel werk gestopt in het verzamelen van patiëntmateriaal en het organiseren van de afname hiervan, waar de hele afdeling en ikzelf de vruchten van hebben kunnen plukken, dank je wel daarvoor. Anke, bedankt voor het helpen opzetten van bloedafname bij bioptpatiënten, voor het aanleveren van bloed en voor je hulp bij het napluizen van ziektescores en diagnoses. Laurens, Monique en Karen (eeh, ik bedoel mevrouw de patroon Wiegant), bedankt voor de gastvrijheid in de oude AIO kamer op D. Maud, dank je voor het gids spelen in Berlijn en Madrid en het meestrijden voor het niet eten in een vreetschuur. Sandhya, you're so incredibly nice it was a joy to be around you. Bart, bedankt voor je vrolijkheid en onze gedeelde liefde voor TARC. Lize, altijd goedlachs en vrolijk, ik ben trots dat je eindelijk wat eigen data hebt gevonden om te presenteren. Maartje, een beetje vreemd maar wel gezellig, bedankt voor je "mopjes". Nick, Huub en Thijmen, bedankt voor de échte gezette koffie en jullie nietsontziende humor.

Graag wil ik alle artsen bedanken voor het aanleveren van patiëntmateriaal en het meedenken tijdens researchbesprekingen. Researchverpleegkundigen, bedankt voor alle tijd en moeite die jullie hebben gestoken in het verkrijgen van materiaal van zoveel mogelijk patiënten voor al het onderzoek. Marjolein, dank je voor het inplannen van alle bloedafnames en je flexibiliteit wanneer ik het een keer vergeten was. Ook wil ik graag alle patiënten die bloed hebben afgestaan voor onderzoek hartelijk bedanken.

Everyone in Tim's group, such a huge amount of people now! It is great to be part of such an international group that contains so many talented people. Thanks for all of the answered questions, the many kilos gained due to pie and

cookies twice a week, the dinners, drinks, jokes and dancing on pooltables. Kim, Lenny, Marta, Annelies, Mark, Jasper, Renoud and Alsya, when I joined the group halfway through my PhD you were not only welcoming, but also very stimulating. Thank you for making the transition effortless and enjoyable! Rina en Cornelis, dank jullie wel voor alle hulp bij het moleculaire biologie werk. Sanne, dank je voor het runnen van de vele Luminex platen. Jullie staan samen met de andere analisten altijd klaar voor iedereen en zonder jullie allen zou het niet half zo soepeltjes lopen op het lab!

Sofie, van onzekere student tot eigenwijze en leergierige AIO, je bent het bewijs dat artsen ondanks het stereotype hele goede onderzoekers kunnen zijn. We hebben samen talloze dagen en nachten cellies geïsoleerd, bollies geteld en youtube filmpjes bekeken (en gootstenen ontstopt). Dank je voor alle hulp en gezelschap! Eefje, je bent heel erg precies en intelligent, mooi om te zien hoe al je werk straks gaat worden omgezet in de daadwerkelijk trial. Dank je wel voor alle antwoorden op mijn domme vragen over lab waardes. Jouw temperatuurhuishouding en vochtbalans zijn misschien niet helemaal in orde (non-existent?), je bent een plezier om mee samen te werken. Marzia, you were essential for the performance of all the miRNA experiments and RNA sequencing analyses. Thanks for all of your help and time! You are extremely dedicated, precise and smart, a perfect recipe for a molecular biologist. In addition, we had enormous amounts of fun during meetings and labwork. Most of that was me making fun of you because of your Italian "English", but it was fun nonetheless. Please don't kill me in your sleep... Elena, you have put a lot of time and effort into optimizing protocols for the miRNA analyses to ensure smooth experiments, thanks for all your help! Of course, smoothness in science is never as hoped so we spent hours figuring out what we had to change to make things work. I don't think I have ever met someone who is as easy to hunt onto the closet as you, it was a joy to tease you about throwing plates on the floor, dancing the sirtaki and your somewhat alternative means of communication (ftoe ftoe ftoe!)

Current and previous roommates in the WKZ, thank you for the great atmosphere for both working and laughing. At first I was afraid that a room with 12 people would be impossibly loud and distracting, but it is actually a perfect environment for me. Most of the time it is quite silent and ideal for working, but when Ruben starts explaining about his addiction to Japanese tea (tisane?), Tessa says something smart about Thapsigargin or Maarten (the one with the cowboy boots) is venting about failed experiments again, there is some good fun to be had.

Everyone at the LTI, it is wonderful to be a part of such a large department that contains so many bright people. Thank you for all of the great scientific input during meetings, the collegial atmosphere in the lab and the friendliness during drinks and parties. Barbara, thanks for all of your help with the qPCR analyses and other TSLP work. Medewerkers van de luminex core facility, bedankt voor het runnen van alle analyses. Stefan en Karin, bedankt voor alle energie die jullie in de FACS analyses hebben gestopt, wanneer alles is doorgemeten hebben we echt een hele waardevolle dataset.

Voorschotengang, Paerlen aan de Vliet, ik vind het heel erg mooi dat we na zo'n lange tijd van vrienden zijn nog steeds onwijs veel lol hebben samen. Sommige dingen veranderen niets (de snuffelstages van Emmelotte, het agenda beheren van Bob), sommige dingen veranderen volledig (David weg bij Bol.com, Ron Gastrobar als nieuwe favoriete spot), toch hoop ik nog vele jaren van jullie humor en gezelligheid te mogen genieten.

Sequoia, mooie mannen, ik heb nog nooit in een groep gefunctioneerd waar zo bliksemsnel wordt geschakeld tussen banale mannendiscussies en het wereldnieuws. De lasten van het promoveren vielen zonder problemen van mijn brede schouders tijdens clubeten, borrels, weekenden en skivakanties (inclusief keer op keer nagenieten van de filmpjes). Na 10 jaar zien we elkaar nog steeds wekelijks, 15 zo volstrekt verschillende persoonlijkheden wordt nooit saai!

Huisgenoten, tijdens het begin van mijn wetenschappelijke leven woonden we nog in één huis, maar onze wegen en woonplaatsen scheidden zich snel daarna. De feestjes, drankspelletjes, stamppotten, filmavondjes (durka durka!), 24 en Off Center marathons, Mario party avonden en Dota potten zullen me nog lang heugen. Mooi dat we nog veel contact hebben, wel jammer van die rugpijn. Misschien moeten jullie je eigen gewicht gaan leren dragen, stelletje feeders.

Decapentaplegic, na TSLP toch een goede tweede plaats op mijn lijstje van favoriete eiwitten. Onze studieuren in de medische bieb waren misschien niet de meest productieve, het was wel ongelooflijk gezellig. De heerlijke biomedisch-geëngageerde grappen, that's-what-she-saids en scherpe woordspelingen worden wat mij betreft nooit oud. We zijn misschien wat gescattered over de wereldbol op dit moment, elk bezoek aan Nederland met bijbehorend diner is top! Ook mooi om te zien dat we toch best wel goed terecht zijn gekomen, na Balthasar en Han promoveer ik als derde binnen een half jaar, niet verkeerd. Jasper is vet laat zeg maar.

Mijn paranimfen, Sarita en Sander, dank dat jullie me willen bijstaan tijdens de verdediging. Sarita, je was erbij toen ik op het ontstekingslab begon en toen ik naar het WKZ verhuisde kwam je me achterna. Ik heb van je geleerd, met je gepipetteerd en met je gepubliceerd. Maar ik heb vooral heel erg veel met je gelachen. Je bent een van de meest goedlachse personen die ik ken en nooit gepikeerd als je zelf de (massive) butt van the joke bent. Ik weet zeker dat ik niet de wong fook hing paranimf heb gekozen! Sander, we kennen elkaar al heel erg lang, het lijkt pas een paar jaar geleden dat ik wekelijks het tapijt van je ouders verdronk in omgegooide biertjes. Jouw onwijze droge humor is altijd een genot en jouw enorme expertise op het gebied van next-gen sequencing en crowdfunding zijn een ware aanwinst!

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Curriculum vitae

Maarten Reinier Hillen was born on October 27th 1986 in Leiderdorp, the Netherlands. In 2005, he graduated from the Stedelijk Gymnasium in Leiden (VWO Gymnasium) and started his undergraduate studies Biomedical Sciences at Utrecht University. In 2008, he attained his Bachelor's degree and enrolled in the research master Infection & Immunity at the same university. He performed his major research project in the lab of Dr. Kiki Tesselaar at the department of Immunology at the UMC Utrecht on the role of CD27-CD70 interactions in humans and mice. He graduated in 2010 and started his PhD training under supervision of Dr. Joel van Roon, Prof. Erik Hack and Prof. Floris Lafeber in Utrecht, the results of which are presented in this thesis. Maarten is currently employed as a postdoctoral researcher in the group of Prof. Timothy Radstake at the UMC Utrecht, investigating dysregulation in dendritic cells of patients with primary Sjögren's syndrome. To further study this disease, at the end of 2015 he will start to work in the lab of Dr. Francesca Barone in Birmingham, United Kingdom.

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