Monocytes and Dendritic Cells in Sjögren's Syndrome

Monocyten en dendritische cellen in het syndroom van Sjögren

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Voor mijn oma's

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CONTENTS

1

GENERAL INTRODUCTION

1.1 THE IMMUNE SYSTEM

1.1.1 Innate and adaptive immunity

Humans are continuously exposed to external dangers such as bacteria, viruses, and parasites. Given the fact that the human population worldwide is outnumbered by bacteria alone by an estimated 830 million trillion fold (1), it is not hard to envisage that a good defense mechanism is an absolute necessity. This mechanism is provided by the immune system.

The immune system is divided in two main branches (2, 3), called innate and adaptive immunity. The basic difference between the two lies in the specificity and memory of the response. The adaptive immune system responds in a highly specific fashion to a particular antigen and has a memory function, while the innate immune system responds more broadly to a large variety of stimuli and does not develop memory. In most cases, both branches are used and interact with each other to create an effective immune response.

The adaptive immune response mainly consists of T- and B-cells, each specific for a particular antigen, resulting in a large array of specificities (4). In non-exposed individuals these cells are in a naïve state, meaning they do not respond to the antigen. In order to become active, T- and B-cells have to be stimulated by other immune components, such as dendritic cells (5). After activation, part of the T- and B-cell compartment develop into memory cells. Unlike naïve cells, these cells are capable of responding to antigenic stimulation without the need for dendritic cell stimulation. This ensures a faster and more effective response to a subsequent exposure to the same antigen.

The innate immune system contains a number of different cell types (6), such as granulocytes, natural killer (NK) cells, macrophages and dendritic cells (DC). Furthermore, several families of proteins, including complement and defensins are part of this system. This thesis will focus on the role of DC in the pathogenesis of Sjögren's Syndrome.

1.1.2 Macrophages

Macrophages are for a large part derived from monocytes circulating in the peripheral blood. In the early phases of inflammation, monocytes are recruited to the tissue by chemoattractants such as CCL2 and C5a (7, 8). Once *in situ*, monocytes develop into macrophages with a high phagocytic capacity (9). This phagocytosis can be random, but can also be mediated by a number of pattern recognition receptors, including Toll-like receptors (TLR), mannose receptors and scavenger receptors (10-12). The activation of macrophages is influenced by the cytokines present in the local environment. In the case of 'type I' cytokines such as IFN-γ and TNF-α, classically activated macrophages are generated (13, 14). Classical macrophages take up pathogens in endosomal vesicles, which then fuse with lysozomes containing strongly proteolytic enzymes, antimicrobial peptides and reactive oxygen species (15, 16). This finally results in degradation of the pathogen. Pathogen-associated proteins are then processed for presentation on the cell

membrane. As classical macrophages also express chemotactic factors for T-cells, such as CCL3, -4 and -5 (17, 18) as well as co-stimulatory molecules CD80 and CD86 and the Th1 stimulatory cytokine IL-12 (19), antigen presentation will lead to the recruitment and activation of T-cells, and the generation of an adaptive immune response. The immune response is further augmented by the secretion of inflammatory mediators such as TNF-α, IL-1β and IL-6 (13, 20), inducing both increased activation of other local macrophages, and systemic effects such as fever and increased vascular permeability.

In the absence of 'type I' cytokines, but in the presence of 'type II' cytokines such as IL-4, IL-10, IL-13 and TGF-β or glucosteroids, alternatively activated macrophages are generated (19). These play a role in the resolution of inflammation by producing anti-inflammatory cytokines including TGF-β, IL-10 and IL-1R antagonist (13). Furthermore, alternative macrophages inhibit Th1 responses by skewing the immune system towards a Th2 type of response (19). Finally, alternative macrophages stimulate angiogenesis and tissue remodeling, thus counteracting inflammation induced tissue damage (21).

1.1.3 Dendritic cells

Dendritic cell subsets and origin

DC are the major type of antigen presenting cells (APC) and contain two major subsets, conventional dendritic cells and plasmacytoid dendritic cells (22). Conventional dendritic cells (cDC) comprise a heterogeneous group of APC characterized by expression of molecules such as DC-SIGN, CD1a, BDCA-1 and BDCA-3 (human) or CD11c (mouse) and can be found in almost all tisues, both lymphoid and non-lymphoid (23, 24). The origin of cDC has been the subject of debate in many recent papers (25). Adoptive transfer studies indicate that cDC are derived from the blood. However, since numbers of cDC in the blood are low, it was presumed that they derive from a blood borne precursor which differentiates once in the tissue. Indeed a number of transplantation studies as well as studies tracking labeled cells suggest that a large part of the cDC present in secondary lymphoid tissues are derived from blood monocytes (26, 27). DC are generally considered to be part of the myeloid compartment, and therefore as derived from common myeloid progenitors in the bone marrow. However, it has been shown that in mice, common lymphoid progenitors also give rise to DC in the spleen and particularly in the thymus (28). Similarly, human DC could be derived both from common myeloid and common lymphoid precursors, with no differences in surface marker or gene expression profile (29). Additionally, several reports describe the presence of a local precursor for cDC in for example the spleen and the skin, indicating that not all cDC populations are dependent on replenishment from the blood (30-32). Furthermore, although cDC were initially considered an end-stage of differentiation, some reports now show that (limited) cDC proliferation takes place in peripheral tissue, thus contributing to local cDC presence (33).

Plasmacytoid dendritic cells (pDC) are characterized by expression of CD123, BDCA-2 and BDCA-4 (human) or expression of B220, Gr-1 and PDCA-1 (mouse) (24, 34).

They are found in almost all lymphoid tissues, including spleen, lymph nodes and liver, but also in non-lymphoid organs such as the kidneys (35-37). Contrary to cDC, pDC are found in peripheral blood in a relatively mature form. Plasmacytoid DC are therefore not considered to be derived from monocytes, but rather directly from a precursor in the bone marrow. This precursor may be shared with at least some cDC, as precursors with developmental potential for both lineages have been described (38, 39).

Dendritic cells in immunity

Once differentiated into DC in the tissue, cDC are relatively immature. Immature cDC are highly phagocytic and take up potential antigen by random endocytosis or through the use of Fc receptors or pattern recognition receptors such as TLR and C-type lectins (40, 41). Upon phagocytosis, antigen is digested into small peptides and coupled to MHC molecules for presentation on the cell membrane (42). To generate an effective immune response, cDC have to be activated and differentiated into mature cDC. This can be accomplished by a variety of stimuli, including bacterial or viral components, cytokines produced by other immune cells such as IFN-γ and TNF-α, CD40L expressed on activated T-cells or endogenous products released under cellular stress, e.g. heat shock proteins (40). In the context of a proper activating signal, cDC upregulate expression of MHC class II molecules, thereby increasing the chance of a specific T-cell recognizing its antigen. Furthermore, co-stimulatory molecules CD80 and CD86 are upregulated, which bind to CD28 on the surface of interacting T-cells, resulting in T-cell activation. Finally, production and secretion of cytokines IL-1, IL-6, IL-12, IL-15 and, under particular circumstances, IL-4 is induced. Depending on the type of signal received, cDC produce either high or low amounts of IL-12 and occasionally IL-4. This will skew the responding T-cell to a Th1 or Th2 phenotype, and thus strongly influence the outcome of the immune response (43, 44). Usually cDC present endogenous antigen in the context of an MHC class II molecule, leading to the activation of $CD4^+$ T-cells. However, cDC are also capable of cross presentation, meaning that exogenous antigen is presented in the context of an MHC class I molecule, leading to a cytotoxic $CD8^+$ T-cell response (45).

After the initial phase of immune activation, cDC play a role in the resolution of the immune response by expression of inhibitory molecules. After activation, T-cells upregulate surface expression of CTLA-4, ICOS and PD-1. These receptors negatively regulate T-cell activity after binding to B7 family members expressed on cDC including CD80/86, ICOS-L and PD-L1/2 (46, 47).

Although direct interaction between cDC and B-cells have been clearly shown to occur in lymph nodes, the importance of this interaction is less well studied. cDC may capture antigen and transfer it to B-cells, increasing the likelihood of a B-cell cognate response (48, 49). Furthermore, the contact between a DC and a B-cell makes interactions between B-cells and $CD4^+$ helper cells more efficient, as it places the B-cell near a T-cell specific for the same antigen (50). Finally, cDC produce cytokines which influence the survival and differentiation of B-cells, as well as the secretion of antibodies, for example through the

production of B-cell activating factor (BAFF) (51, 52).

Plasmacytoid DC mainly function in the response against viruses and have a less wide mode of activation. Instead of the large array of TLR expressed on cDC, pDC mainly express TLR7 and TLR9 (53). These TLR recognize viral products such as unmethylated DNA and double stranded RNA and lead to the rapid activation of pDC (54). After activation, pDC in both blood and tissue produce large amounts of IFN-α, which is an important factor for the differentiation and activation of cDC as well as T-cells (35, 36). Furthermore, pDC produce IL-6, which together with IFN-α induces differentiation of memory B-cells to plasma cells (55, 56). In the immature state, pDC have a low endocytic capacity and low expression of co-stimulatory molecules and MHC class II. Consequently, these cells are poor T-cell stimulating cells. After activation, expression of CD40, CD80 and 4-1BB is upregulated, together with MHC class II (36). Plasmacytoid DC activated by a viral stimulus induce expansion of both $CD4^+$ and $CD8^+$ T-cells, albeit at a lower level than cDC (57). Activated pDC can produce IL-12 and induce a Th1 response, although the exact amount is under debate and may depend on the specific stimulus. Interestingly, activated pDC also induce modest Th2 responses, probably due to the expression of OX40L rather than production of IL-4 (58).

Dendritic cells in tolerance

The human body is exposed to a large number of harmful microorganisms, but also to many harmless exogenous substances as well as endogenous proteins. Immune responses against these substances are unfavourable, as they do not serve to protect the body but can cause damage to tissues instead. To prevent the induction of unwanted immune reactions, the immune system has several mechanisms to induce tolerance.

Conventional DC play a role in both central and peripheral tolerance. Central tolerance takes place during the generation of T-cells in the thymus. Thymocytes in the double positive stage ($CD4^+CD8^+$) are tested for their reactivity against local cDC loaded with self-peptides. In the case of strong recognition, the thymocyte is depleted and does not enter the circulation (59). T-cells with a low recognition of self-proteins may escape this mechanism, and are further controlled in the periphery.

Peripheral tolerance can be achieved by immature cDC as well as cDC activated in the presence of a tolerizing stimulus through a number of mechanisms. Firstly, immature cDC which have taken up antigen in a non-inflammatory environment, express low levels of co-stimulatory molecules. If a cDC in this state presents antigen to a T-cell, this does not result in T-cell activation, but rather cause anergy (60). Secondly, immature, cDC or cDC tolerized by cytokines such as IL-10 and TGF-β can actively induce tolerance by depletion of reactive T-cells. This depletion is usually characterized by a rapid proliferation of T-cells, followed by apoptosis. The mechanisms mediating this effect are not completely clear thus far, but include signaling through Fas-FasL interactions and depletion of tryptophan by the enzyme indolamine 2,3-dioxygenase (IDO) (61-63). Thirdly, immature or tolerized cDC can induce several subsets of regulatory T-cells, including Tr1, $CD8^+$ regulatory cells and

$CD4^+CD25^+Foxp3^+$ cells. IL-10 production by tolerized cDC plays a role in the induction of Tr1, while antigen presentation by cDC under non-inflammatory conditions results in expansion of potent antigen specific $CD4^+CD25^+Foxp3^+$ cells (64, 65). Furthermore, interaction between CD80/86 on cDC and CTLA-4 on regulatory T-cells is important for proper functioning of these cells (66). Finally, tolerogenic cDC may skew the immune response to a Th2 type, as has been shown in a collagen induced arthritis model (67). This is of particular benefit in Th1 mediated diseases, as it counteracts harmful Th1 responses.

Plasmacytoid DC also have tolerogenic properties. In a mouse model for asthma, depletion of pDC led to an increased Th2 response and more severe disease, while disease could be prevented by adoptive transfer of pDC (68). The functional properties of pDC mediating this effect have not been elucidated, but other studies have shown that pDC can induce and support the function of $CD4^+CD25^+$ cells and Tr1 cells, even after activation (69, 70). Furthermore, pDC activated by CTLA-4 produce IDO, inhibiting T-cell proliferation and survival (71).

Trafficking of dendritic cells

Conventional DC exert their function both by stimulating activated T-cells in non-lymphoid tissues, and by priming naïve T-cells in lymph nodes. In order to properly exert this function, immature cDC have to migrate to the site of inflammation, take up antigen, and then migrate to the lymph nodes. Migration of cDC is regulated by a large number of proteins, including the expanding family of chemokine receptors (72). After differentiation from monocytes, immature cDC express the chemokine receptors such as CCR1, -2 and -5, and CXCR4. The ligands for these receptors are chemokines CCL3, -4 and -5 and CXCL12, respectively. These chemokines are produced at sites of inflammation. After uptake of antigen and appropriate activation, expression of these chemokine receptors is downregulated and cDC start to express CCR7 (73, 74). This receptor mediates migration towards CCL19 and CCL21, which are expressed in lymphatics and lymph nodes. In lymph nodes, expression of these chemokines is mainly found in T-cells zones, thus directing activated cDC to the most efficient location for T-cell priming.

Migration of pDC is regulated quite differently from migration of cDC, although the expression of chemokine receptors is largely similar. Firstly, pDC migrate to lymph nodes through high endothelial venules (HEV) rather than the lymphatic system (75). This migration is partly mediated by expression of CD62L and CD62E. Secondly, pDC migrate in response to CXCL12 but not other inflammatory chemokines, indicating that chemokine receptors such as CCR1, CCR2 and CCR5 are non-functional in this cell type (76). Also CCR7, which is expressed by both immature and activated pDC is not functional in humans in this cell type. Instead, pDC express functional CXCR3, the receptor for CXCL9 and CXCL10. These ligands are expressed in inflamed lymph nodes, and CXCR3 is the major chemokine receptor mediating pDC migration under inflammatory circumstances (75). Under non-inflammatory conditions, pDC may be recruited through

chemerin, which is expressed on the luminal side of HEV. Plasmacytoid DC express high levels of chemerin receptor and efficiently respond to this ligand *in vitro* (77).

Dendritic cells in autoimmunity

Initial studies into a number of autoimmune disorders concentrated on T- and B-lymphocytes as these cells appeared to be the harmful effector cells. Although altered skewing of the T-cell response as well as B-cell hyperactivity have been described, the question remained whether these aberrancies were intrinsic to the respective cell type, or caused by an underlying defect in antigen presentation. In diseases such as diabetes, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS) and Sjögren's Syndrome (SjS), both pDC and cDC were present in tissue lesions in increased numbers (78-84). Furthermore, these DC often displayed signs of increased activation such as expression of CD83 and HLA-DR, supporting the idea that increased APC function resulted in increased immunity and finally autoimmunity. In the case of RA, successful treatment and decrease in clinical symptoms was strongly correlated to the number of DC present in synovial tissue (85). In animal models for diabetes and SjS, DC were the first cells to infiltrate the affected tissues, suggesting that these cells play a role in the initiation of the pathogenesis (78, 86). In fact, autoimmunity including experimental autoimmune encephalitis and diabetes could be induced by transfer of autoantigen presenting DC (87, 88). Conversely, development of diabetes could be prevented by ablation of the cDC population in the nonobese diabetic (NOD) mouse (89).

Although all these data strongly suggest a role for DC in both initiation and perpetuation of autoimmune responses, it is less clear whether this is due to DC intrinsic aberrancies or the inflammatory environment. Culturing monocytes from healthy donors in the presence of serum from SLE patients results in the generation of aberrantly activated DC, suggesting an environmental cause (90). However, a number of abnormalities have been described in peripheral blood of patients, DC generated *in vitro* and DC obtained from pre-diseased animals, which suggests that these DC have intrinsic defects. One interesting example is provided by patients suffering from Wiskott Aldrich syndrome. This syndrome is caused by a mutation in one specific protein involved in cytoskeletal rearrangement, resulting in a defect in DC migration and recruitment (91). Around 40% of patients suffering from this syndrome develop an autoimmune disorder, including autoimmune hemolytic anemia, vasculitis and inflammatory bowel disease (92). Defects in adhesion and migration of monocytes and DC were detected in diabetic patients as well as the NOD mouse model (93-95). Furthermore, in the NOD mouse, a defective generation of DC from monocytes was described *in vitro*, resulting in more macrophage-like cells (96). Although this may appear unexpected, this is not necessarily at odds with the increased autoimmunity seen in these animals. DC are not only important in the induction of immunity, but also in the proper maintenance of tolerance. If antigen presentation by DC is hampered, this could result in decreased thymic presentation of self-antigens, resulting in a failure of central tolerance.

Dendritic cell aberrancies in rheumatic diseases have mainly been described in animal models, although some human data is available. In an SLE mouse model, increased T-cell activity was shown to be the result of increased antigen presentation by DC (97). This increased APC function was maintained after *in vitro* culture, suggesting an intrinsic property of DC. Furthermore, DC cultured from the bone marrow of MRL/lpr mice showed defects in uptake and processing of apoptotic cells, resulting in lupus-like symptoms (98). A similar deficiency in clearance of apoptotic cells was seen in NOD DC (99). This may contribute to the pro-inflammatory environment due to aberrant presentation of self-antigens. In RA, peripheral blood monocytes show an altered subset distribution as well as increased activation leading to increased adhesion to various surfaces (100, 101). DC generated from RA monocytes *in vitro* showed an increased expression of Fc receptor CD32 and produced more TNF-α when stimulated (102), again suggesting intrinsic defects.

In summary, both cDC and pDC strongly impact the outcome of an immune response, as they are capable of inducing either immunity or tolerance depending on the type of stimulus received. The specific type of immunity can be skewed by both cell types, determining the efficiency of the response as well as the extent of tissue damage. Aberrancies in cDC or pDC populations as well as in the molecular networks regulating their migration and activation may strongly impact immune responses and lead to either immune deficiency or autoimmunity. Alterations in monocytes, cDC and pDC have been described in various autoimmune diseases, suggesting these cells are important players in the pathogenesis of these disorders.

1.2 SJÖGREN'S SYNDROME

1.2.1 Pathology

The first report of what is now known as Sjögren's Syndrome (SjS) comes from the French ophthalmologist Henri Gougerot in 1926, who described a patient with a combination of dry eyes and a dry mouth (103). This finding was further confirmed by the Swedish physician Henrik Sjögren who described 19 patients with a similar combination of complaints in his thesis published in 1933 (104). Initially the disease was named 'Gougerot-Sjögren Syndrome', but given the high number of patients Henrik Sjögren described during his career, it is now known under his name only.

As mentioned above, SjS is characterized by dryness of the eyes (xerophtalmia) and the mouth (xerostomia) (105). Patients usually complain of a sandy feeling in the eyes, difficulty swallowing food and sometimes a change of taste. Due to the lack of lubrication, the incidence of opportunistic infections in both the eyes and the mouth is increased and a number of patients suffers from dental problems such as frequent caries (106). SjS occurs in a primary form in the absence of other autoimmune diseases, or in a secondary form, in which case it accompanies conditions such as RA or SLE. This thesis will focus on

primary SjS (pSjS). Estimates of the prevalence of pSjS range around 3-4%, with a nine fold predominance of female patients (107-109).

Extraglandular manifestations

Although SjS is characterized by the ocular and oral complaints, symptoms can also be observed in many other organs as well as systemically. Skin problems are a common feature of SjS and include dryness, Raynaud's phenomenon (abnormal vasoconstriction in digits) and cutaneous vasculitis (110). The latter may be of prognostic importance since SjS patients displaying symptoms of cutaneous vasculitis are at an increased risk of lymphoma and disease related death (111). Also increased mucosal dryness is found in many patients, in particular in the mouth and vagina, increasing the risk of local infections (112).

Another frequent extraglandular manifestation is found in the joints. Up to 50% of patients complain of arthralgia, which may or may not be accompanied by arthritis (113). Although mild myopathies are found, muscular involvement is relatively rare.

Partly as a result of the decrease in salivary flow as well as salivary proteins, SjS patients suffer from a number of gastrointestinal symptoms. These include dysphagia, nausea and dyspepsia, as well as epigastric pain (112). Besides the lack of salivary proteins, these complaints may be caused by autoimmune activity in the stomach itself as anti-parietal antibodies and lymphocytic infiltrations of the stomach are found in a subset of patients (114).

Inflammatory processes can also be found in the bladder (interstitial cystitis), kidney (interstitial nephritis and tubular dysfunction), pancreas (pancreatitis), lung (interstitial lung disease) and in rare cases in the heart (acute pericarditis) (112).

A lack of control of inflammatory processes may also lead to the formation of lymphomas, in particular Non-Hodgkin lymphoma, which is found 16-44 fold more often in SjS patients than in the general population (115, 116). In most cases these lymphomas are benign and require little or mild treatment, but in a small proportion of patients, they may develop into a severe malignancy. This risk emphasizes the need for continuous monitoring of SjS patients.

Neuropathies are found in a relatively large subset of SjS patients, although the estimated prevalence remains under debate and ranges from 10-45% (117-119). Most patients suffer from peripheral neuropathies, including mainly sensory problems, although motorical deficits have also been described (120). Even though only a small proportion of patients shows symptoms of central nervous system involvement, this is of great importance in monitoring of the disease, as symptoms can be severe. Focal brain lesions may lead to sensory or motor deficits or seizures (121), but patients may also present with MS-like symptoms or cognitive problems stretching to full dementia. Additionally, psychiatric disorders are associated with SjS, mainly consisting of various degrees of depression (122). Although this may partly be explained by the decreased quality of life of patients, neurological involvement is likely. Finally, it is estimated that over 50% of patients suffer from extreme fatigue (123), in many cases leading to inability to work, and

a strong decrease in quality of life. This may be partly the result of inflammatory processes in for example the thyroid gland, but may also originate from sleep disturbances (124).

Diagnosis

The symptoms characterizing SjS usually develop gradually over a period of time and are not disease specific. Since in a large proportion of patients disease onset occurs between 40 and 50 years of age, symptoms may initially be explained as menopausal complaints. This further delayes diagnosis and treatment. The lack of a disease specific marker complicates the study of SjS as patient groups described in various studies may differ. Therefore, a set of criteria was established to confirm and unify the diagnosis of SjS. The latest set of criteria, the 2002 European-American classification criteria (105), consists of six elements, both subjective and objective in nature. The subjective criteria consist of complaints of dry eyes and mouth, such as 'sandy eyes' and problems swallowing dry food. The objective criteria include clinical tests of salivary and lachrymal function such as measurements of stimulated and unstimulated salivary flow, tear flow and tear break-up time. Two laboratory measurements regarding the the presence of antibodies against SS-A and/or SS-B and the presence of focal lymphocytic infiltrates in a biopsy of the minor salivary gland are also included. To score positive for SjS, patients have to fulfill at least four of these six criteria, including at least one of the laboratory findings. Other causes of oral and ocular dryness such as previous radiation in the neck region, infection, malignancy and the use of specific medications have to be excluded.

Although these criteria may help physicians in properly characterizing patients, they were initially devised for unification of the various studies into SjS. A number of patients therefore does not completely fulfill the criteria, even though the symptoms cannot be attributed to another illness. These patients may either reach the total criteria at some point in the future, or remain classified as 'incomplete' SjS. This group is likely to benefit from the common treatment for SjS, and may therefore be considered as SjS for clinical practice.

SjS is a heterogeneous disorder, with symptoms ranging from barely detectable to severe and changing over time in the same patient. To further unify the patient groups included in SjS studies, measurement of disease activity would be usefull. A recent report described the development of the first SjS Disease Activity Index (SSDAI) (125), which includes a large array of possible complaints such as pulmonary and articular involvement, fatigue, salivary gland swelling and neuropathy. Although this index requires further validation, it may ultimately prove useful in stratification of patients for therapeutic purposes.

1.2.2 Treatment

Stimulation of salivary and lachrymal flow

As some salivary and lachrymal function is still present in most patients, initial therapy is aimed at stimulating flow. Stimulation of salivary flow can be achieved by sucking on

sugar-free sweets, in particular those containing an acidic component. In patients where this type of stimulation is not sufficient, pharmacological intervention may be needed. Possible treatments include pilocarpine, a muscarinic agonist that stimulates M3 receptors, including those resulting in salivary production. Pilocarpine has indeed been shown to increase both labial and whole salivary flow as well as to improve subjective measurements of oral dryness (126). Furthermore, improvement of ocular dryness was reported, although lachrymal flow was not increased (127). Side effects mainly include sweating and abdominal pain and are tolerable in most patients. More recently a second M3 agonist, cevimeline was described for the treatment of SjS. This compound has a higher affinity for salivary gland M1 and M3 receptors than those expressed in the heart. Positive effects on both objective salivary and objective lachrymal flow were observed in various placebo-controlled trials, in particular in those SjS patients with low focal scores (128, 129). Furthermore, an increase in the secretion of antibacterial proteins was seen, which would help prevent oral infections (130). The effect was suggested to be more long-lasting than that obtained by pilocarpine (131). However, a number of patients left the trials due to severe side effects, mainly nausea.

Interferon-α was used in several studies to improve salivary flow in SjS. In some patients an increase in salivary flow was observed, but in larger groups no significant differences in oral dryness could be found between IFN-α and placebo (132). The high cost of this therapy and the low efficacy make this an unattractive treatment.

The anti-inflammatory drug cyclosporine A has been used successfully to induce tear flow. Improvements were observed in Schirmer tests, visual acuity and ocular complaints (133, 134).

This was accompanied by a decrease in inflammatory cells such as T-cells and APC in the conjunctiva (135). When pharmacological intervention does not suffice to increase tear flow, punctual occlusion can be considered. This involves sealing the puncta, which normally drain tear fluid to the nose, either temporarily by insertion of a plug, or permanently by laser induced sealing.

Fluid replacement

Some SjS patients may not have sufficient remaining salivary and lachrymal function or are refractory to stimulation. In this group, the use of replacement fluids may be helpful. For many patients, oral dryness can be alleviated by flushing water. When this method does not suffice, artificial saliva can be used. This is a fluid containing methylcellulose, providing both moisture and lubrication for swallowing. Similarly, artificial tears can be used to treat persistent dry eyes. These consist of methylcellulose to provide moisture, or agents which decrease water tension, thus enabling the remaining fluid to spread over a larger ocular surface.

Systemic treatments

The main systemic treatment for SjS is the use of the anti-malerial hydroxycholoquine. This compound is an anti-inflammatory drug which alters the lysosomal pH, thereby

inhibiting antigen presentation (136). Whether this is also the functional mechanism for the beneficial effect in SjS remains to be elucidated, but improvement of both arthralgia and fatigue have been described in a subset of patients (137). In cases refractory to hydroxychloroquine, the immune suppressant methotrexate may be used. However, a beneficial effect for this drug has only been shown for subjective and not objective symptoms in a small study (138). Moreover, methotrexate treatment increases the risk of pulmonary complications.

Given the autoimmune nature of SjS, the use of immunosuppressants appears to be a logical treatment. However, no positive effects on salivary and lachrymal function could be observed when using these agents (139), with the exception of topical ocular cyclosporin. This, in combination with the associated severe adverse effects, implies that immunosuppressants are not advised for treatment of SjS, except in patients with severe life-threatening extraglandular complaints such as acute pneumonitis and severe neurological symtoms. In these patients, use of glucocorticoids and/or cyclophosphamide may be beneficial (140, 141).

Treatments using biologicals

In recent years, a number of treatments for SjS have been investigated in clinical trials, especially the humanized monoclonal antibodies against various inflammatory molecules. One of the first members of this group to be used in SjS was anti-TNF-α. Given the increase in TNF-α in SjS, and good results in conditions such as RA and psoriasis, this seemed a promising approach. Although the first small study appeared to find a beneficial effect, larger controlled studies could not confirm this and the use of this treatment is no longer recommended (142, 143).

Another option for treatment of SjS is anti-CD20. CD20 is expressed on most B-cells except plasma cells and treatment with anti-CD20 leads to B-cell depletion. In two phase II studies, treatment of SjS with anti-CD20 lead to an increase in salivary flow, particularly in early patients with residual salivary function (144, 145). A redifferentiation of salivary epithelium was observed, indicating a recurrence of salivary function. Beneficial effects on systemic complaints were also seen in SjS patients treated with this agent. After six to nine months, SjS symptoms reappeared and patients needed re-treatment (146). Although this again resulted in an improvement of salivary flow and symptoms, some patients developed a serum-sickness-like phenomenon and had to stop treatment, which may implicate problems for long-term treatment of patients with anti-CD20.

The last treatment proposed for SjS is the inhibition of B-cell activation. SjS patients show an increased expression of B-cell activating factor (BAFF) both in serum and salivary glands. This factor plays a role in the activation, survival and antibody production of B-cells, and is therefore considered of great importance in SjS. A chimeric humanized antibody blocking BAFF has now been developed and shown to decrease the number of B-cells both in peripheral blood and tissues (147). In initial studies in RA, clinical symptoms decreased in patients treated with anti-BAFF while treatment was well tolerated (148).

Although these results are encouraging, no studies regarding the use of anti-BAFF in SjS have been reported thus far.

1.2.3 Etiopathology

Genetic components of SjS

Although SjS is not clearly inherited, some familial clustering of the disease has been observed, suggesting a genetic component. In fact, a family history of autoimmune disease causes a seven fold increased risk of developing SjS (149). Unfortunately, twin studies which would make an estimation of the genetic component possible have not been performed in SjS thus far. A number of studies shows an association between polymorphisms in particular genes and the occurrence and severity of SjS. Initial reports mainly regard the association between HLA serotypes and SjS, and described increased frequency of two particular HLA-I serotypes, HLA-A24 and HLA-B8 (150, 151). Furthermore, associations were found between SjS and HLA-II molecules -DR2, -DR3, -DR5 and -DRw52 (152, 153). Most of these serotypes also correlated with the occurrence of anti SS-A antibodies. In addition, an association between these antibodies and HLA-A1, -DR1 and DQ1 was described (154), although these serotypes were not related to disease incidence. The serotype associating with SjS appeared to differ depending on the ethnic background of the patients, suggesting that HLA variancy may act as a co-factor rather than a causative agent (155).

Other genetic factors which may influence the development of SjS include cytokines and their receptors, but results of polymorphism analyses in these genes are not always consistent. For example, some groups report an increased frequency of the IL-10 GCC haplotype, which resulted in increased IL-10 levels and increased presence of autoantibodies, while others find no association (156-158). A similar discrepancy was found for IL-1R antagonist and IL-6 (159-161). A TNF-α polymorphism leading to higher production of this cytokine was associated with both an increased incidence and the presence of autoantibodies, while TGF-β variants only influenced SS-A antibody levels (161). Further conflicting data was obtained for CTLA-4, where one group showed an association with both the occurrence of disease and the occurrence of extraglandular manifestations, while another found no correlation (162, 163). Finally, polymorphisms in the TAP gene important for antigen presentation are associated with the development of SjS (164, 165).

Sex steroid involvement

Given the high number of women among SjS patients, and the fact that the most common age of onset is around menopauze, a role for sex steroids in the pathogenesis of SjS has been proposed. In a mouse model, estrogen deficiency resulted in a SjS-like disease with increased apoptosis and proteolytic cleavage of alpha-fodrin in salivary and lachrymal glands, suggesting a role in the formation of autoantigens (166).

Estrogen deficiency is also correlated to an increase in B-cell lymphopoiesis (167), contributing to the B-cell hyperactivity and autoantibody production observed in SjS. Furthermore, estrogen inhibits expression of various cytokines including IL-6, IFN-γ and TNF-α (168, 169), possibly through the regulation of SOCS2 and SOCS3 (170). Estrogen also drives both expansion and function of $Foxp3^+$ regulatory T-cells (171), which are important in the maintenance of tolerance against a large array of self molecules. The decrease in estrogen experienced during menopause might cause a decrease in these inhibitory signals and might lead to autoimmunity in predisposed individuals.

Besides a decrease in estrogen, menopausal women may also experience a decrease in androgen levels. In postmenopausal women, androgens are mainly produced in peripheral tissues, and this is regulated by intracrine signaling. Local production can therefore be measured by expression of DHEA responsive proteins such as CRISP-3. In SjS patients, serum levels of the androgen DHEA are indeed decreased (172), as well as local levels of CRISP-3 in the salivary glands (173). Androgens have been shown to protect against the development of autoimmunity, including salivary inflammation in the MRL/lpr model (174).

Autoantibodies in SjS

One of the hallmark features of SjS is the increased presence of autoantibodies against a number of different proteins. As in many other rheumatic diseases, SjS patients often display rheumatoid factor and anti-nuclear antibodies, but this is not discriminatory. More disease specific are antibodies against the Ro/SS-A and La/BB-S antigens. SS-A has a function in the quality control of RNA folding and consists of a complex of two ribonuclear proteins, one of 60 and one of 52 kDa (175). SS-B is a single 48 kDa protein and is involved in the transcription by RNA polymerase III (176). Estimates of the prevalence of anti-SS-A and anti-SS-B antibodies in SjS differ considerably, depending on the detection method used. For SS-A, estimates range from 40-96%, while for SS-B the reported prevalence is 26-87% (177, 178). Antibodies are of both IgA and IgG subtypes and can also be detected in saliva, albeit at lower frequency (179). Although both types of antibodies can be used as part of the diagnostic procedure, they are not completely disease specific as both are also found in SLE patients (180). Whether SS-A and SS-B antibodies exert a pathological function in SjS patients remains to be elucidated, but the presence of these antibodies is associated with increased extraglandular manifestations (178). SS-A/SS-B positive patients should be monitored carefully during pregnancy, as their offspring have an increased risk of congenital heart block (181).

A second type of antibody associated with SjS is anti-α-fodrin. Alpha-fodrin is a 240 kDa cytoskeletal protein which is cleaved aberrantly in salivary glands of SjS patients. As a result, a 120 kDa antigen is generated, leading to the induction of an antibody response (182). The incidence of anti-α-fodrin antibodies is around 30% in primary SjS, although immunoblotting reveals positivity in up to 80-95% (183). Differences may be explained by the specific detection method used, as the majority of anti-fodrin antibodies

appears to be of the IgA rather than IgG class. Immunization of neonatal mice with the aberrant α-fodrin antigen prevents disease development in an SjS model (184), suggesting a role for the anti-α-fodrin response in the pathogenesis of the disease.

The most recently described antibodies in SjS are directed against the muscarinic M3 receptor. These antibodies are of particular interest as they may have a role in the pathology of SjS. The M3 receptor is expressed on salivary acinar cells, and is involved in the nervous stimulation of saliva secretion (185). Mice deficient in M3 receptors show decreased salivary flow upon stimulation (186). The anti-M3 antibodies present in SjS patients can either block receptor-ligand interactions or exert an agonistic effect on these receptors, but finally lead to exhaustion and lack of signaling (187). As a result, salivary flow is decreased, even in the presence of intact acini. Interestingly, salivary flow deficiency in SjS patients does not correlate directly with the degree of infiltration found, suggesting that tissue destruction is not solely responsible for the clinical symptoms. In this light, anti-M3 antibodies may provide an alternative explanation for lack of salivary function.

Epithelial involvement

The salivary epithelium is clearly a target in the SjS autoimmune response, but has also been described as an active player in the pathogenesis itself. Earlier studies imply that the salivary epithelium may function as non-professional APC, as both MHC class I and class II are expressed (188). Furthermore, increased expression of co-stimulatory molecules CD80 and CD86 is found on SjS epithelial cells, as well as adhesion molecules involved in binding of lymphocytes such as ICAM-1 and VCAM (189-191). More recently, TLR expression was detected on SjS epithelial cells in culture, and triggering of these receptors enhances co-stimulatory function (192, 193). A matter of debate has been whether these characteristics are induced by the ongoing inflammation or are intrinsic to the SjS epithelium. Culture experiments show that the antigen-presenting phenotype is preserved in the absence of inflammation, suggesting that this is an intrinsic property. However, upregulation of co-stimulatory markers and MHC molecules after stimulation by IFN-γ suggests that the pre-existing phenotype may be further enhanced by the local immune response (190).

Epithelial cells also produce a number of chemokines involved in the recruitment of lymphocytes as well as the induction of germinal centers. Levels of CXCL13, involved in the organization of B-cell follicles and CCL19 and CCL21, involved in directing lymphocytes to lymphoid structures, are all increased in SjS, and the expression was localized to epithelial cells (194). Also inflammatory chemokines such as CCL3, CCL4 and CCL5 are produced by acinar and ductal cells and stimulate the influx of T-cells and DC (195). Although the B-cell stimulator BAFF is not expressed under normal culture conditions in either SjS or control epithelium, a strong induction is seen in SjS after stimulation with IFN (196), suggesting hypersensitivity of the salivary epithelial cells. Next to these chemokines, SjS epithelium produces increased amounts of matrix metallo-proteases (MMP) -2 and -9 (197). These enzymes are involved in the disintegration of glandular

architecture, increasing the infiltration of lymphoid and myeloid cells, and reducing the production of saliva

Finally, epithelial cells may serve as a source of auto-antigen by virtue of increased apoptosis. In the salivary glands of SjS patients levels of both pro- and anti-apoptotic molecules are elevated (189, 198, 199). The pro-apoptotic molecules such as Fas, FasL and caspase-3 are mainly expressed in epithelial cells, while anti-apoptotic markers including Bcl-2 are found in infiltrating lymphocytes, indicating that both increased and decreased apoptosis may occur, depending on the cell type. The effect of this apoptosis may be threefold. Firstly, epithelial cells undergoing apoptosis no longer contribute to the production of saliva, thus decreasing the total flow. Secondly, the increased apoptosis stimulates the generation of auto-antigens (200). Apoptotic epithelium expresses caspase-3, which is involved in the aberrant cleavage of α-fodrin, resulting in the 120 kDa auto-antigenic fragment. Also the release of perforin and granzyme-B by recruited T-cells leads to epithelial apoptosis and the generation of altered, more antigenic SS-B protein fragments (201). Finally, anti-apoptotic molecules may rescue cells from apoptosis, but still induce saliva hypersecretion. In salivary gland epithelium transfected with Bcl-xL, apoptosis was prevented, but calcium signaling was severely impaired, thus reducing secretion of saliva (202). Next to the function of anti-M3 autoantibodies, this may offer an explanation for the lack of salivary function in patients with relatively little lymphocytic infiltrations.

SjS patients usually present with established disease, complicating the separation of immunological and epithelial specific defects. Therefore, animal models are used to study the early stages of SjS in the absence of gross inflammation. In the NOD mouse model aberrancies in the structural development of the salivary glands occur as early as 1 day after birth and include lower epithelial proliferation, epithelial apoptosis and increasing expression of MMP-2 and MMP-9 (203, 204). Although some of these features disappear after three to seven weeks, they reappear in older mice (>20 weeks). For example, old NOD mice show increased expression of apoptotic molecules such as Fas and FasL, as well as increased proteolytic activity, including that of MMP-2 and MMP-9. The fact that particular alterations occur in mouse models prior to the onset of infiltration corresponds with the notion of intrinsic epithelial aberrancies. This is further supported by the fact that expression of Fas and FasL, increased apoptosis and increased proteolytic activity are also observed in NOD.scid mice lacking functional T- and B-cells (205).

Viral etiology

Several viral infections result in sicca symptoms similar to SjS, and this has lead to several studies focusing on the role of viruses in the induction of SjS. Up to half of hepatitis C virus (HCV) infected patients show lymphocytic infiltrations in their salivary gland, and around 30% complain of xerostomia (206). Furthermore, antibodies to HCV are found in significantly more SjS patients than controls, although the frequency depends on the ethnic origin of the patient population (207, 208). However, although HCV sicca syndrome

and SjS appear similar in clinical presentation, the former is not associated with increased levels of anti-nuclear antibodies, anti-SS-A or anti-SS-B, suggesting that the two are in fact pathologically different (209).

Human T-cell leukemia virus (HTLV) has also been associated with the occurrence of SjS. In initial studies, partial sequences of the HTLV gene *tax* were found in the salivary glands of 4/14 SjS patients, but not healthy controls (210). Mice transgenic for this gene exhibit exocrinopathy similar to SjS (211). Antibodies against various HTLV proteins could be detected in both serum and saliva of SjS more frequently than in healthy controls (212). However, in areas where HTLV is endemic, prevalence of SjS is not increased, suggesting that although there may be an association, the two are not causally related (213).

Earlier, the presence of Epstein-Bar virus has been associated with SjS. The EBV protein early antigen-D was detected in 8/14 SjS salivary glands, but not in controls (214). EBV DNA sequences were found both in SjS and control glands, but were more common in patients (50 versus 8%). B-cell lines derived from SjS patients produce EBV after transformation *in vitro* (215). This last finding may be of relevance to the development of lymphomas in SjS patients, as these are usually of the B-cell type and EBV is a known cause of lymphoproliferative disorders.

Murine cytomegalovirus (CMV) induces a SjS like phenomenon in C57BL/6 *lpr/lpr* mice, which do not spontaneously develop sialoadenitis (216). Antibodies against CMV are present in serum of SjS patients, but the frequency does not differ much from that in the general population (217). The fact that CMV can be detected in SjS salivary gland more often than in controls is probably not due to a causal influence of CMV in SjS, but is more likely to be the result of reactivation of the virus due to the ongoing inflammation.

The most recent addition to the list of viruses associated with SjS is coxsackievirus, in particular the B4 serotype. In a study preformed in Greece, viral RNA was detected in 7/8 SjS patients, while it was not present in controls (218). Furthermore, crossreactivity was detected between anti-SS-A and anti-SS-B antibodies and a coxsackievirus derived protein (219), suggesting a role for this virus in the development of SjS through molecular mimicry. However, in a study performed in France including larger numbers of patients, these findings could not be confirmed (220), and further research will be necessary to establish whether this virus indeed plays a pathogenic role in SjS.

B-cell hyperactivity

The most characteristic histological finding in SjS is the formation of focal lymphocytic infiltrates. These infiltrates contain both $CD3^+$ T and $CD19^+$ B lymphocytes, as well as $CD83^+$ dendritic cells and $RDF9^+$ macrophages (83). In an estimated 17-28% of SjS patients these infiltrates show a high degree of structural organization, resembling germinal centers with follicular dendritic cells, B-cells and also some T-cells (221, 222). Germinal center formation is probably driven by expression of lymphoid organizing factors such as CXCL13, CXCL12 and CCL21. The main function of these germinal centers is to activate B-cells by contact with primed T-cells. B-cells then proliferate and finally mature

into plasma cells. This process is important for the pathogenesis of SjS, as increased B-cell proliferation and antibody production have been observed in SjS salivary glands (222).

B-cell activity is not only increased by the presence of germinal centers, but also by increased local and systemic expression of BAFF. BAFF is a survival factor for B-cells mainly produced by monocytes, neutrophils, DC and macrophages (52). Mice that overexpress BAFF develop autoimmune phenomena, including infiltration of the salivary glands and decrease in salivary flow (223). In SjS, increased expression of BAFF was strongly correlated to the serum level of autoantibodies (224). Furthermore, increased expression of BAFF was detected in salivary glands of SjS patients, both by infiltrating leukocytes and by salivary epithelium (223, 225), suggesting a role in the expansion of autoreactive B-cells *in situ*.

T-cells in SjS

Infiltrates not only contain B-cells, but also considerable numbers of T-cells (226), with a predominance of $CD4^+$ cells (227). These $CD4^+$ cells produce a number of cytokines, although these differ somewhat between studies. Several reports describe expression of IL-2 and IFN-γ, both *in situ* and in T-cell clones isolated from salivary glands (228-232). In some of these studies, IL-4 and IL-10 were found, while these could not be detected in others. Together the data suggests the presence of both Th1 and Th2 type cells (233), resulting in stimulation of both cellular and humoral responses. The role of T-cells in boosting antibody responses was further emphasized in a recent publication showing increased production of BAFF by SjS T-cells (234). Activity of $CD8^+$ cytotoxic T-cells was shown by the presence of granzyme B and perforin in SjS salivary glands, but not controls (231, 235). These cells may play a role in the destruction of glandular epithelium, as well as the generation of novel antigenic epitopes (236).

The role of regulatory T-cells in SjS has only been studied in a limited number of papers, with conflicting results. Two studies report a decrease of Treg in peripheral blood of SjS patients (237, 238), while one reports a significant increase (239). These differences may stem from a difference in cut-off value of CD25 as a marker of Treg. Additional studies including the more recently described marker Foxp3 may shed more light on the frequency of Treg in SjS blood. One study did include Foxp3 in their analysis of SjS salivary glands, and describes a relatively small number of Treg compared to virally infected glands (237). Whether this has pathological relevance remains to be determined, as all three studies agree that Treg functionality is not impaired in SjS patients.

The role of DC in SjS

Infiltrates in the salivary glands of SjS patients contain large numbers of T- and B-lymphocytes. Both cell types have been the focus of many studies on SjS. These cells display increased activity, as evidenced by the high production of IFN-γ and the frequent occurrence of autoantibodies. Whether the so-called hyperactivation is an

intrinsic property of these cell types or the result of an underlying more general immune activation has received much less attention. As DC play a key role in the initiation and perpetuation of immune responses, alterations in this cell population result in aberrant immune activation and a break of tolerance to self. In SjS patients, increased numbers of both $CD1a^+$ and $CD83^+$ DC are present in the salivary glands (83). These cells are found both in the infiltrates, but also dispersed throughout the parenchyma. Focal lymphocytic infiltrations can occasionally be missed during biopsy, something which is much less likely for the presence of DC due to their scattered presence. Therefore, this cell type is an interesting candidate for the improvement of the diagnostic procedure. Furthermore, the expression of CD83 shows that salivary gland DC are activated and capable of stimulating T-lymphocytes, thus boosting the inflammatory response and providing an explanation for the increased T-cell activity observed in SjS patients. DC also play a role in the formation of auto-antibodies, as DC present antigen to B-cells in germinal centers as seen in SjS salivary gland tissue (240). This leads to proliferation and class-switching of B-cells and is an important step in antibody formation. In the NOD animal model for SjS, DC accumulate in the salivary glands prior to the infiltration of T- and B-cells (86), suggesting that DC are not only important in the perpetuation of the immune response, but may also be involved in the initiation phase.

High levels of IFN-α are found locally in the salivary glands of SjS patients (241). As numbers of NK cells are low, pDC are conderided the primary source of this cytokine. Stainings for the pDC markers CD123 and BDCA-2 indeed revealed the presence of pDC in diseased but not in healthy salivary gland tissue (84). As serum levels of IFN-α in SjS did not appear increased in initial studies, the increased pDC activation was considered a strictly local phenomenon.

Although the increased presence of DC in salivary glands in SjS has now been clearly established, the mechanisms leading to this accumulation and the functional role of cDC in the pathogenesis of the disease remain to be elucidated.

1.3 THE NOD MOUSE

1.3.1 Autoimmunity in the NOD mouse strain

Autoimmune phenomena in the NOD mouse

The NOD mouse strain is derived from a single female CTS mouse who spontaneously developed glucosuria (242). After inbreeding the offspring of this animal for six generations, an inbred mouse strain called the nonobese diabetic (NOD) mouse was generated which shows a diabetes incidence of around 80% in females and 20% in males at 30 weeks of age. Ultrastructural studies show that the NOD mouse developed lymphocytic infiltrations in the submandibular gland, similar to those seen in SjS patients (243). Since salivary flow also declined in NOD but not control mice with increasing age, this mouse strain

was proposed as a model for SjS (244). Similar to human SjS patients, NOD mice show increased frequencies of anti-nuclear antibodies as well as anti-SS-A antibodies (245). Although dacryoadenitis, which is common in SjS patients, also occurs in NOD mice, the gender distribution is different. In humans, SjS, including both sialoadenitis and dacryoadenitis, occurs nine times more frequently in females, while in mice, dacryoadenitis occurs more frequently in males (246).

The NOD mouse develops numerous other autoimmune phenomena, amongst others in the kidney, colon and, under the appropiate diet, thyroid gland (247). Furthermore, hemolytic anemia is observed, as well as immune mediated hearing loss (248). Since NOD mice are also susceptible to experimental autoimmune encephalitis, it was usually regarded as a typical Th1 biased animal (249). However, it is now known that the NOD mouse also shows exacerbation of the Th2 condition asthma in an OVA induced model (250), indicating that the immune aberrancies in this animal are more complex than merely a strong Th1 skewing.

Genetics of NOD sialoadenitis

Although a number of genetic regions involved in the pathogenesis of diabetes have been described in the NOD mouse, the genetic background of sialoadenitis is less obvious. For the development of diabetes, expression of the NOD specific MHC allele MHC I-A^{g7} is crucial. Replacing the allele by the MHC I-A^{b} or MHC I-A^{q} did not abrogate the development of SjS like symptoms including the formation of lymphocytic infiltrations (251). Furthermore, replacement of a large number of diabetes associated gene regions (termed *Idd* loci) did not result in a decrease in sialoadenitis (252). To date, only three genetic loci appear to influence the developent of SjS like disease in NOD animals. The first is located on chromosome 4 and is now termed the *Nss1* locus (251). This locus contains among others the genes for TLR12 and C1q components, which are upregulated in NOD animals. The other loci are located on chromosomes 1 and 3 and are termed *Idd5* or *Aec2* and *Idd3* or *Aec1*, respectively (253). NOD mice in which these loci are replaced by C57BL/6 alleles do not develop sialoadenitis, while C57BL/6 congenic for these two loci exhibit SjS like phenomena similar to NOD. *Idd3* contains the gene for IL-2 and IL-7 but does not induce SjS on a healthy background, while *Idd5* does and can overcome a number of protective alleles (254). Candidate genes responsible for the effect of *Idd5* include those for Bcl-2, IL-10 and extracellular matrix components such as fibronectin and laminin. Interestingly, normal mice congenic for *Idd5* and *Idd3* do not develop insulitis and diabetes.

Genetic susceptibility to thyroiditis of the NOD mouse can be enhanced with the introduction of the $H2^{h4}$ allele (255). This mouse also develops SjS like disease with lymphocytic infiltrations of the salivary and lachrymal gland, as well as a decrease in salivary flow. Additionally, an increased presence of anti-Ro and anti-La antibodies is seen in this animal when compared to the parental NOD strain (256).

1.3.2 Immune aberrancies in NOD sialoadenitis

The lymphocytic infiltrations seen in NOD submandibular salivary gland mainly contain $CD4^+$ T-cells, but also $CD8^+$ T-cells, B-cells, macrophages and DC. All of these cells likely play a role in the development of the pathology, either at initiation, at the effector stage, or both.

T-cell abnormalities

$CD4^+$ cells produce cytokines such as IFN-γ, IL-2, IL-4 and IL-13, supporting the development of $CD8^+$ T-cells and B-cells and stimulating the activity of the innate immune system. Blocking $CD4^+$ cells resulted in a lack of salivary gland pathology in NOD mice, indicating the importance of this subset in the development of sialoadenitis (257). Reports on intrinsic abnormalities in NOD $CD4^+$ cells are relatively few, but include decreased proliferation in response to concanavalin A and anti-CD3, reduced secretion of IL-2 and IL-4 and an enhanced Th1/Th2 balance (258-261). Submandibular $CD8^+$ cells express perforin and granzyme B (262) and have direct cytotoxic effects by releasing these products. Furthermore, $CD8^+$ derived enzymatic activity may play a role in the increased proteolysis observed in NOD animals, leading to the generation of altered self proteins and increased immunogenicity (201). In the NOD mouse, increased numbers of both CD4 and CD8 T-cells are present, in particular in the lymph nodes, although relative proportions seem normal when compared to control strains (263). One explanation for this is the resistance to apoptosis described in NOD thymocytes, leading to defective negative selection and increased thymic output. In addition, peripheral T-cells are less sensitive to activation induced cell death, resulting in higher numbers of both populations (264).

In initial studies, levels of the regulatory $CD4^+CD25^+$ T-cell subset were described to be decreased in NOD mice (265, 266). The suppression mediated by these cells *in vitro* was deficient, thus further hampering the regulatory properties of this subset. In contrast, one recent study reports normal levels of thymic and peripheral Treg in NOD mice, a difference possibly resulting from the use of additional Treg markers or a difference between the various NOD colonies used (267). A second study, however, describes NOD derived $CD4^+CD25^+$ Treg to be fully capable of inducing suppression *in vitro*, and suggests that the earlier findings of deficient Treg function may have been influenced by the type of APC used in these studies (268). Whether quantitative or qualitative Treg deficits are indeed involved in the pathogenesis of sialoadenitis in NOD remains unsolved for now.

B-cell abnormalities

B-cells are present in the infiltrates in NOD salivary glands and produce auto-antibodies which are important for the pathogenesis of salivary dysfunction. Depleting functional B-cells from NOD mice by antibodies or the use of transgenic techniques reduces the infiltration of salivary glands and prevents the loss of secretory function (187, 269). Transfer of IgG from normal NOD to B-cell depleted animals results in a clear decrease in salivary function. B-cells may also serve as APC, capturing antigen by cell surface

bound immunoglobulins. Similar to T-cells, increased numbers of B-cells are present in NOD lymphoid organs (263). In thymus, where only a small number of B-cells can be detected, levels are increased up to five fold in NOD mice. Both these findings may be the consequence of a relative increase in cell survival, which is found in NOD B-cells (270). The presence of autoreactive B-cells suggests a defect in tolerance induction in this subset as well as in T-cells (271).

NK and NKT cells

Activated NK cells release granules containing granzyme and perforin, and appear therefore as a candidate for the induction of cytotoxicity in NOD salivary glands. However, only few NK cells are found among the infiltrating cells (86). NK cell activity has been shown to be decreased in NOD mice, probably due to the lack of expression of the NK activating receptor NKG2D (272). NK cells are therefore not likely to play a role of importance in the pathogenesis of sialoadenitis in NOD.

Different results are obtained for the population of NKT cells. This population shows characteristics of both NK cells and T-cells in that it expresses the invariant T-cell receptor α chain as well as the NK cell markers NK1.1 and CD16 (273). NKT cells are CD1d restricted, and respond to glycolipids such as the stereotypical ligand α-galactoseceramide. NKT cells have been implicated in the induction of tolerance, and significantly lower numbers are found in young NOD animals (274). Furthermore, NKT cells present in NOD mice produce less IL-4, which is relevant since part of their regulatory function depends on skewing the immune system towards a more Th2 like phenotype by IL-4 secretion. Transferring additional NKT cells or stimulating the remaining population resulted in a therapeutic effect on diabetes development, but the effect on salivary gland inflammation has not been studied.

Macrophages and DC

Although many abnormalities have been described in the B-, T- and Treg cell populations in NOD mice, it remains to be determined whether these are the result of intrinsic aberrancies in these populations themselves or the consequence of abnormal stimulation by APC, including macrophages and DC. Macrophages and DC are generally considered to be at least partly derived from blood monocytes, and alterations in this cell type have been described in NOD mice. Among these are an increased proportion of Ly-6C^{low} monocytes, which is considered to be a relatively mature monocyte subset (275). NOD monocytes show increased adhesion to fibronectin and ICAM-1 *in vitro* as well as a defective recruitment to thioglycollate induced peritonitis (93, 95). These alterations may lead to altered monocyte trafficking and abnormal accumulation of monocyte-derived cell populations such as macrophages and DC.

Macrophages are present in the infiltrates in both pancreas and salivary gland of NOD mice. NOD macrophages produce higher levels of IL-12 upon LPS stimulation, but less glutathion and MHC class I, resulting in decreased antigen processing and presentation

(276-279). NOD macrophages are less efficient in clearing apoptotic cells and produce elevated amounts of IL-1β and TNF-α during this process (280), resulting in presentation of self-antigens in an immunogenic environment.

DC are of particular interest given their key role in the induction of both immunity and tolerance. *In vitro* DC generation from bone marrow in the presence of GM-CSF results in a lower yield in NOD mice, although one study reported an increased yield (96, 281, 282). This lower yield is due to a decreased proliferation as well as an increased apoptosis and does not result from a genetic defect in receptor expression as the generation of granulocytes is normal. The generated DC exhibit a relatively macrophage-like phenotype, with decreased expression of class II and co-stimulatory molecules CD80 and CD86 and increased expression of acid phosphatase (96, 283, 284). Upon stimulation with LPS, co-stimulatory markers were upregulated in NOD DC, but to a lower extent than in control DC. In most studies, T-cell stimulatory capacity is reduced in NOD BMDC, but not spleen DC (283, 285-287). Other reports describe an elevated antigen presenting function of NOD BMDC (288, 289). However, these studies only compared the effect of DC on T-cell proliferation and not on cytokine production, leaving the possibility that NOD DC have a specific skewing capacity on T-cell effector function, which was suggested in a recent report (282). Interestingly, NOD DC produce more IL-12 when stimulated with LPS, an effect which is mediated by increased activity of IKK and NFκB (290). This increase in IL-12 in combination with faulty antigen presentation may lead to a lack of tolerance induction and an increased immunogenicity. Treg stimulated in the presence of NOD DC were not able to suppress $CD4^+$ proliferation, whereas Treg stimulated by control DC were potent suppressors (268). Deletion of DC using a diphteria toxin model showed that cDC are crucial to the development of diabetes in the NOD mouse (89). Unfortunately, the effect of DC depletion on sialoadenitis was not reported in this study.

The relevance of DC in the pathogenesis of sialoadenitis became clear from kinetic studies into the development of the infiltrations in NOD submandibular glands.

While T-and B-cells start infiltrating the glands around 7-10 weeks of age, this was preceded by an accumulation of DC at the age of 3-5 weeks (86). DC accumulation was also seen in NOD.scid mice, showing that this phenomenon is independent of lymphocyte infiltration. The mechanism behind the DC accumulation has not been elucidated thus far, but may include increased migration from the blood as well as increased retention in the gland. Indeed, decreased migration and increased adhesion to fibronectin has been observed for NOD BMDC, suggesting a less active migratory phenotype (93, 95).

The effects of the accumulation of DC in NOD salivary gland remain to be determined, but may include priming and activation of autoreactive T-cells, promoting maturation and antibody production by plasma cells and chemoattraction of other leukocytic cells.

1.4 SCOPE OF THE THESIS

The accumulation of DC in NOD submandibular glands prior to the infiltration of T- and B-cells, the presence of DC in salivary glands of SjS patients and the fact that DC play a key role in initiating and steering immune responses lead to the hypothesis that DC and DC precursors (or monocytes) play a role in the initiation and perpetuation of SjS. To explore this hypothesis, monocytes, local salivary gland DC, pDC and a relevant chemoattractant were studied in SjS patients and NOD mice.

In chapter two, we describe that increased numbers of mature $CD16^+$ monocytes were found in SjS patients. These monocytes are shown to preferentially develop into mature DC similar to those found in SjS salivary glands, indicating that the increased frequency of $CD16^+$ monocytes may result in the accumulation of DC in salivary glands and contribute to the pathology.

In chapter three, an elevated expression of type I interferon associated genes is shown in SjS peripheral blood monocytes. This expression is induced by increased type I interferon activity in SjS serum, and possibly due to the increased activity of circulating pDC in these patients. The increased type I interferon activity correlates to the presence of anti-SS-B and may influence systemic pathology in SjS patients.

In chapter four, DC isolated from NOD salivary glands are characterized and a complete lack of CCR5 surface expression on these cells is described. This lack is partly due to the microenvironment as DC obtained from other sources only show a partial decrease. Possibly due to the lack of surface expression, available levels of CCR5 ligands are increased, and DC are more sensitive to the induction of IL-12, leading to a more pro-inflammatory environment in the salivary gland.

In chapter five, the expression of fractalkine, a chemoattractant relevant for mature monocytes, is studied in the NOD mouse model. Expression is detected both in NOD and control mice, but NOD mice show an increased and aberrant cleavage, resulting in generation of an autoantigenic products and the formation of anti-fractalkine autoantibodies.

In summary, this thesis shows alterations in monocyte populations, local cDC and circulating pDC populations and the expression of a monocyte chemoattractant, all resulting in the promotion of local or systemic inflammation and contributing to the pathogenesis of SjS.

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2

SYSTEMIC INCREASE IN TYPE I INTERFERON ACTIVITY IN SJÖGREN'S SYNDROME - A PUTATIVE ROLE FOR PLASMACYTOID DENDRITIC CELLS

Manon E. Wildenberg, Cornelia G. van Helden-Meeuwsen, Joop P. van de Merwe, Hemmo A. Drexhage and Marjan A. Versnel

Dept. of Immunology, Erasmus MC, Rotterdam, The Netherlands

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SUMMARY

In the salivary glands of primary Sjögren's Syndrome (pSjS) patients, type I interferon activity is increased, but systemic levels of type I interferon proteins are rarely detected. This study focused on the systemic activity of type I interferon in pSjS, as well as the role of peripheral plasmacytoid dendritic cells (pDC).

Monocytes obtained from pSjS patients showed an increased expression of 40 genes. Twenty-three of these genes (58%), including *IFI27*, *IFITM1*, *IFIT3* and *IFI44* were inducible by type I interferon. pSjS serum had an enhanced capability of inducing *IFI27*, *IFITM1*, *IFIT3* and *IFI44* in the monocytic cell line THP-1, likely due to the action of IFN-beta. This effect could be inhibited by blocking the type I IFN receptor, supporting a high type I interferon bioactivity in pSjS serum. In addition, circulatory pDC showed increased expression of CD40. This expression was correlated to the expression level of the type I interferon regulated genes *IFI27* and *IFITM1* in monocytes of the same individual.

This study indicates that the increased type I interferon activity observed in pSjS patients is not only a local but also a systemic phenomenon and points to pDC as a possible source of this activity.

INTRODUCTION

Sjögren's Syndrome (SjS) is an autoimmune disease characterized by lymphocytic infiltrates in the salivary and lachrymal glands. Patients mainly suffer from dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca), while multiple extraglandular manifestations such as fatigue, myalgia and arthralgia are frequently present and form a major cause of morbidity. SjS can occur in the absence of other auto-immune diseases (primary SjS, pSjS) or together with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) (secondary SjS).

The pathogenesis of pSjS remains unclear, but studies have indicated a role for aberrancies in both the immune system and the target organs. Regarding the immune system, focus has been on the B-cells, due to the high prevalence of autoantibodies (1-3).

Although B-cell hyperactivity has indeed been described (4, 5), the question remains whether these alterations are B-cell intrinsic or caused by alterations in other parts of the immune system, such as innate immunity. Recently, aberrancies have been described in the type I interferon system in salivary glands of pSjS patients. Type I interferon plays an important role in the innate immune system by inhibiting viral replication, activating natural killer cells, boosting the generation and activation of dendritic cells (DC) and enhancing antibody responses (6-10). In salivary glands of pSjS patients, an increased activation of the type I interferon was found by gene array studies (11, 12). Also, the presence of the stereotypical type I interferon IFN-alpha was described in pSjS labial biopsies (13).

However, since serum IFN-alpha could only be detected in very few patients, the increased type I interferon was believed to be a local phenomenon.

One of the main producers of type I interferon is the plasmacytoid dendritic cell (pDC) (14). As pDC are readily detectable in salivary glands of pSjS patients, but not in healthy controls, these cells are considered an important source of local type I interferon (11). In SLE, a similar influx of pDC is seen in inflamed skin sections, while numbers of peripheral pDC are decreased (15, 16). Also in other immune disorders such as multiple sclerosis and diabetes alterations in number and activation status of peripheral pDC have been reported (17, 18), but thus far, no data are available on the status of peripheral pDC in pSjS.

In the current study, the systemic activity of the type I interferon system in pSjS was studied by gene expression profiling on the two main subtypes of peripheral blood monocytes. Both subsets showed a significant upregulation of type I interferon inducible genes, which correlated with an increased type I interferon activity in the serum. Additionally, although the number of pDC was decreased in the blood of pSjS patients, the remaining pDC showed signs of increased activation, pointing to these cells as a possible source of the increased systemic type I interferon activity in pSjS.

MATERIALS AND METHODS

Patient samples

Heparinized blood and serum samples were obtained from 22 female primary pSjS patients (mean age 52.2 +/- 15.7) at the Erasmus MC, Rotterdam, The Netherlands. Inclusion criteria consisted of a positive diagnosis according to the criteria of the American-European consensus group (19). Patient characteristics are summarized in Table I. Fourteen healthy female controls not suffering from autoimmune diseases were included (mean age 46.2 +/- 6.0). Informed consent was obtained from all patients and controls, and the study was approved by the Medical Ethics Committee of the Erasmus MC.

Microarray analysis

Microarray analysis was performed to compare two pooled monocyte samples of patients with those of gender and age matched controls. Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll gradient (Amersham, Little Chalfont, England). PBMC of 5 subjects were pooled and stained using CD14-APC and CD16-PE (BD, San Jose, CA). Subsequently, $CD14^{+}CD16^{-}$ and $CD14^{low}CD16^{+}$ cells were sorted on a FACS Vantage cell sorter (BD, purity >95%) and total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). Quality of RNA was checked using a 2100 Bioanalyzer (Agilent technologies, Amstelveen, The Netherlands). RNA was processed to cDNA and aRNA as described previously (20). Gene expression profiles were generated using Affymetrix Human Genome U133 Plus 2.0 chips and analyzed as described previously (20).

Table I. Patient characteristics.

Patient	Age	Duration of illness	SS-A	SS-B	Hydroxy-chloroquine	Pilocarpine	Extraglandular manifestations	Affymetrix	RQ-PCR
1	25	11	+	+	+	-			
2	66	5	+	+	+	-			
3	42	1	+	+	+	+		+	
4	36	13	-	-	+	+	polyneuropathy		
5	48	18	+	+	+	-		+	
6	65	33	+	-	-	-	Raynaud	+	
7	25	3	+	+	+	+			+
8	39	8	+	+	+	-		+	+
9	75	1	+	+	+	-			
10	68	10	+	+	-	+			
11	75	1	+	-	-	-			+
12	36	2	+	+	+	+		+	
13	70	2	+	-	+	-	Raynaud		
14	58	5	+	+	+	+	polyneuropathy	+	+
15	49	4	+	+	+	-		+	
16	36	1	+	-	+	-			+
17	64	5	+	+	-	-			+
18	72	3	+	+	-	+	polymyalgia		+
19	50	5	+	-	+	-	Raynaud		
20	44	3	+	+	-	+		+	
21	51	13	+	-	+	-		+	
22	55	1	+	-	-	-		+	

Briefly, probe intensity background was removed using robust multichip analysis (21) and intensity levels were quantile normalized (22). Array groups corresponding to pSjS and controls (two arrays per group, 5 pooled individuals per array) were compared based on the perfect match probe intensity levels only (21), by performing a per-probe set two-way analysis of variance (ANOVA, with factors probe and group). This resulted in average expression levels over the two repeats for each probe set in each group as well as a *p*-value for the significance of the difference between the groups (23). The *p*-values were adjusted for multiple testing using Sidak step-down adjustments (24), and differences with adjusted *p*-values <0.05 were considered significant. Microarray data were deposited in the ArrayExpress database (http://www.ebi.ac.uk/), and are available under accession number E-MEXP-1242.

Real time Quantitative Polymerase Chain Reaction (RQ-PCR)

Monocytes were isolated using CD14 microbeads (Miltenyi, Bergisch Gladbach, Germany) and AutoMacs sorting (Miltenyi). Total RNA was isolated using a RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was generated using Superscript II (Invitrogen, Carlsbad, CA) and random hexamer primers (Amersham). RQ-PCR analysis was carried out using predesigned primer/probe sets (Applied Biosystems) according to the manufacturers suggestions. For calculation of relative expression, all samples were normalized against expression of the household gene *Abl* (25).

Flow cytometry

PBMC were stained using BDCA-2-APC (Miltenyi), CD40-FITC (Serotec, Oxford, England), CD83-PE (Immunotech, Marseille, France), CD86-FITC and isotype controls (all BD). Fluorescence was measured on a FACS Calibur and analyzed using CellQuest software (both BD). BDCA-2^{+} cells were gated and expression values of activation markers were calculated as MFI specific staining - MFI isotype control. Relative expression was calculated as expression level relative to the average expression level in controls.

IFN-alpha and IFN-beta protein level and bio-assay

IFN-alpha protein levels were determined using the Human IFN-alpha ELISA kit (Biosource, Camarillo, CA), which is based on the international reference standard for IFN-alpha as supplied by the National Institutes of Health, Bethesda, MA. IFN-beta protein level was determined using the Human IFN-beta ELISA kit (PBL InterferonSource, Piscataway, NJ). For measurements of type I interferon bio-activity, $2.5x10^5$ THP-1 cells were cultured in RPMI 1640 medium containing 25 mM HEPES and L-glutamine supplemented with 10% FCS, 60 ug/mL penicillin and 100 ug/mL streptomycin (all BioWhittaker, Verviers, Belgium). An equal volume of patient serum was added and incubated for 16 hours. As a positive control, recombinant human IFN-alpha (5 ng/ml, Peprotech, London, UK) was added. For blocking purposes, THP-1 cells anti-IFN alpha/beta R2 as well as IgG2a antibody control were added at 5 ug/ml for the entire duration of the assay (both R&D Systems Inc., Minneapolis, MN). Gene expression was determined by RQ-PCR.

Statistical analysis

Data were analyzed by Mann-Whitney U test and Spearman Rho correlations using SPSS software (SPSS Inc., Chicago, IL) and considered statistically significant if $p<0.05$. Box plots represent median and upper and lower quartiles with whiskers representing range.

RESULTS

Gene expression profile in pSjS monocytes

Expression profiles of the major subpopulation of $CD14^{+}CD16^{-}$ monocytes isolated from pSjS patients were compared with those of gender and age-matched controls. When the threshold for minimal fold change was set to 2, significant upregulation was found for 54 probe sets, representing 40 genes (Table II). Furthermore, 7 probe sets representing 7 individual genes were downregulated in pSjS (Table III).

A clustering of type I interferon related genes was observed, in that 23 (58%) of the genes with increased expression in pSjS have been described to be upregulated by type I interferon (Table II). When the smaller subset of $CD14^{low}CD16^{+}$ monocytes was analyzed, a similar type I interferon signature was observed (data not shown). Therefore, validation of these results was carried out on total CD14 monocytes instead of monocyte subsets. For this purpose, four of the genes identified by microarray analysis were tested by RQ-PCR on individual pSjS patients and controls (Figure 1). To validate both genes upregulated strongly and genes upregulated to a lesser degree, two type I interferon related genes with a differential expression >4 fold were chosen (*IFI27* and *IFITM1*), as well as two genes showing a differential expression <4 fold (*IFIT4* and *IFI44*). *IFI27*, *IFIT4* and *IFI44* again showed significantly increased expression in pSjS, while *IFITM1* showed a similar trend (p=0.07). When the mean of all controls plus 2 standard deviations was used as a cut-off value, 86% of pSjS scored positive for *IFI27*, 57% for *IFITM1*, 67% for *IFIT1* and 71% for *IFI44*, while none of the controls scored positive for any of these genes.

Increase in type I interferon activity in pSjS serum, probably due to IFN-beta

As the type I interferon signature found in pSjS monocytes might be explained by an increased concentration of these cytokines in the blood, ELISA analyses of the stereotypic type I interferons IFN-alpha and IFN-beta were performed in pSjS and control serum samples. IFN-alpha could only be detected in a small number of pSjS patients (5/23) and not in controls (Figure 2A). IFN-beta could be detected in most samples, and was increased in pSjS serum compared to controls, although this did not reach statistical significance (Figure 2B).

Difficulties in detecting serum type I IFN have been described earlier, and are probably due to the large number of subtypes. Therefore, the total activity of type I interferon was measured using a monocyte bio-assay approach. THP-1 cells were cultured in the presence of pSjS or control serum, and expression of the type I interferon regulated genes *IFI27*, *IFITM1*, *IFIT4* and *IFI44* was determined by RQ-PCR. As a control, exogenous type I interferon was used, which upregulated all four genes in this cell line (Figure 2C). A significantly higher expression of *IFI27* and *IFIT4* was found when THP-1 cells were incubated with pSjS serum (Figure 2C), with a similar trend for *IFITM1* and *IFI44* (p=0.09 and p=0.07 respectively). To test if this was indeed mediated by type I interferon, the effect of blocking the type I interferon receptor was studied. When THP-1 cells were incubated

Table II. Genes upregulated in pSjS $CD14^{+}CD16^{-}$ monocytes.

Fold	Gene symbol	Description
12.4	IFI27	**interferon alpha inducible protein**
4.4	IFI44L	**interferon inducible protein 44 like**
4.1	IFITM1	**interferon induced transmembrane protein 1**
4	SIGLEC	sialoadhesin
3.8	CCL2	CCL2
3.5	RSAD2	**iron ion binding**
3.5	IFIT4	**interferon induced protein with tetratricopeptide repeats 4**
3.4	USP18	**ubiquitin specific protease 18**
3.3	SERPING1	**serine protease inhibitor, clade G, member 1**
3.3	IFIT1	**interferon induced protein with tetratricopeptide repeats 1**
3.1	LGALS3BP	galectin 6 binding protein
3.1	HERC5	**Hect domain and RLD 5**
2.9	LOC129607	hypothetical protein 129607
2.9	EPSTI1	epithelial stromal interaction 1
2.9	OASL	**2-5 oligoadenylate synthetase like**
2.9	ISG15	**ISG15 ubiquitin-like modifier**
2.7		transcribed locus
2.6	KLHDC7B	kelch domain containing 7B
2.6	LY6E	**lymphocyte antigen 6 complex, locus E**
2.6	NEXN	nexilin
2.6	IFIT2	**interferon induced protein with tetratricopeptide repeats 2**
2.5	IFI44	**interferon inducible protein 44**
2.4	OAS3	**2-5 oligoadenylate synthetase 3**
2.4	ANKRD22	ankyrin repeat domain 22
2.4	CDC42SE2	cdc42 small effector 2
2.4	GBP5	**guanylate binding protein 5**
2.4	CXCL10	**chemokine ligand 10**
2.4	MX1	**myxovirus restistance 1**
2.4	GBP1	**guanylate binding protein 1**
2.4	S100P	S100 calcium binding protein P
2.3		transcribed locus
2.3	SAMD9L	sterile alpha motif domain containing 9-like
2.3	OAS2	**2-5 oligoadenylate synthetase 2**
2.2	RGC32	response gene to complement 32
2.2	BIRC4BP	**XIAP associated factor 1**
2.2	GMPR	guanosine monophosphate reductase
2.2	DNAPTP6	DNA polymerase-transactivated protein 6
2.1	FLJ20035	hypothetical protein FLJ20035
2.1	STAT1	**Stat 1**
2	IFITM3	**interferon induced transmembrane protein 3**

Gene profiling was performed on pooled samples of pSjS patients and controls using microarray analysis. Genes with a significant (p<0.05) >2 fold differential expression are shown. Genes regulated by type I interferon are shown in bold.

Table III. Genes dowregulated in pSjS CD14^{+}CD16^{-} monocytes.

Fold	Gene symbol	Description
2.5	NOTCH2	notch homologue 2
2.3	HLADQA1	human leukocyte antigen DQ-A1
2.3	NEBL	nebulette
2.2		transcribed locus
2.2	GRAP	GRB2 related adaptor protein
2.1	PELI1	pellino homolog 1
2.1	FLJ22662	hypothetical protein 2

Gene profiling was performed on pooled samples of pSjS patients and controls using microarray analysis. Genes with a significant (p<0.05) >2 fold differential expression are shown.

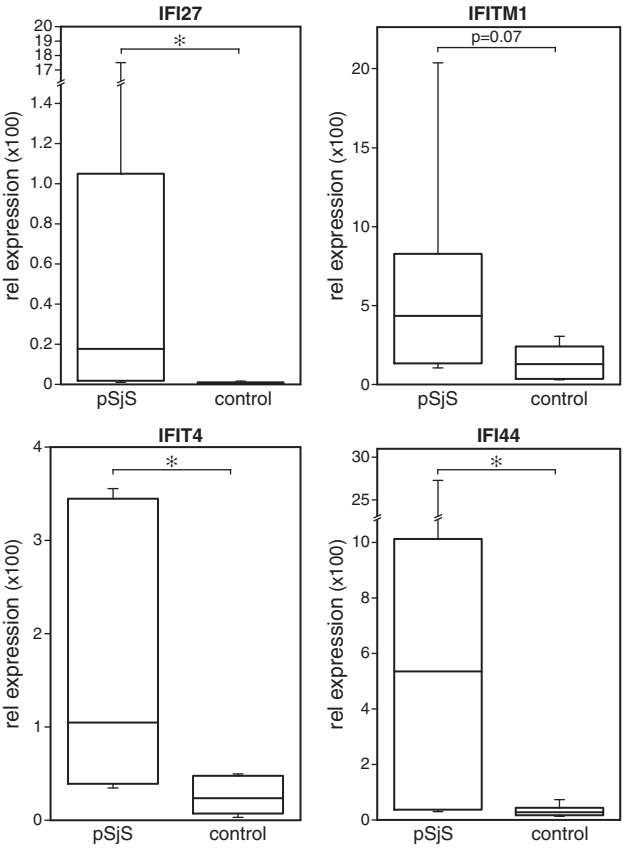

Figure 1. Type I interferon related genes are upregulated in pSjS.
Monocytes of pSjS and controls (n=7) were isolated using anti-CD14 beads, RNA was isolated and gene expression was determined by RQ-PCR. Box plots represent median with upper and lower quartiles, whiskers represent range, *p<0.05.

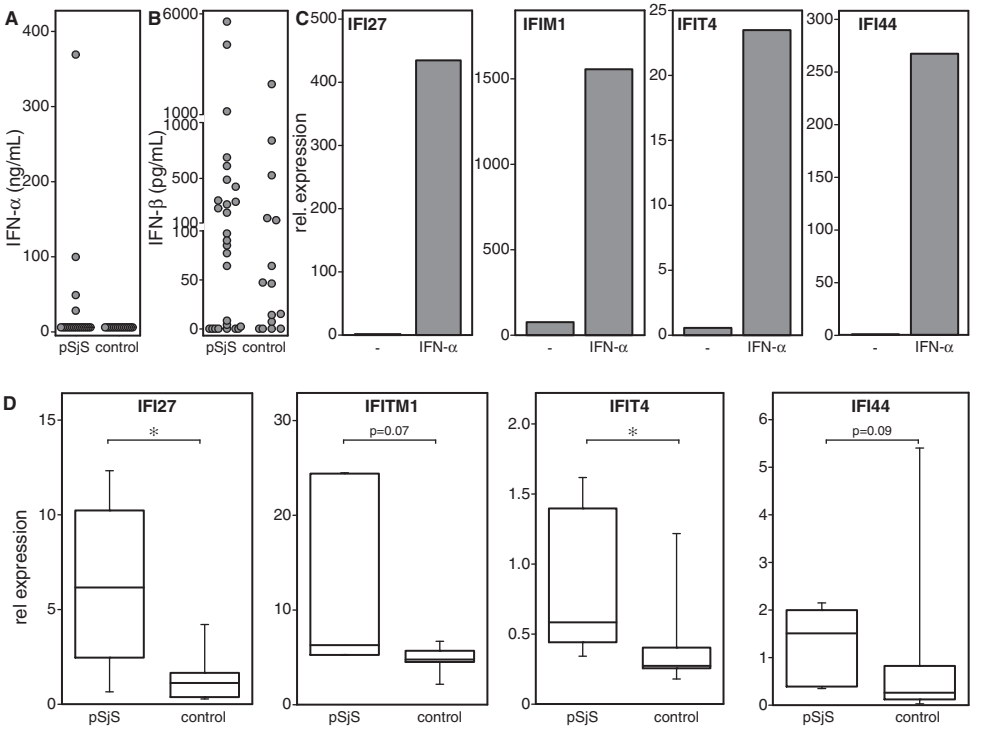

Figure 2. IFN-alpha and -beta protein and type I interferon activity in pSjS and control serum. IFN-alpha (**A**) and IFN-beta (**B**) protein in serum were measured by commercial ELISA (pSjS *n*=20, controls *n*=15). (**C**) THP-1 cells were stimulated with recombinant IFN-alpha (100U/ml) for 16 hours, gene expression levels were measured by RQ-PCR. (**D**) Type I interferon activity was measured using a reporter cell assay. THP-1 cells were incubated with pSjS or control serum (*n*=6-8) and expression of type I interferon induced genes was determined by RQ-PCR. Box plots represent median with upper and lower quartiles, whiskers represent range, **p*<0.05.

with pSjS serum in the presence of a blocking antibody, induction of *IFI27*, *IFITM1* and *IFI44* was completely inhibited, while induction of *IFIT3* was partially inhibited (Figure 3A). As an isotype control did not exert this effect, this indicates that the increased expression of these genes is indeed caused by an increased type I interferon activity in the serum.

Given the increased expression of IFN-beta in pSjS serum, we hypothesized that this cytokine was mainly responsible for the type I IFN effect seen in these patients. To test this, expression of genes differentially regulated by IFN-alpha (*PDXK* and *VEGF-C (26)*) and IFN-beta (*CXCL11* and *BCLAF-1 (27)*) was determined. Expression of *CXCL11* was significantly increased in pSjS, with *BCLAF-1* following the same trend (Figure 3B). IFN-alpha regulated genes did not differ between pSjS and controls, strongly suggesting that the increased systemic type I IFN activity observed in these patients is due to IFN-beta.

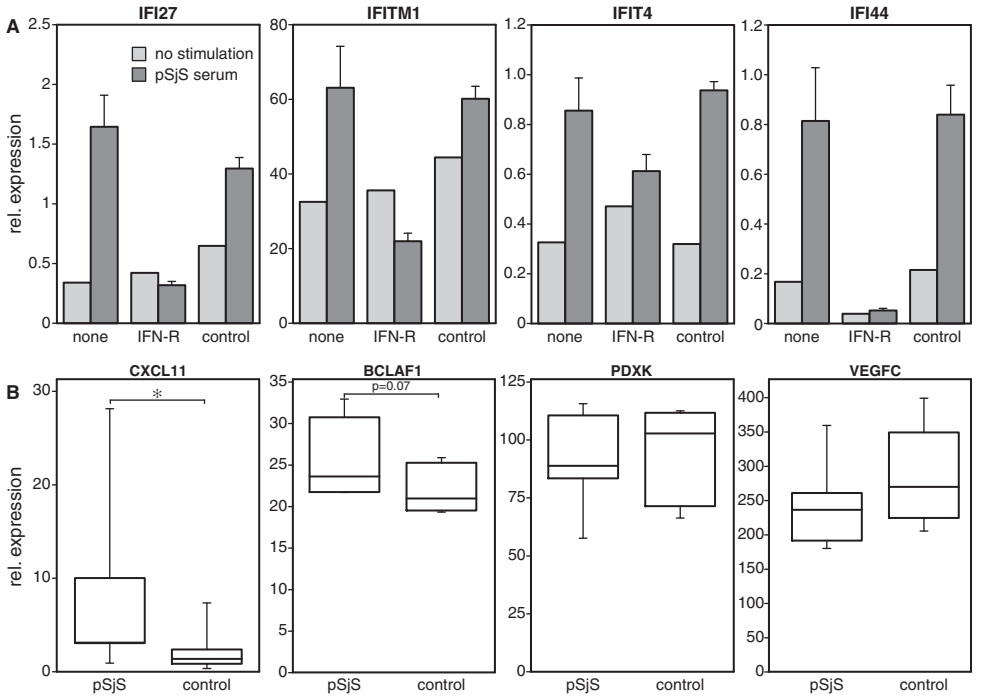

Figure 3. Type I IFN activity is mediated through the type I IFN receptor.
(**A**) THP-1 cells were incubated with pSjS serum in the presence of a blocking IFN-alpha/beta receptor antibody or isotype control. Gene expression levels were determined using RQ-PCR. (**B**) Expression of genes differentially regulated by IFN-alpha and beta were measured in pSjS monocytes using RQ-PCR (n=7). Box plots represent median with upper and lower quartiles, whiskers represent range, *p<0.05.

Increased activation of pDC in pSjS

The main type I interferon producer in the blood is the pDC (28), making this an interesting candidate for the increased type I interferon activity observed in pSjS serum. The relative number of pDC was significantly decreased in peripheral blood of pSjS patients when compared to controls (median 0.31 and 0.46% respectively, p=0.006, Figure 4A). In SLE, a similar decrease has been observed and here the general hypothesis is that decreased pDC numbers in peripheral blood are the result of an increased systemic activation of these cells, followed by increased migration to peripheral sites (15). Since increased numbers of pDC are found in pSjS salivary glands (11), this mechanism may also account for the decreased numbers of pDC in pSjS. Therefore, activation of pDC was measured by determining cell surface expression of activation markers CD40, CD86 and CD83 (17, 29). Indeed, expression of CD40 was significantly higher in pDC obtained from pSjS, with expression of CD86 showing a similar trend (Figure 4B-C). No difference in CD83 expression was found (Figure 4D).

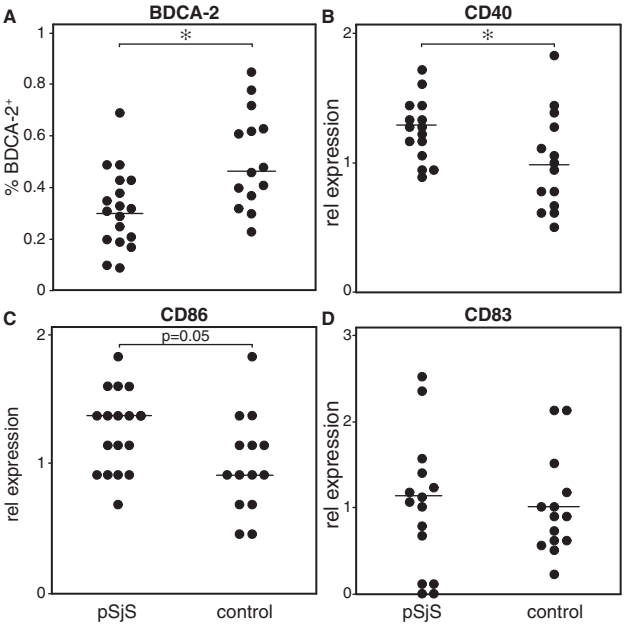

Figure 4. Relative number of pDC is decreased and activation is increased in pSjS.
PBMC of pSjS (*n*=19) and controls (*n*=15) were stained for BDCA-2, CD40, CD86, CD83 and control IgG. (**A**) The percentage of BDCA-2$^+$ cells of all nucleated cells was calculated. (**B**) BDCA-2$^+$ cells were gated and expression level of co-stimulatory markers was defined as MFI of specific staining - MFI of control, relative expression is calculated as expression level relative to the average expression level of controls, **p*<0.05.

Interestingly, expression of CD40 on pDC correlated significantly with the expression level of *IFI27* and *IFITM1* in monocytes of the same individual (Spearman's Rho 0.708 and 0.664 respectively, $p<0.05$), supporting the hypothesis that the systemic type I interferon signature in patients is linked to a higher systemic pDC activation.

DISCUSSION

The data presented here show that pSjS monocytes display upregulation of a large number of genes associated with interferon exposure. Although it should be pointed out that some of these genes can also be upregulated by IFN-gamma, the specific combination of genes points to a 'type I interferon signature'. A similar signature has been observed in PBMC of patients suffering from dermatomyositis and SLE (30-33). Also, in SLE, serum levels of type I interferon have been found to be increased in SLE patients (34, 35), thus explaining the specific upregulation of the genes in question. In pSjS, the existence of a type I interferon signature has been reported in the salivary glands (11-13). This type I

interferon signature in pSjS was thought to be a strongly local phenomenon in the salivary glands, as serum type I interferon could only be detected in very few patients. The current study shows that the type I interferon signature is in fact present in peripheral blood monocytes of pSjS patients, clearly indicating that the interferon activity is also increased systemically.

It has been shown previously that some monocyte isolation procedures may lead to activation. Therefore, two different isolation methods were used for the Affymetrix analysis and the subsequent RQ-PCR validation, with both producing similar results, indicating that the increased activation is not due to the particular type of isolation.

The main source of type I interferon in the blood is generally considered to be the pDC. Upon activation, these cells produce large amounts of type I interferon, stimulating both innate and adaptive immune responses. The systemic type I interferon signature in SLE patients led to the hypothesis that pDC play a role in this disease. Numbers of blood pDC are decreased in SLE patients, while an influx is observed in inflamed skin, indicating that the decreased presence of pDC in blood might be the result of increased peripheral activation and subsequent migration to the skin (15). Similarly in pSjS, the increase in pDC in the salivary glands was hypothesized to be caused by increased activation in the periphery (11), but data supporting this idea was not available. This study shows that in pSjS, the relative number of pDC in the blood is decreased significantly, while the remaining pDC indeed show signs of increased activation Although exposure of pDC to type I interferon *in vitro* did not result in downregulation of BDCA-2, we cannot formally exclude the possibility that prolonged exposure to type I interferon *in vivo* leads to a decreased expression of BDCA-2 on a per cell basis, rather than decreased numbers of pDC. However, this appears unlikely, as BDCA-2 levels expressed on pSjS pDC were higher than those on control pDC (data not shown).

Interestingly, an earlier report showed that pSjS serum combined with apoptotic material is capable of inducing pDC activation, probably mediated by immune complexes consisting of auto-antibodies and nucleic acids (13, 36). Whether this mechanism is also responsible for the results of this study remains to be established. The correlation between CD40 expression on pDC and expression of type I interferon inducible *IFI27* and *IFITM1* in monocytes of the same individual supports the idea that the systemic type I interferon signature in pSjS patients may be linked to a systemic increase in pDC activation. However, this does not exclude the possibility that pDC present in the salivary glands are not only responsible for the local expression of type I interferons, but also contribute to the systemic activity. Additionally, myeloid DC have been reported to produce type I interferon, mainly in the context of specific viral infections (37, 38). To determine if the myeloid DC present in pSjS salivary glands also exhibit this phenotype will require further investigation.

The systemic upregulation of the type I interferon pathway may have several consequences. Type I interferon increases the differentiation of monocytes into highly immunogenic dendritic cells, thus increasing the ongoing immune response (39).

Monocytes obtained from pSjS patients showed normal expression of chemokine receptors CCR2 and CX3CR1 and adhesion molecule CD62L, indicating that the type I interferon stimulation did not alter the migratory phenotype of these cells. Protein levels of HLA-DR on peripheral monocytes did not differ between pSjS and controls (M.E.W., manuscript in preparation).

Furthermore, type I interferon stimulation of DC has been reported to increase the production of BAFF and APRIL (40). Interestingly, BAFF was found to be upregulated significantly in pSjS monocytes on the mRNA level (data not shown). BAFF plays a role in survival of (auto-reactive) B cells and promotes CD40 independent class switch recombination and have indeed been found to be increased both systemically and in the salivary glands of pSjS patients (41-44). Interestingly, expression of CD40 by pDC was significantly higher in pSjS patients positive for anti-SS-B antibodies (data not shown). Finally, type I interferon activates other immune cells, including natural killer (NK) cells, leading to increased lytic activity and cytokine production. The systemic type I interferon signature described in this study could therefore lead to a general increased activation of the immune system, as well as a specific increase in the survival of autoreactive B-cells. Recently, the development of a pSjS specific disease activity index has been described (45). Further research will have to show if disease activity is related to serum activity of type I interferon in pSjS patients.

Additionally, the increase in systemic type I interferon activity may be important in the pathogenesis of the extraglandular manifestations in pSjS. One of the main systemic complaints of pSjS patients is the occurrence of a debilitating fatigue, a symptom which is also present in the majority of patients receiving IFN-alpha therapy for various diseases (46). Similarly, complaints such as myalgia and arthralgia, frequently occurring in pSjS, have been found to increase strongly in patients receiving exogenous type I interferon. Interestingly, treatment with anti-TNF antibodies has been shown to be ineffective in pSjS patients (47). Given the cross regulation of TNF-alpha and type I interferon (48), it could be envisaged that this is at least partly due to a consequent increase in type I interferon activity.

This study clearly shows that the upregulation of the type I interferon pathway in pSjS is a systemic phenomenon and not just a local effect, thus expanding the role for type I interferon in the pathogenesis of this disease. Since the data presented here indicate pDC as a possible source for the increased type I interferon activity, this cell type may be a promising target for future drug development.

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The authors declare no conflicts of interest.

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3

INCREASED FREQUENCY OF CD16^{+} MONOCYTES AND THE PRESENCE OF ACTIVATED DENDRITIC CELLS IN SALIVARY GLANDS IN PRIMARY SJÖGREN'S SYNDROME

Manon E. Wildenberg[1], Jojanneke M.C. Welzen-Coppens[1], Cornelia G. van Helden-Meeuwsen[1], Hendrika Bootsma[2], Arjan Vissink[3], Nico van Rooijen[4], Joop P. van de Merwe[1], Hemmo A. Drexhage[1] and Marjan A. Versnel[1]

[1]Dept. of Immunology, Erasmus MC, Rotterdam, The Netherlands
[2]Dept. of Rheumatology and Clinical Immunology, University Medical Center Groningen, The Netherlands
[3]Dept. of Oral and Maxillofacial Surgery, University Medical Center Groningen, The Netherlands
[4]Dept. of Molecular Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands

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ABSTRACT

Objectives

In the salivary glands of primary Sjögren's Syndrome (pSjS) patients an accumulation of dendritic cells (DC) is seen, which is thought to play a role in stimulating the local inflammation. Aberrancies in subsets of monocytes, generally considered the blood precursors for DC, may play a role in this accumulation of DC. This study aimed at determining the level of mature $CD14^{low}CD16^{+}$ monocytes in pSjS and their contribution to the accumulation of DC in pSjS.

Methods

Levels of mature and immature monocytes in pSjS patients (*n*=19) and controls (*n*=15) were analyzed by flow cytometry. The reverse transmigration system was used for generation of DC generated from monocyte subsets. The phenotype of DC in pSjS salivary glands was analyzed using immunohistochemistry. *In vivo* tracking of monocyte subsets was performed in a mouse model.

Results

Increased levels of mature $CD14^{low}CD16^{+}$ monocytes were found in pSjS patients (14.5% +/- 5.5 vs 11.4% +/- 3.4). These cells showed a normal expression of chemokine receptor and adhesion molecules. Mature monocytes partly developed into $DC\text{-}LAMP^{+}$ (19.6% +/- 7.5) and $CD83^{+}$ (16% +/- 9) DC, markers also expressed by DC in pSjS salivary glands. Monocyte tracking in the NOD mouse showed that the homologue population of mature mouse monocytes migrated to the salivary glands, and preferentially developed into $CD11c^{+}$ DC *in vivo*.

Conclusion

Mature monocytes are increased in pSjS and both patient and mouse data support a model where this mature monocyte subset migrates to the salivary glands and develops into DC.

INTRODUCTION

Primary Sjögren's Syndrome (pSjS) is an autoimmune disorder characterized by focal lymphocytic infiltrates in the salivary and lachrymal glands (1). Although the pathogenesis remains to be fully elucidated, several findings indicate involvement of the innate immune system. These include increased expression of Toll-like receptors and upregulation of the type I interferon system (2-5). Furthermore, in labial biopsies, increased numbers of dendritic cells (DC) were observed (6). Interestingly, in the NOD animal model for pSjS, a strong accumulation of DC was observed prior to the appearance of infiltrates (7).

DC are potent antigen presenting cells and play a key role in the development of immune responses by stimulating T-cells and boosting B-cell responses (8-10). Therefore, the increased presence of DC is likely to play a role in the initiation and perpetuation of local immunity.

The increased presence of DC in pSjS salivary gland may involve aberrancies in the precursor population of blood monocytes. Interestingly, monocyte alterations have been described in other autoimmune conditions such as RA and diabetes, and include increased expression of Fc receptors, increased adhesion to extracellular matrix and shifts in monocyte subsets (11-13). In humans, monocytes can be separated in two populations, based on expression of CD14 and CD16. The majority of monocytes is $CD14^{+}CD16^{-}$, but approximately 10% is $CD14^{low}CD16^{+}$ and this is considered to be a relatively mature population (14, 15). $CD16^{+}$ mature monocytes are further characterized by high levels of CX3CR1 and low levels of CCR2 and CD62L (16). $CD16^{-}$ immature monocytes have been hypothesized to convert into mature $CD16^{+}$ (17), which in turn develop into DC. Indeed, $CD16^{+}$ monocytes have been shown to preferentially develop into tissue trafficking DC in an *in vitro* model (17). In the mouse system, the populations of Ly-$6C^{high}$ and Ly-$6C^{low}$ monocytes are considered to be homologous to the human $CD16^{-}$ and $CD16^{+}$ subsets (15). Supporting the idea of a kinetic relationship between the two subsets, in mice it has been shown that *in vivo*, Ly-$6C^{high}$ monocytes develop into Ly-$6C^{low}$ monocytes (18).

Previously, increased levels of mature monocytes were described in RA and infectious diseases such as HIV and sepsis (13, 19-21). In human pSjS, no data has been reported on the levels of mature monocytes. However, the NOD mouse (22), the mature monocyte subset is increased significantly (23). The purpose of this study was to determine if the mature monocyte subset is expanded in pSjS and if DC derived from this subset contribute to the accumulation of activated DC observed in the salivary glands of these patients.

MATERIALS AND METHODS

Patients and controls

Heparinized blood samples were obtained from 19 female pSjS patients fulfilling the American-European consensus criteria (24) (mean age 52.5 +/- 16.8, mean disease duration 8.2 yrs +/- 9.1) at Erasmus MC, Rotterdam, The Netherlands (Supplementary table 1). Patients treated with corticosteroids were excluded. Disease duration was determined as time from initial diagnosis to time of inclusion. Control samples were obtained from 15 female volunteers (mean age 46.2 +/- 6.0) not suffering from autoimmune diseases or taking immunosuppressive medication. Labial biopsies of six subjects were obtained during diagnostic biopsy procedures at the Erasmus MC. Two patients were diagnosed as pSjS according to the criteria mentioned above, four did not meet any of the objective criteria for pSjS and were considered controls. Parotid biopsies of two patients fulfilling the criteria for pSjS were obtained at the University Medical Center Groningen,

The Netherlands in the course of another study (25). All subjects gave informed consent and experimental procedures were approved by the Medical Ethical Committees of Erasmus MC and University Medical Center Groningen.

Mice

NOD/LTj mice were bred and housed in our own facility under specified pathogen free conditions. All experimental procedures were approved by the Erasmus University Animal Ethical Committee.

Cell isolations

PBMC were isolated from peripheral blood using a Ficoll gradient (Amersham, Little Chalfont, England). When appropiate, $CD16^+$ and $CD16^-$ populations were separated using CD16-microbeads and AutoMACS separation (Miltenyi, Bergisch Gladbach, Germany).

Flow cytometry

Human cell samples were stained using FITC anti-CX3CR1 (MBL International, Woburn, MA, USA), PE-anti-CCR2, PE-anti-DC-SIGN (all R&D Systems Inc., Minneapolis, MN, USA), PE-anti-HLA-DR, PE-anti-CD62L, PE-anti-CD80, PE-anti-CD86, PE-anti-CD16, and APC-anti-CD14 (all BD Bioscience, San Jose, CA, USA), PE-anti-CD83 and PE-DC-LAMP (both Immunotech, Marseille, France). Murine samples were stained using biotinylated-anti-CD11c, followed by APC-streptavidin (both BD bioscience).

Reverse transmigration assay

Development of DC from monocytes was studied in a reverse transmigration assay (17). Briefly, HUVEC cells were grown to confluency on a collagen matrix (Allergan, Irvine, CA, USA). PBMC were depleted of NK cells using CD56-microbeads (Miltenyi). $CD16^+$ cells were then isolated using CD16-microbeads and labeled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (Sigma Aldrich, Deisenhofen, Germany). $CD16^+$ and $CD16^-$ cells were remixed and applied to the endothelial layer for two hours. Afterwards, non-adherent cells were washed away and culture was continued for 48 hours. Reverse transmigrated cells were then removed using 1mM EDTA. All cultures used Ham's F12 culture medium (BioWhittaker, Verviers, Belgium), supplemented with 20% FCS (BioWhittaker), 50 µg/mL endothelial cell growth supplement (R&D systems Inc.) and 100 µg/mL heparin (Sigma).

Cytokine measurements

Levels of IL-10, IL-1beta, TNF-alpha and IFN-gamma were determined using a human cytokine 25-plex AB Bead kit (Biosource, Camarillo, CA, USA). Measurements were performed on a Luminex XYP (Luminex, Austin, TX, USA) and analyzed using StarStation software (Applied Cytometry Systems, Sheffield, UK).

Immunohistochemistry

Labial and parotid biopsies were snap-frozen in Tissue-Tek (Sakura, Zoetermeer, The Netherlands). Cryostat sections (6 µm) were prepared and fixed with cold methanol and acetone. To limit background staining, an avidin/biotin block was used (Vector Laboratories, Burlingame, CA, USA). Slides were incubated with anti-DC-LAMP (Immunotech, Marseille, France), anti-DC-SIGN or IgG control (both BD Biosciences) followed by biotinylated rabbit-anti-mouse IgG and subsequent streptavidin-ABComplex-AP (both Dako A/S, Glostrup, Denmark). AP label was developed using new fuchsine staining (Chroma, Stuttgart, Germany). Slides were counterstained with hematoxylin (Merck, Whitehouse Station, NJ, USA).

***In vivo* monocyte tracking**

Monocyte subsets were labelled as described previously (26). Briefly, for labelling of Ly-6C^{low} monocytes, mice were injected with Fluoresbrite plain YG 0.5 micron microspheres (Polysciences Inc. Warrington, PA, USA) intravenously in the tail vein. Before labelling of Ly-6C^{high} monocytes, mice were injected with Cl2MDP (clodronate) liposomes intravenously in the tail vein. Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. It was encapsulated in liposomes as described previously (27). This results in a transient depletion of blood monocytes, followed by repopulation of Ly-6C^{high} monocytes (18). Twenty hours later, these mice also received Fluoresbrite microspheres intravenously, thus labelling the Ly-6C^{high} population. Four days later, submandibular glands (SMG) were removed and cleared of all adipose tissue and lymph nodes. Tissue was then digested using Liberase (Roche, Woerden, The Netherlands) treatment and cells were washed twice in PBS containing 10% FCS. Cell suspensions were then analyzed by flow cytometry.

Statistics

For comparison of unpaired data, Mann-Whitney U test was used. For comparison of paired data, Wilcoxon Signed Ranks Test was used. Correlations were calculated using Spearman's rank test. All analyses were carried out using SPSS software (SPSS Inc., Chicago, IL, USA), results were considered statistically significant if $p<0.05$.

RESULTS

Increase in mature monocytes in pSjS

PBMC were stained for CD14 and CD16 and frequencies of mature and immature monocyte subsets were determined by flow cytometry. The frequency of CD16^{+} mature monocytes was significantly increased in pSjS compared to healthy controls (Figure 1A), while the frequency of CD16^{-} immature monocytes was not altered significantly (data not shown). Several cytokines have been reported to induce the upregulation of CD16

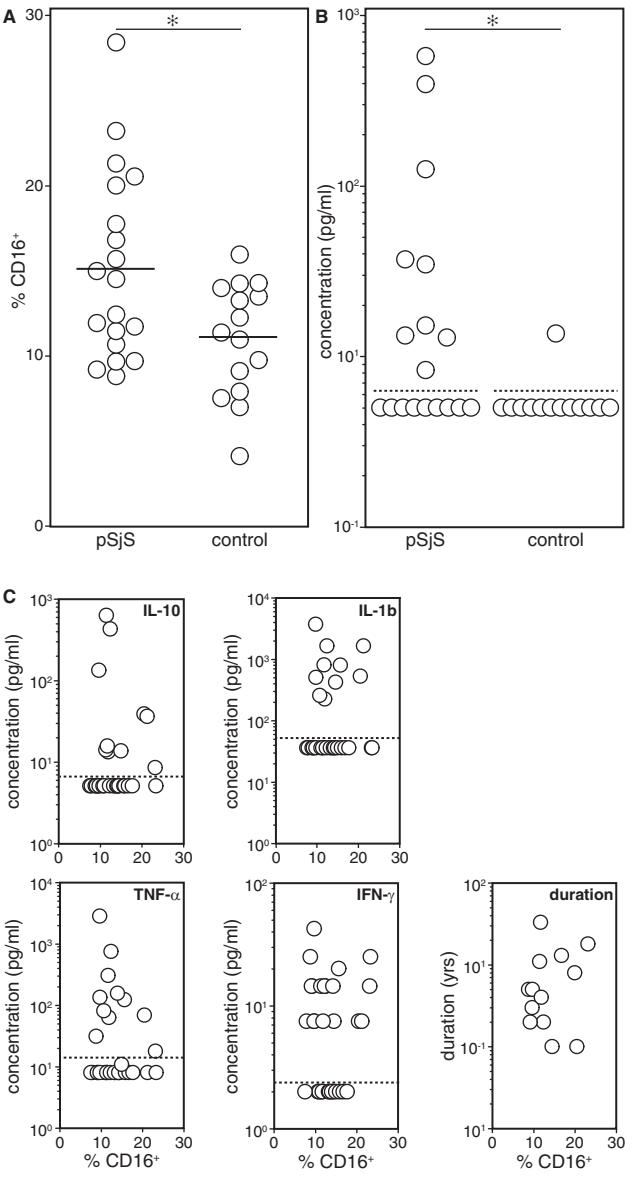

Figure 1. Increased frequency of mature monocytes in pSjS does not correlate to cytokine levels. (**A**) PBMC were isolated, stained for CD14 and CD16 and analyzed by flow cytometry. Each data point represents one individual patient, **p*<0.05. (**B, C**) Serum cytokine levels were measured by 25-plex AB Bead kit. Disease duration was measured as time from diagnosis to inclusion in the study. Each data point represents one individual patient, dashed lines represent detection limits.

on monocytes *in vitro*, including IL-10 (28). Although levels of IL-10 were increased in the serum of pSjS patients (Figure 1B), no correlation was found between the level of IL-10 and the frequency of $CD16^+$ monocytes (Figure 1C, Spearman's rho -0.385, p=0.19). Also, levels of the inflammatory cytokines IL-1beta, TNF-alpha and IFN-gamma did not correlate to $CD16^+$ frequency (Figure 1C, Spearman's rho 0.067, -0.061 and -0.086, p=0.72, 0.74 and 0.65 respectively). Furthermore, no correlation between frequency of mature monocytes and disease duration was found (Figure 1C, Spearman's Rho 0.094, p=0.76).

Normal phenotype of mature monocytes in pSjS

Besides expression of CD16, mature monocytes are characterized as $CCR2^{low}$ $CD62L^{low}CX3CR1^{high}$. Expression of these markers was determined, as an aberrant expression pattern would have implications for the migration of this subset. Mature monocytes expressed low levels of CCR2 and CD62L and high levels of CX3CR1 (Figure 2A). For comparison, immature monocytes expressed low levels of CX3CR1 and high levels of CCR2 and CD62L (Figure 2B). No differences were observed between pSjS and controls, indicating that the monocyte subsets are phenotypically normal in pSjS with regard to these migration markers and would be expected to show a normal migration pattern.

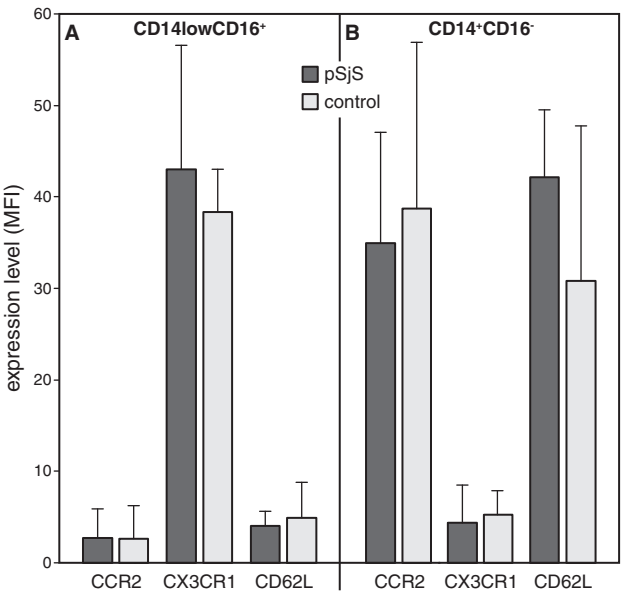

Figure 2. Normal chemokine receptor and adhesion molecule expression on pSjS monocyte subsets.

PBMC were separated into $CD16^+$ and $CD16^-$ populations using magnetic bead isolation and stained for CD14, CCR2, CD62L and CX3CR1. (**A**) $CD14^{low}CD16^+$ and (**B**) $CD14^+CD16^-$ monocytes were gated and analyzed by flow cytometry. Expression was calculated as MFI specific antibody -MFI isotype control, (n=4-6).

Mature monocytes are capable of developing into DC-LAMP+ dendritic cells

The mature monocyte subset has been described to preferentially develop into tissue trafficking DC (17), but the exact phenotype of these DC is unknown. To determine the phenotype of DC derived from the mature monocyte subset, the reverse transmigration model was used. In this system, monocytes develop into DC while traversing through endothelial cells as well as extracellular matrix, thus abolishing the need for addition of exogenous cytokines (17). To specifically study DC originating from mature monocytes, this subset was isolated from PBMC obtained from controls, labeled with CFSE and remixed with the original PBMC. This total population was then used in the reverse transmigration system. After 48 hours, the reverse transmigration fraction contained a clear population of DC with cell surface ruffling and dendrites (Figure 3A). This population contained both CFSE+ and CFSE− cells, indicating that these cells are both of CD16+ mature and CD16- immature origin. DC derived from both monocyte subsets uniformly expressed HLA-DR at high levels (Figure 3B). Previously, our group has reported the increased presence of CD83+ and CD1a+ DC in the salivary glands of pSjS patients (6). CD83 was present on a considerable proportion of DC generated, especially in those generated from mature monocytes (Figure 3B). CD1a+ DC were only rarely generated (2%), and originated solely from immature monocytes (data not shown).

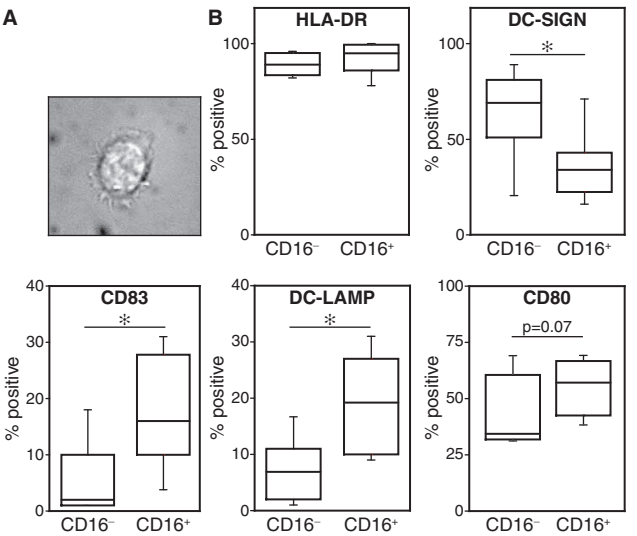

Figure 3. Mature monocyte derived DC partly express CD83 and DC-LAMP.
CD16+ monocytes were isolated from control PBMC by magnetic isolation and labeled using CFSE. Cells were then remixed and applied to the reverse transmigration system. (**A**) After 48 hours, reverse transmigrated cells were harvested and showed ruffled membranes. (**B**) DC were harvested and CFSE+ and CFSE- DC were analyzed separately for marker expression by flow cytometry.

Recently, two more DC markers were described, being DC-SIGN and DC-LAMP (29, 30). Expression of DC-SIGN was observed in up to 70% of DC derived from mature monocytes, but was more common in DC derived from the immature subset (up to 89%, Figure 3B). Contrary to this, expression of DC-LAMP was present in significantly larger proportions of DC derived from mature monocytes (Figure 3B). Similarly, expression of activation marker CD80 also tended to be higher in $CD14^{low}CD16^{+}$ derived DC (Figure 3B).

DC-LAMP^{+} DC are present in lymphocytic infiltrates in pSjS salivary gland

Data on the expression of DC-SIGN and DC-LAMP in the salivary glands of pSjS patients has not been reported thus far. Therefore, expression of these markers was studied in labial or parotid biopsies obtained from four pSjS patients. Expression of DC-SIGN could readily be observed in all four patients, both in the lymphocytic infiltrates and scattered throughout the parenchyma (Figure 4A). DC-LAMP^{+} cells were clearly present as well, with an expression pattern restricted to the lymphocytic infiltrates (Figure 4B).

Mouse homologue subset of mature monocytes migrates to salivary gland and preferentially develops into DC

To monitor monocyte trafficking *in vivo*, the NOD mouse model was used. This strain spontaneously develops lymphocytic infiltrates in the salivary glands similar to those seen in pSjS patients (7) and exhibits an expansion of $Ly6C^{low}$ mature monocytes (23). Ly-$6C^{low}$ mature and Ly-$6C^{high}$ immature monocytes were labeled *in vivo* using latex beads

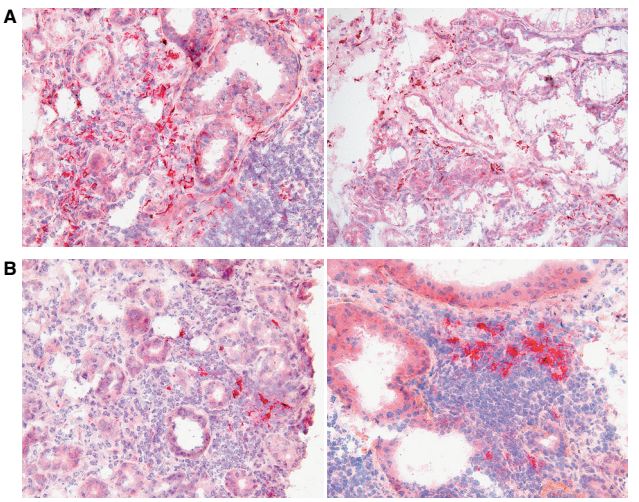

Figure 4. DC-SIGN and DC-LAMP have different expression patterns in pSjS salivary gland.
Frozen parotid (left) or labial (right) biopsies of four pSjS were analyzed by immunohistochemistry, representative stainings are shown. (**A**) DC-SIGN^{+} cells are scattered throughout the parenchyma (magnification 320x). (**B**) DC-LAMP^{+} cells are restricted to the focal infiltrates (magnification 320x).

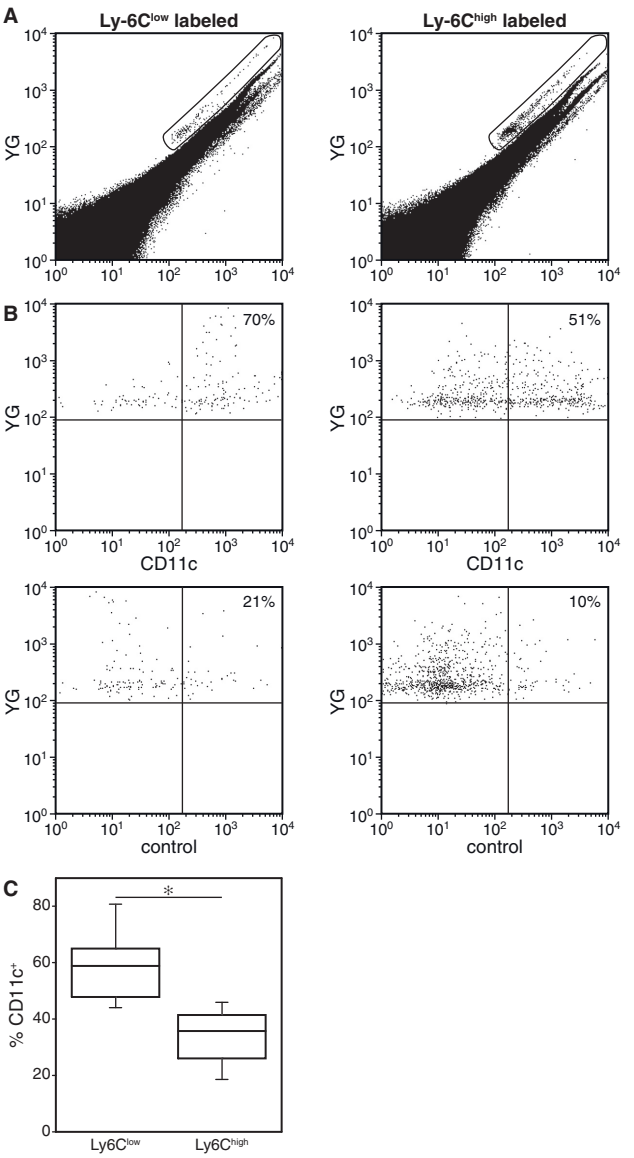

Figure 5. Mature mouse monocytes develop into salivary gland CD11c⁺ DC.
(**A**) Ly-6C^{low} (left) and Ly-6C^{high} (right) monocytes were labeled using YG dyed beads and presence of YG⁺ cells in the SMG was analyzed after 4 days. (**B**) YG⁺ cells were gated and analyzed for expression of CD11c, a representative picture of each labeling is shown. (**C**) YG⁺ cells isolated from SMG were gated and analyzed for expression of CD11c⁺, boxplots represent median with upper and lower quartiles, whiskers represent range (n=7-10), *p<0.01.

labeled with the fluorescent marker Yellow-Green (YG) (26). Four days later, submandibular glands (SMG) were removed and analyzed for presence of YG^+ cells by flow cytometry. Although SMG total cell suspensions show some autofluorescence, a clear population of YG^+ cells could be observed in all mice (Figure 5A), regardless of the monocyte subset labeled. The YG^+ population in the SMG was then further analyzed for expression of the dendritic cell marker CD11c. YG^+ cells derived from mature monocytes cells were largely positive for CD11c (mean 59% +/- 14), indicating their dendritic nature (Figure 5B). In contrast, expression of CD11c on YG^+ cells derived from immature monocytes was significantly lower (mean 36% +/- 8, Figure 5B-C). This supports the idea that although both populations migrate to the SMG, mature monocytes preferentially develop into tissue DC.

DISCUSSION

This study shows that in pSjS the frequency of $CD16^+$ mature monocytes is increased compared to controls. Similar data has been described for RA patients (13, 19), although in our hands, the increase in $CD16^+$ monocytes in RA was less pronounced than that in pSjS (data not shown). The mature subset expresses normal levels of chemokine receptors and adhesion molecules. DC derived from mature monocytes partly express DC-LAMP and CD83, a phenotype similar to DC in pSjS salivary gland.

Increased levels of $CD16^+$ monocytes have been described in systemic infection and sepsis, where cytokines were suggested to be the causal factor (21, 31, 32). Since in our patient cohort no correlation between $CD16^+$ frequency and these cytokines could be found, a straightforward cytokine effect appears to be unlikely. Additionally, levels of mature monocytes did not correlate with disease duration, thus contradicting the idea that the increased frequency of this subset is simply due to an ongoing systemic inflammation.

The fate of mature monocytes after leaving the circulation has been poorly elucidated. Most data on the developmental fate of monocyte subsets has been derived from mouse studies which focused on the homologue population of Ly-$6C^{low}$ mature monocytes (33). A recent report has shown that Ly-$6C^{low}$ monocytes can develop into lung DC but not spleen DC (34), thus indicating that this subset may be mainly involved in the seeding of DC in non-lymphoid organs. In the NOD mouse, a spontaneous model for pSjS (22), an increase in both mature monocytes and salivary gland infiltrating $CD11c^+$ DC was observed (7, 23). This study shows that mature monocytes indeed have the capacity to migrate into the submandibular glands, and develop into $CD11c^+$ cells more often then their immature counterpart. In mice, expression of CD11c is mainly found on DC and alveolar macrophages, with only low levels being expressed by other macrophage subsets (35, 36). Given the high expression of CD11c on Ly-$6C^{low}$ monocyte derived cells in the NOD salivary gland, these cells are most likely DC. Recently, the NOD mouse model was reported to lack longitudinal stability with regard to the development of SjS-like symptoms (37).

In the current study, the decrease in salivary flow was not analyzed, so a direct link between monocyte infiltration and decreased salivary flow could not be established. However, the results do show that Ly-6C^{low} monocytes are capable of migrating to the salivary glands and preferentially develop into DC. Although the populations of CD16$^+$ and Ly-6Clow monocytes cannot be taken as completely identical, this strongly supports the idea that mature monocytes preferentially differentiate into tissue DC.

A marker which has been implicated in maturation of DC from monocytes in humans is the inhibitory FcGRIIb. Blocking this receptor results in increased DC maturation and activation, suggesting the FcGRIIb is a marker of relatively immature monocytes (38). Indeed, expression of this receptor is decreased in CD16$^+$ monocytes compared to CD16$^-$ monocytes as determined on the mRNA level (data not shown), confirming the hypothesis that CD16$^+$ monocytes are more prone to develop into DC than their CD16$^-$ counterpart.

Previously our group has shown that increased numbers of CD83$^+$ and CD1a$^+$ DC are present in labial salivary glands of pSjS patients (6). In addition to this, it is here shown that the more recently described markers DC-SIGN and DC-LAMP (29, 30) are expressed by DC in pSjS labial and parotid salivary glands, with expression of DC-LAMP restricted to the lymphocytic infiltrates. The importance of DC for the formation of pathological infiltrates is shown by the fact that insulitis does not develop in the absence of macrophages and DC. Furthermore, specific expansion of the DC population in a mouse model results in the formation of lymphocytic infiltration of the salivary glands (39, 40). To test if mature monocytes can develop *in vitro* into DC displaying the phenotype seen in salivary glands, the reverse transmigration system was used (17). This system allows for monocytes to rapidly differentiate into DC without the need for addition of cytokines. The cells used in this assay are obviously still capable of producing cytokines themselves, and these may impact the process in a paracrine fashion. Therefore, this system should not be considered as 'cytokine-free', but rather as a closer resemblance of the physiological environment than cultures in the presence of excess amounts of exogenous cytokines. Both monocyte subsets developed into DC expressing high levels of HLA-DR, with a large proportion of cells also expressing DC-SIGN. Expression of CD83 and DC-LAMP was detected in particular on DC derived from mature monocytes. A similar trend was observed for CD80, indicative of a more activated DC phenotype. This is in line with previous observations, where increased expression of CD86 was observed in CD16$^+$ derived DC (41). Only low numbers of CD1a$^+$ DC were detected, and originated solely from immature monocytes. This indicates that the CD1a$^+$ DC present in SjS salivary gland may be a separate subset from the DC displaying the activated phenotype. These results originate from *in vitro* experiments, which lack the local influence of the salivary gland. Cytokines produced by the glandular epithelium or infiltrating immune cells may also impact the differentiation of DC, leading to different expression patterns than those described here. Therefore, although this data shows that mature monocytes have the capacity to develop into activated DC, whether they preferentially do so specifically in salivary glands requires further confirmation *in vivo*.

In summary, pSjS patients show increased numbers of mature $CD16^+$ monocytes, which have the capacity to develop into $CD83^+$ and DC-$LAMP^+$ DC, similar to the type of DC observed in salivary gland infiltrates in pSjS patients. Direct tracking of monocyte subsets is not possible in humans due to ethical restraints, but our studies in the NOD mouse support the model of the mature monocyte subset migrating to the salivary glands and preferentially developing into DC.

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Supplementary Table 1. Primary SjS patient characteristics.

Patient	Age	Duration of illness (years)	SS-A	SS-B	Extraglandular manifestations
1	25	11	+	+	
2	66	5	+	+	
3	42	1	+	+	
4	36	13	-	-	polyneuropathy
5	48	18	+	+	
6	65	33	+	-	Raynaud
7	25	3	+	+	
8	39	8	+	+	
9	75	1	+	+	
10	68	10	+	+	
11	75	1	+	-	
12	36	2	+	+	
13	70	2	+	-	Raynaud
14	58	5	+	+	polyneuropathy
15	49	4	+	+	
16	36	1	+	-	
17	64	5	+	+	
18	72	3	+	+	polymyalgia
19	50	5	+	-	Raynaud

4

LACK OF CCR5 ON DENDRITIC CELLS PROMOTES A PRO-INFLAMMATORY ENVIRONMENT IN SUBMANDIBULAR GLANDS OF THE NOD MOUSE

Manon E. Wildenberg[1], Cornelia G. van Helden-Meeuwsen[1], Joop P. van de Merwe[1], Christophe Moreno[2], Hemmo A. Drexhage[1] and Marjan A. Versnel[1]

[1]Dept. of Immunology, Erasmus MC, Rotterdam, The Netherlands; [2]Division of Gastroenterology and Hepatopancreatology, Erasme Hospital, Brussels, Belgium

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ABSTRACT

Sjögren's Syndrome is an autoimmune disease characterized by lymphocytic infiltration of the salivary glands. In the NOD mouse, a model for this disease, the development of lymphocytic infiltrates in the salivary glands is preceded by an accumulation of dendritic cells (DC). Given the key importance of DC in regulating the immune response, we characterized the DC isolated from NOD salivary glands. These DC lacked membrane expression of CCR5, whereas DC from control salivary glands did express this molecule. The lack of expression was present already prior to the onset of lymphocytic infiltration, indicating that this was not the result of ongoing inflammation. DC from other sources in the NOD mouse also showed a decrease in CCR5 expression. The lack of CCR5 expression in NOD salivary gland was accompanied by an increase in inflammatory chemokines. Furthermore, DC from CCR5$^{-/-}$ animals or DC treated with a CCR5 antagonist showed increased secretion of IL-12. Interestingly, in Sjögren's Syndrome patients, CCR5 expression on circulating monocytes was decreased and correlated to increased levels of IL-12. This data indicates that CCR5 has regulatory properties and that the lack of CCR5 in NOD DC contributes to the pro-inflammatory environment in the salivary glands.

INTRODUCTION

The nonobese diabetic (NOD) mouse is frequently used as a model to study the development of primary Sjögren's Syndrome (pSjS) (1, 2). Similar to human patients, these mice develop lymphocytic infiltrates in the salivary glands, and finally show a decreased salivary flow. The lymphocytic infiltrates start to develop at around 7 weeks of age and show an increasing structural organization (2). Earlier research in our laboratory has shown that the influx of lymphocytes is preceded by an accumulation of dendritic cells (DC) (2).

DC are potent antigen presenting cells capable of stimulating both naïve and memory T-cells and boosting B-cell responses, thus playing a key role in the initiation of the immune response (3, 4). Furthermore, DC are crucial to the development of tolerance, preventing harmful immune responses against harmless antigens (5, 6). To fully exert their sentinel function, DC migrate from the blood into tissues and further to the draining lymph nodes. This migration is a complex multi-step process, tightly regulated by numerous molecules including chemokines and their receptors (7, 8).

The chemokine family contains a large number of proteins, characterized by their small size and conserved cysteine residues (9). Most chemokines bind and signal through several receptors, and one receptor usually has multiple ligands (9, 10), creating a highly complex network of interactions. Chemokine receptors are G-protein coupled, 7 transmembrane receptors, which have differential expression patterns on various leukocyte subsets (11). On DC, the expression of chemokine receptors is linked to their

state of maturation. For example, immature DC express CXCR4, CCR1, -2 and -5, most of which are downregulated after exposure to an activating stimulus (12, 13). Besides cell trafficking, chemokine receptors have been described to regulate several other processes in DC, including endocytosis, dendrite formation, cell survival and maturation of DC (14-18).

Given the importance of DC in the immune response, the expression of chemokine receptors in the DC accumulating in the NOD salivary glands was determined. Most strikingly, a complete lack of CCR5 expression was observed in these cells. Earlier it has been shown that mice deficient in CCR5 display excessive immune responses, for example in an influenza model (19). Therefore, we hypothesized that the lack of CCR5 results in a proinflammatory environment. The results indeed show an immunoregulatory role for CCR5 in DC, both by scavenging of inflammatory ligands and by regulating the secretion of IL-12.

MATERIALS AND METHODS

Animals

Female C57BL/6 and CCR5$^{-/-}$ mice were obtained from Harlan (Horst, The Netherlands) and Jackson ImmunoResearch Laboratories (Bar Harbor, ME) respectively. NOD/LTj mice were bred in our own facility under specified pathogen free conditions. Animals were used at the age of 5-20 weeks. All mice were supplied with water and standard chow ad libitum. Experimental procedures were approved by the Erasmus University Animal Ethical Committee.

Human materials

Heparinized blood and serum samples were obtained from 20 female pSjS patients (mean age 52.7 +/- 16.4, mean disease duration 6.6 years +/- 7.8) at Erasmus MC, Rotterdam, The Netherlands. Inclusion criteria consisted of a positive diagnosis according to the criteria of the American-European consensus group (20) and the absence of other rheumatic or autoimmune disorders. Control blood samples were obtained from 14 female volunteers (mean age 45.8 +/- 6.1) not suffering from autoimmune diseases or taking immunosuppressive medication. This study was approved by the Medical Ethical Committee of Erasmus MC and all subjects gave informed consent.

PBMC were isolated from peripheral blood using a Ficoll gradient (Amersham, Little Chalfont, England).

Isolation of CD11c^{+} cells from tissue

Organs were removed and cleared of adipose tissue and lymph nodes. Tissue was digested by Liberase (Roche, Woerden, The Netherlands) treatment at 37^{o}C and washed twice in phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS). Subsequently cells were cleared from debris using a Ficoll (Amersham) gradient.

Cells appearing at the interface were incubated with mouse CD11c-microbeads (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturers protocol and isolated using the AutoMACS (Miltenyi) system.

Flow cytometry

Cells were resuspended in PBS containing 1% bovine serum albumin (BSA) and incubated with primary antibody. Primary antibodies used were biotinylated MHC class II (C57BL/6 clone ER-TR3; NOD clone 10.2.16), biotinylated CD11c, CD8alpha-FITC, CD80-PE, CD86-FITC, CD40-PE, CXCR4-PE and CCR5-PE (all BD Bioscience, San Jose, CA). For human material, CCR5-PE-Cy5 (BD Bioscience) was used. Afterwards cells were washed twice and, when appropiate, incubated with streptavidin-APC (BD Bioscience). For intracellular CCR5 staining, surface CCR5 was blocked by incubation with a tenfold excess of unlabeled anti-CCR5 antibody (BD Bioscience). Next, cells were fixed using 2% paraformaldehyde, and permeabilized using 0.5% saponin. Cells were then incubated with labeled CCR5 antibody diluted in 0.5% saponin, washed and resuspended in 1% BSA. Cell suspensions were analyzed using a FACSCalibur (Becton Dickenson, San Diego, CA) and WinMDI software (Microsoft Co., Redmont, WA). Expression was calculated as MFI specific staining – MFI isotype control, for calculation of relative expression levels expression of C57Bl/6 was set to 1 and NOD levels were calculated accordingly.

Cytokine determination

Levels of IL-12p40 in supernatant were determined using an IL-12p40 Duoset kit (R&D Systems Inc.) and serum levels of IL-12 were determined using a human cytokine 25-plex AB Bead kit (Biosource, Camarillo, CA) according to the manufacturers suggestions. Induction of IL-12 was calculated as secretion after LPS stimulation devided by secretion without stimulus.

Bone marrow derived dendritic cell (BMDC) culture

Bone marrow was isolated from femur and cultured in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (BioWhittaker), 50 µM beta-mercaptoethanol (Sigma Aldrich, Deisenhofen, Germany) and 20 ng/ml recombinant mouse GM-CSF (Biosource, Camarillo, CA) for nine days. To enrich immature DC, cells were incubated with anti-CD86 antibody (clone GL-1), washed, labeled with goat anti-rat microbeads (Miltenyi) and separated using the AutoMACS (Miltenyi) system. For stimulation experiments, BMDC were incubated with Met-RANTES (10 ug/ml, R&D Systems Inc.) where appropiate and stimulated using LPS (5 ng/ml, Sigma Aldrich) for 16 hours, after which supernatants were collected.

RNA isolation and RT-PCR

RNA was isolated from submandibular glands using the Rneasy Mini kit (Qiagen, Valencia, CA) according to the manufacturers suggestions. cDNA was generated using

Superscript II (Invitrogen, Carlsbad, CA) and random hexamer primers (Amersham). RQ-PCR analysis was carried out using predesigned primer/probe sets (Applied Biosystems) according to the manufacturers suggestions. For calculation of relative expression, all samples were normalized against expression of the household gene *Abl*.

Statistics

Data was analyzed using Students T-test, except for relative data (C57BL/6 set to 1), where Mann Whitney U-test was used. Error bars represent standard error of the mean for original data and standard deviation for relative data. Correlations were calculated using Spearman's Rank test. All statistical analysis were performed using SPSS software (SPSS Inc., Chicago, IL) and considered significant if $p<0.05$.

RESULTS

Lack of CCR5 expression on CD11c$^+$ cells in NOD salivary gland

CD11c$^+$ cells were isolated from SMG of NOD and control mice, and the chemokine receptor expression pattern was analyzed by flow cytometry. CD11c$^+$ cells isolated from control mice showed expression of both CXCR4 and CCR5, (Figure 1A), while NOD CD11c$^+$ showed a somewhat decreased expression of CXCR4 and completely lacked expression of CCR5 (Figure 1A). As CCR5 is downregulated during the activation of DC, the lack of CCR5 could be due to an increased activation of CD11c$^+$ cells in NOD SMG. However, expression of the activation markers CD40, CD80 and CD86 did not show a significant pattern of upregulation (Figure 1A and B).

As expression of CCR5 can be influenced by inflammatory environmental stimuli, expression levels may be influenced by the ongoing inflammatory process in the salivary glands of NOD mice. Therefore, CD11c$^+$ cells isolated from SMG prior to the development of lymphocytic infiltrates (5 weeks) were compared to CD11c$^+$ from SMG with full blown inflammatory infiltrations (15 weeks). At both ages CD11c$^+$ from control mice showed clear expression of CCR5 (Figure 1C), whereas expression in NOD was already below detection level at 5 weeks of age. The absence of activation marker upregulation in combination with the fact that CCR5 expression is undetectable already prior to the onset of lymphocytic infiltration indicates that the lack of expression is not caused by the ongoing inflammation in the SMG.

Decreased CCR5 expression in NOD DC derived from other sources

To determine if the lack of CCR5 expression is tissue specific or a general phenomenon in NOD mice, CD11c$^+$ cells from other *in vitro* and *in vivo* sources were studied. CD11c$^+$/autofluorescence^{low} DC derived from the lungs of NOD mice showed a clearly decreased expression of CCR5 when compared to control mice (Figure 2A). Similarly, DC cultured

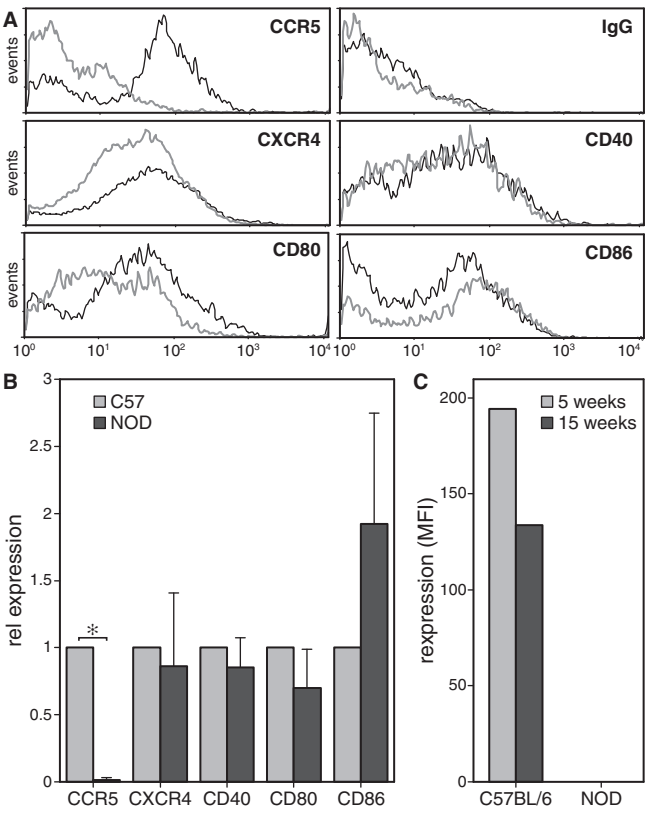

Figure 1. Chemokine receptor and activation markers on SMG CD11c⁺ cells.
CD11c⁺ cells were isolated from SMG by Ficoll gradient followed by magnetic bead isolation. Cells were then stained for surface markers and analyzed by flow cytometry. (**A**) Grey lines represent NOD mice, black lines represent C57BL/6 mice, one representative sample of each strain is shown. (**B**) Average expression in C57BL/6 mice was set to 1 and expression levels of NOD mice were calculated accordingly (*n*=4-5), error bars represent standard deviation, **p*<0.05. (**C**) CD11c⁺ cells were isolated from SMG of mice at 5 or 15 weeks of age. Expression of CCR5 was calculated as MFI specific staining - MFI control.

from bone marrow (BMDC) in the presence of GM-CSF showed a significantly decreased expression of CCR5 (Figure 2B).

Lack of surface CCR5 is accompanied by increased intracellular CCR5 in NOD SMG

CCR5 is a cycling receptor, undergoing rounds of ligand binding, internalization, uncoupling of the receptor and relocalization to the cell surface (21). Therefore, the lack of CCR5 on CD11c⁺ cells in NOD SMG could be due to an altered localization of this molecule. Indeed, when intracellular expression of CCR5 was studied, an increased expression was found in CD11c⁺ cells isolated from NOD SMG when compared to CD11c⁺

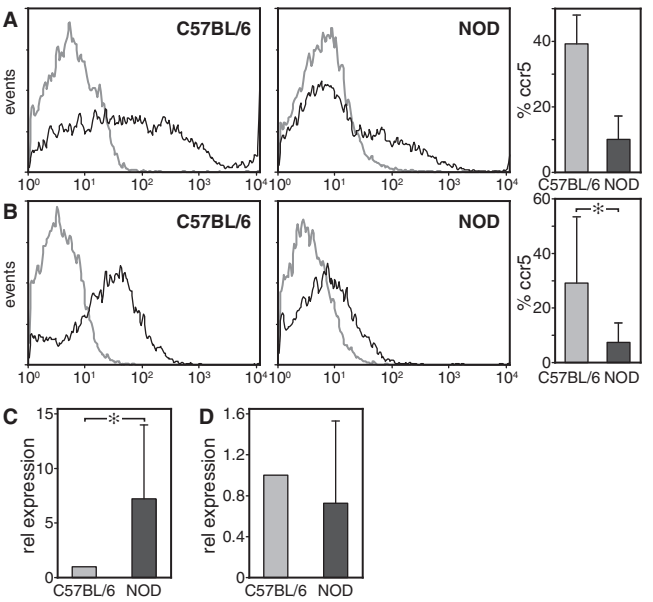

Figure 2. CCR5 expression on lung and bone marrow CD11c⁺ cells.
CD11c⁺ cells were isolated from lungs of C57BL/6 and NOD mice (**A**) or generated *in vitro* from bone marrow (**B**) and analyzed by flow cytometry. Results of gated autofluorescentlow cells (**A**) or gated CD11c⁺ cells (**B**) are shown, black lines represent specific staining, grey lines represent isotype control. One representative image for each strain is shown. Bars represent average expression, error bars represent standard deviation. (**C, D**) CD11c⁺ cells were isolated from salivary glands (**C**) or cultured from bone marrow (**D**) and analyzed for intracellular expression of CCR5 by flow cytometry. Average expression in C57BL/6 mice was set to one and expression levels of NOD mice were calculated accordingly (n=3), error bars represent standard deviation, *p<0.05.

from control mice (Figure 2C). However, in BMDC, no increase in intracellular CCR5 was found (Figure 2D), indicating that the altered CCR5 localization in SMG may be dependent on the microenvironment.

Lack of CCR5 receptor expression may lead to increased free ligand

The lack of CCR5 expression on the surface of CD11c⁺ cells in the SMG of NOD mice may influence biologically available levels of CCR5 ligands CCL3, CCL4 and CCL5. When measured in total salivary gland, mRNA levels of these chemokines tended to be somewhat higher in NOD than in control mice (Figure 3A). Furthermore, in control mice these chemokines can be scavenged from the environment by surface CCR5 on tissue CD11c⁺ cells, thus decreasing the level of free, active ligand. This results in a balanced ratio between CCR5 ligands and CCR5 itself. Due to the lack of surface CCR5 expression, this mechanism is not available in NOD mice, which would be indicated by an increased

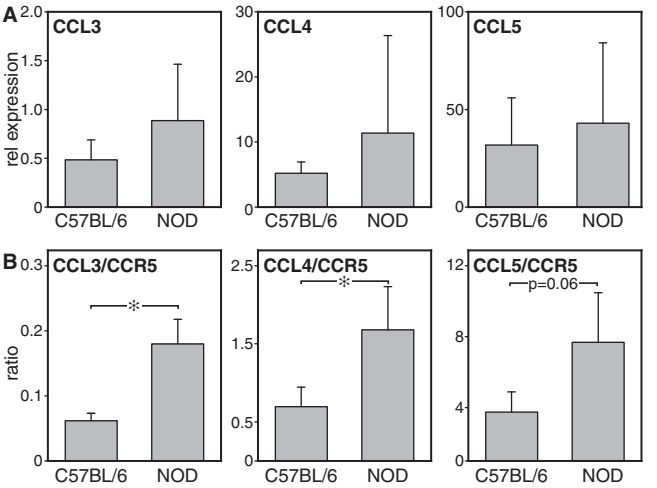

Figure 3. Ratio of CCR5 ligands to CCR5 is increased in NOD SMG.
Total mRNA was isolated from SMG and expression levels of CCL3, CCL4, CCL5 and CCR5 were determined by RT-PCR (*n*=5). Error bars represent standard deviation, **p*<0.05

CCR5 ligand to CCR5 ratio. Indeed, ratios of CCL3, CCL4 and CCL5 relative to the level of CCR5 were increased significantly in NOD salivary gland (Figure 3B).

Lack of CCR5 signaling leads to increased IL-12 secretion

CCR5 has been linked to the regulation of IL-12 secretion in an oral tolerance model (22). To test whether CCR5 also regulates IL-12 specifically in DC, BMDC were cultured from both C57BL/6 and CCR5$^{-/-}$ mice and stimulated with LPS. Secretion of IL-12, as well as relative induction, indeed was higher in CCR5$^{-/-}$ mice (Figure 4A). To exclude a role for the knock out procedure, wild type BMDC treated with the CCR5 antagonist Met-RANTES (23) were compared to untreated cells. Again, secretion and induction of IL-12 was higher in BMDC treated with Met-RANTES (Figure 4B). This supports the idea that CCR5 is involved in the regulation of IL-12 secretion.

Decreased levels of CCR5 in primary Sjögren's Syndrome patients

The relevance of decreased CCR5 expression in human pSjS was explored by measuring CCR5 expression on monocytes, which are generally considered the most important blood precursors for DC. Similar to the mouse model, a significant decrease in surface expression of CCR5 was found in monocytes of pSjS patients when compared to monocytes of controls (Figure 5A). Interestingly, the level of CCR5 expression was negatively correlated to the serum concentration of IL-12 (Figure 5B, Spearman's Rho -0.483, *p*=0.004).

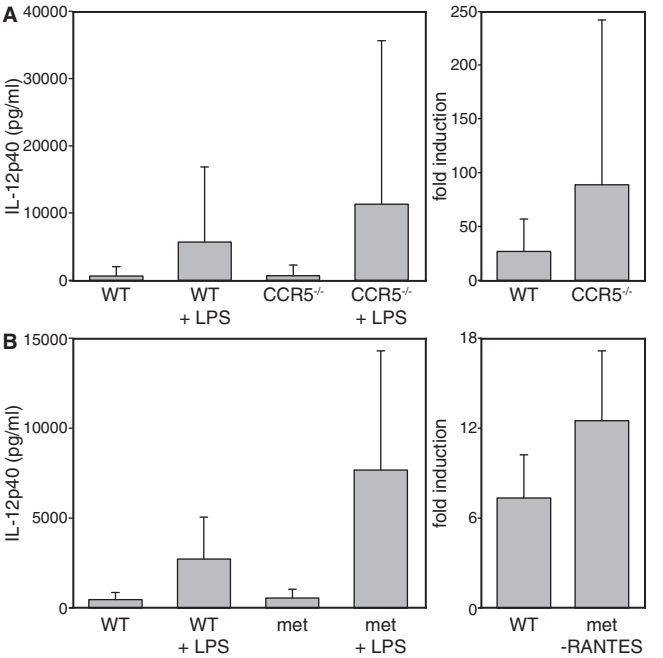

Figure 4. IL-12 secretion in the absence of CCR5 signalling.
(**A**) BMDC were generated in the presence of GM-CSF from wild type (WT) and CCR5$^{-/-}$ animals. Cells were then stimulated using LPS (5 ng/ml) and IL-12p40 was measured in the supernatant by ELISA (*n*=6). Error bars represent standard deviation. (**B**) BMDC were generated in the presence of GM-CSF from wild type animals, treated with met-RANTES (met) and stimulated using LPS (5 ng/ml). IL12p40 levels in the supernatants were measured by ELISA (*n*=3). Bars represent mean values, error bars represent standard deviation.

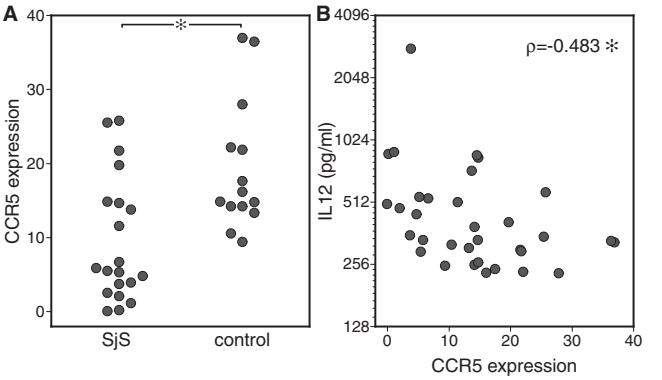

Figure 5. Decreased CCR5 expression in pSjS patients.
(**A**) PBMC were isolated from pSjS patients and healthy controls and analyzed by flow cytometry. CCR5 expression was determined as MFI specific staining - MFI isotype control. Each dot represents one individual patient, ***p*<0.01 (**B**) Serum levels of IL12p40 were determined by AB Bead kit and correlated to the monocyte expression of CCR5 as determined by flow cytometry. Each dot represents one individual patient, **p*<0.05.

DISCUSSION

No expression of CCR5 was found on $CD11c^+$ cells in the salivary glands of NOD mice, while control mice did show expression. As the lack of CCR5 was present prior to the development of lymphocytic infiltrates and expression of activation markers was not increased, this was not the result of an increased state of DC activation due to the ongoing inflammation. In $CD11c^+$ cells isolated from NOD mice, an increase in intracellular CCR5 was detected, indicating that the lack of surface expression may at least partly be explained by an altered subcellular localization of the molecule. However, BMDC and lung DC also showed decreased CCR5 expression, which was not accompanied by increased intracellular levels, suggesting a different cause such as a genetic deficiency. Therefore, the complete lack of CCR5 on SMG $CD11c^+$ cells might be partially explained by an altered subcellular localization and partially by another, possibly genetic factor. The lack of surface CCR5 expression appears to have functional consequences, as NOD mice show defective recruitment in response to a CCR5 ligand in an *in vivo* migration model (24).

CCR5 was initially thought to be involved in directing immature DC to tissue sites (13). Surprisingly, in the steady state, $CCR5^{-/-}$ mice develop normally and show cell numbers and lymphoid architecture similar to wild type animals (25). Furthermore, several reports show normal macrophage recruitment in $CCR5^{-/-}$ mice during inflammation (19, 25-27). These findings may be explained in the light of the high redundancy in the chemokine system. In CCR5 deficient cells, migration is probably regulated by other chemokine molecules, such as CCR1 and CCR2. RNA levels of these receptors were comparable in NOD and control SMG (data not shown), which may explain the presence of DC in NOD SMG despite the lack of CCR5.

In accordance with previous studies in $CCR5^{-/-}$ mice (19, 26, 28), mRNA of CCR5 ligands tended to be increased in NOD SMG. In addition, the lack of expression of CCR5 in DC isolated from NOD SMG leads to a lack of scavenging by this receptor, thus contributing to increased amounts of free, biologically available ligand. Such scavenging by CCR5 has been reported to play a role in the control as well as the resolution of immune responses (29, 30). The increased availability of free CCL3, -4 and -5 may lead to an enhanced influx of other leukocytes, such as T-cell and macrophages, as is seen in the salivary glands of NOD mice. One could argue that the increased levels of CCR5 ligand are causative rather than the result of decreased expression of CCR5 due to receptor internalization. However, since DC cultured from bone marrow of NOD mice *in vitro* also show strong defects in expression of CCR5, this appears to be unlikely. Furthermore, extended exposure of wild-type DC to CCR5 ligands does not lead to decreased expression of the receptor, with the exception of CCL3 at very high concentrations (data not shown). However, even in this case, expression of CCR5 remained at clearly detectable levels, unlike NOD DC *in vivo*.

In a mouse model for experimental autoimmune encephalitis, oral tolerance could be induced in wild type, but not in $CCR5^{-/-}$ mice (22). Furthermore, in the $CCR5^{-/-}$ mice increased levels of IL-12 were observed in the GALT. The current study shows that DC derived from

CCR5$^{-/-}$ or DC treated with a CCR5 antagonist show increased secretion of IL-12p40 compared to wild type BMDC. Although levels were low, results showed a similar trend for IL12p70 (data not shown). Earlier, increased secretion of IL-12 in response to various stimuli was reported in both NOD DC and NOD macrophages, which also show decreased expression of CCR5, and this was indeed the result of autocrine signaling (31, 32). Interestingly, in pSjS patients, decreased levels of CCR5 were observed in peripheral monocytes, and this correlated negatively with the level of IL-12 in the serum, supporting the hypothesis that CCR5 plays a role in the regulation of IL-12 in DC.

IL-12 secreted by DC is a well known inducer of Th1 cells and plays an important role in the development of various immune responses (33). In the NOD mouse model, addition of IL-12 leads to acceleration of the development of diabetes whereas inhibition of IL-12 using an antagonist has a protective effect (34, 35). Unfortunately, the salivary glands were not studied in these experiments, but increased expression of IL-12 is found in the salivary glands of pSjS patients (36). Furthermore, transgenic mice overexpressing IL-12 develop infiltrations in their salivary glands similar to those seen in NOD mice, indicating an important role for this cytokine in the pathogenesis of pSjS (37).

In a recent report, CCR5$^{-/-}$ mice showed an increased antibody production in the setting of a cardiac transplant (38). These antibodies were highly functional, as CCR5$^{-/-}$ mice showed acute humoral rejection which could be transferred by serum (39). Similar increases in levels of antibody were observed earlier in an infection model in CCR5$^{-/-}$ animals (25). In both studies, numbers of IL-4 producing T-cells were increased, suggesting a skewing of the immune system towards antibody production in the absence of CCR5. Increased levels of autoantibodies are a well known phenomenon in both human pSjS patients as well as NOD mice (40-42). The lack of CCR5 on DC may play a role in this by increasing the numbers of IL-4 producing T-cells, thus stimulating the generation of autoantibodies. Indeed, in NOD mice, IL-4 is of crucial importance to the development of pSjS like symptoms, as NOD.IL-4$^{-/-}$ mice do not develop salivary dysfunction (43). Interestingly, in the patient cohort described in this study, CCR5 expression was lower in patients who were positive for the well known autoantibody SS-B, although this did not reach statistical significance (p=0.067, data not shown).

The hypothesis that the lack of CCR5 plays a role in the increased expression of IL-12 as well as IL-4 appears somewhat at odds with the original Th1/Th2 paradigm. However, it has now been clearly shown that the two responses are not as mutually exclusive as previously thought and in pSjS patients, increased numbers of both IFN-gamma and IL-4 producing T-cells are found (44-46). Furthermore, the NOD mouse, which is regarded as a strongly Th1 biased animal, displays a more aggressive form of the stereotypical Th2 condition asthma (47).

In humans the role of CCR5 polymorphisms (48, 49) has been studied in various autoimmune diseases. In diabetes and systemic lupus erythematosus, no association was found between the frequency of CCR5 polymorphisms and disease incidence (50-52),

but more complications and interestingly, higher levels of autoantibodies were found in patients with polymorphic CCR5 alleles (52-54). Only one earlier study focused on CCR5 in pSjS and concluded that a CCR5 mutation may somewhat protect against pSjS, contradicting our results (55). However, in that study, only one polymorphism was included, leaving the possibility that other polymorphisms influenced the results. In the current study, CCR5 was determined at the protein level, thus resulting in actual expression levels rather than genetics.

The function of CCR5 in the migration of various cell types has been extensively studied. This study shows that in addition, CCR5 is a regulatory molecule and that its expression on immature DC is likely to contribute to the tolerogenic phenotype of this subset. In the salivary gland of the NOD mouse model for pSjS, the lack of CCR5 on dendritic cells contributes to a more pro-inflammatory environment.

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ALTERED FRACTALKINE CLEAVAGE POTENTIALLY PROMOTES LOCAL INFLAMMATION IN NOD SALIVARY GLAND

Manon E. Wildenberg, Cornelia G. van Helden-Meeuwsen, Hemmo A. Drexhage and Marjan A. Versnel

Dept. of Immunology, Erasmus MC, Rotterdam, The Netherlands

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ABSTRACT

Background

In the nonobese diabetic (NOD) mouse model for Sjögren's Syndrome, lymphocytic infiltration is preceded by an accumulation of dendritic cells (DC) in the submandibular glands (SMG). NOD mice also display an increased frequency of mature, fractalkine receptor (CX3CR1) expressing monocytes, which are considered to be precursors for tissue DC. To further unravel the role of fractalkine-CX3CR1 interactions in the salivary gland inflammation, we studied the expression of fractalkine in NOD SMG.

Methods

Protein expression was studied by Western blot analysis of whole tissue lysates. Protease activity was measured in salivary gland tissue lysates using fluorimetric substrates. Digestive capacity of enzymes was determined by *in vitro* incubation of recombinant enzyme and fractalkine, followed by protein staining and Western Blot.

Results

Fractalkine was detected in salivary glands of both NOD and control mice at all ages. Western blot analysis showed fractalkine cleavage with increasing age, which was more pronounced in NOD mice. This cleavage resulted in a decrease of the 31 kDa form of the protein, and the generation of a 19 kDa band. Furthermore, NOD animals over 15 weeks of age showed the presence of a unique 17 kDa fragment. This cleavage was organ specific, as it did not occur in brain or pancreas. Increased gelatinase and alpha-secretase activity were detected in NOD SMG and contribute to the cleavage of the 31 kDa protein. Since aberrant cleavage products may induce autoimmunity, we studied the presence of autoantibodies against fractalkine. Indeed, NOD mice showed significantly more antibodies against fractalkine than control animals.

Conclusions

This data indicates that aberrant proteolytic activity in the NOD SMG results in increased fractalkine cleavage and the generation of a unique fractalkine fragment. This specific cleavage may contribute to autoimmunity and putatively enhanced attraction of mature monocytes.

INTRODUCTION

The nonobese diabetic (NOD) mouse is a frequently used spontaneous animal model for the development of Sjögren's Syndrome (SjS). Similar to SjS patients, these mice develop lymphocytic infiltrates in their salivary glands, leading to gland destruction and decreased salivary flow. The development of infiltrates is preceded by an accumulation

of dendritic cells (DC). Given the key role of DC in the initiation of immune responses, their accumulation as one of the early events indicates a role for DC in the pathogenesis of the disease (1, 2). The exact cause of DC accumulation remains to be elucidated, but alterations in monocytes, which are considered to be a precursor population for DC, have been described in the NOD mouse (3, 4). Particularly, the subset of Ly-6C^{low} monocytes is significantly expanded in the NOD circulation (3). This subset is thought to be a mature population preferentially developing into tissue DC (5), suggesting a link between these cells and the DC accumulation observed in the salivary gland of NOD mice.

Besides the low expression of Ly-6C, the mature monocyte subset is further characterized by low expression of CCR1 and CCR2, and high expression of CX3CR1, the fractalkine receptor (6). Fractalkine is the sole member of the CX3C chemokine family, first described in brain tissue (7, 8). It differs from other chemokines not only by its structure and relatively large size, but also by the fact that it occurs in both a membrane bound and soluble form. The membrane bound form functions as an adhesion molecule, whereas the soluble form is strongly chemotactic for monocytes and T-cells (9, 10).

Given the increase of mature Ly-6C^{low} monocytes in the NOD circulation, their propensity to develop into DC and the importance of fractalkine in the chemoattraction of Ly-6C^{low} monocytes, we studied the expression of fractalkine in NOD salivary glands. Although fractalkine was present in salivary glands of both NOD and control mice, a unique fragment of the protein was observed in NOD mice. Such a fragment may contribute to the break of tolerance against fractalkine resulting in an autoimmune response. Indeed, an anti-fractalkine antibody response is shown here in the NOD mouse.

MATERIALS AND METHODS

Animals

NOD/LTj mice were bred in our own facility. Mice were tested for diabetes twice weekly and excluded from experiments when positive. C57BL/6 and BALB/c mice were obtained from Harlan (Horst, The Netherlands). All mice were housed under specific pathogen free conditions and were fed standard chow and water ad libitum. For all experiments, female mice at the ages of 5-25 weeks were used. All experimental procedures were approved by the Erasmus University Animal Ethical Committee.

Tissue lysates and protease activity

Submandibular glands and pancreases were removed and cleared of adipose tissue and lymph nodes. Tissue was placed in PBS or PBS containing a protease inhibitor cocktail (Complete mini protease inhibitor tablets, Roche, Woerden, The Netherlands) where indicated and homogenized by mechanical disruption followed by ultrasound sonification. Finally lysates were cleared by centrifugation. Caspase-3 and gelatinase (MMP-2/MMP-9) activity were measured using EnzCheck assay kits (Invitrogen, Eugene, OR) according to

the manufacturers instructions. Alpha-secretase activity was measured using a fluorogenic substrate (10 µg/reaction, alpha-secretase substrate II, Merck, Whitehouse Station, NJ) as indicated by the manufacturer. All results are shown as arbitrary units (U) relative to the total amount of protein as measured by Bradford analysis (Biorad, Hercules, CA).

Western Blot

Protein content of lysates was measured by Bradford analysis (Biorad) and 50 µg of each lysate were loaded on a 15% SDS-PAGE gel and run under reducing conditions. For determination of protein size, the Kaleidoscope prestained standard was used (Biorad). Afterwards, protein was transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Membranes were then incubated with anti-fractalkine antibody (C-20, 1 µg/mL, Santa Cruz, Santa Cruz, CA) followed by Donkey-anti-Goat-HRP (80 ng/mL, Santa Cruz). Expression was detected by ECL analysis (Amersham, Piscataway, NJ). For detection of auto-antibodies, tissue lysates known to contain specific forms of fractalkine, were loaded on a 15% SDS-PAGE gel. After transfer, membranes were incubated with IgG purified from mouse serum by protein G-column separation or with total serum. This was followed by Rabbit-anti-mouse-HRP (1:20000, Dako, Glostrup, Denmark) and ECL analysis.

***In vitro* digestion assay**

MMP-2 (65 ng/reaction, Chemicon, Temecula, CA) and MMP-9 (140 ng/ reaction, R&D systems Inc., Minneapolis, MN) were activated by 1 mM APMA in 10mM CaCl2, 100 mM NaCl, 50mM Tris/HCl pH 7.5. Caspase-3 (10 ng/reaction, Gentaur, Brussels, Belgium) was diluted in 10mM PIPES, 2 mM EDTA, 0.1% CHAPS and 5 mM DTT, pH 7.4. ADAM-10 and ADAM-17 (both 500 ng/reaction, R&D Systems Inc.) were diluted in 50mM HEPES, 5 µM ZnCl2, 0.01% Brij-0, pH 7.5). Enzyme activity was confirmed by fluorimetric assay as described above. Active enzyme was then incubated with brain lysate (20 µg protein/reaction) for two hours at 37°C. Digestion was stopped by adding reducing sample buffer and heating to 99°C. Protein analysis was carried out by Western Blot as described above.

Statistics

Bars represent mean, error bars represent standard deviation. For comparison of means Student's t-test was used, for comparison of frequencies, Fishers exact test was calculated, both using SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Fractalkine is present in NOD submandibular glands and cleaved with increasing age

To determine the presence of fractalkine on the protein level, expression was studied in SMG tissue lysates by Western Blot. As expression of fractalkine has been clearly described in normal brain (11, 12), this was used as a reference sample. Analysis of SMG of young (5 wk) NOD mice revealed expression of the protein at a molecular weight similar to that observed in brain lysates (31 kDa). However, when salivary glands of 15 week old mice were studied, additional bands were observed at 19 kDa and 17 kDa (Figure 1A). Also, the 31 kDa band was less intense or even undetectable at these time points, indicating the cleavage of fractalkine with increasing age in the salivary glands of NOD mice.

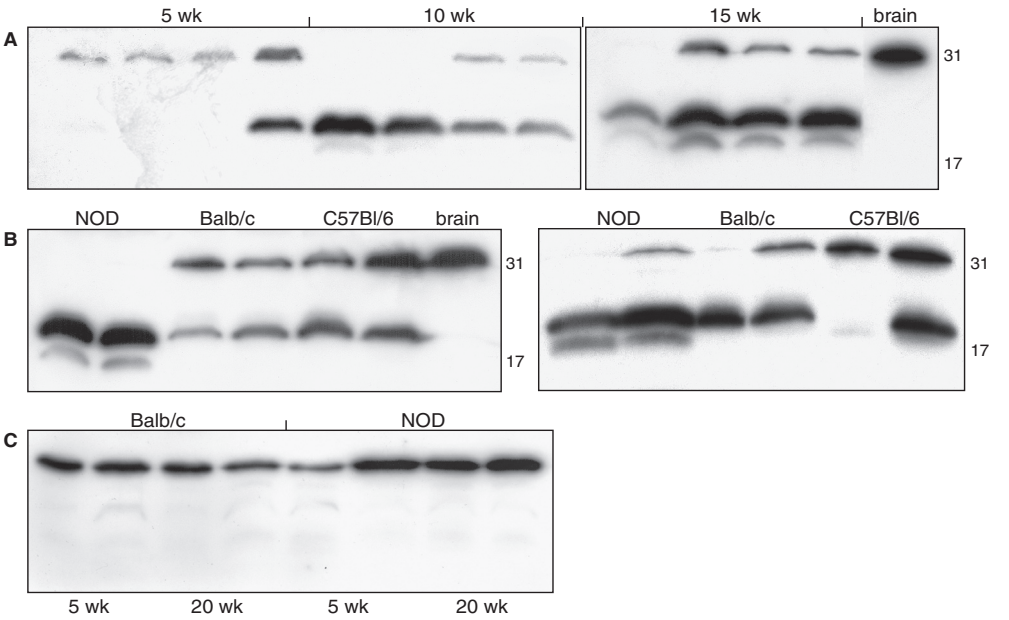

Figure 1. Altered fractalkine cleavage in NOD salivary gland.
Whole tissue lysates were prepared in PBS supplemented with protease inhibitor by mechanical homogenisation followed by sonification. Lysates were then analysed by Western Blotting. A representative picture of six individual experiments is shown. (**A**) SMG of 5,10 and 15 week old NOD mice. (**B**) SMG of 10 week old (left) and 20 week old (right) NOD, C57BL/6 and Balb/c. (**C**) Pancreas of 5 and 20 week old NOD and Balb/c. Numbers represent mass (kDa) as determined using a molecular weight marker.

Fractalkine cleavage is altered in NOD compared to control strains

When control mice were studied, the kinetics of fractalkine cleavage were remarkably different from that in NOD mice. At 5 weeks, no clear differences were found, with the expression of only the 31 kDa form of the protein in both control strains. At 10 weeks, the 19 kDa band started to appear in C57BL/6 and BALB/c mice. However, whereas expression of the 19 kDa form was abundantly found in NOD mice at this age, this band was clearly less intense in the control strains (Figure 1B). The additional 17 kDa form of fractalkine, which was found in NOD mice, never appeared in neither C57Bl/6 nor BALB/c mice at any time point up to 20 weeks (Figure 1B). These results indicate that the kinetics of the cleavage process differ between NOD and control strains and that the specific cleavage in NOD SMG results in the generation of a unique fractalkine fragment in the NOD mouse.

Cleavage of fractalkine does not occur in NOD pancreas

The NOD mouse does not only develop sialoadenitis, but also autoimmune insulitis. Similar to the salivary glands, lymphocytic infiltration in the pancreas is preceded by an accumulation of DC. Since the sialoadenitis and pancreatitis observed in NOD mice are usually considered to be the result of at least partly overlapping defects, expression of fractalkine in the pancreas was studied and compared to that of the SMG. In both NOD and control mice, expression of the 31 kDa form of fractalkine was detected at all ages tested. However, smaller products were not observed at any age, neither in the NOD nor in the control strains (Figure 1C). This indicates that the increase in protease activity is SMG specific.

ADAM-17 induces fractalkine cleavage, but not ADAM-10 or caspase-3

Altered protein breakdown has been described previously in the SMG of NOD mice, for example regarding the autoantigen alpha-fodrin and this was linked to caspase-3 activity (13-15). Therefore, caspase-3 activity was measured in whole salivary gland lysates. Although activity could be detected at all ages, no increase in caspase-3 activity was observed in the NOD mouse (Figure 2A). Furthermore, when brain lysates containing the 31 kDa form of fractalkine were incubated with recombinant caspase-3, no digestion of fractalkine was observed (Figure 2A, insert).

Another family of proteases involved in regulating fractalkine levels are the so-called alpha-secretases. These include ADAM (a disintegrin and metalloprotease)-10 and ADAM-17,which have been shown to cleave fractalkine under steady-state and inflammatory conditions respectively (16, 17). When measured in whole salivary gland lysates, alpha-secretase activity was significantly increased in NOD mice from the age of 15 weeks on (Figure 2B). When incubated with brain lysate *in vitro*, ADAM-17 but not ADAM-10 was capable of cleaving 31 kDa fractalkine. However, the 19 and 17 kDa bands did not appear, indicating that ADAM-17 is not responsible for the specific fractalkine cleavage observed in NOD SMG (Figure 2B, insert).

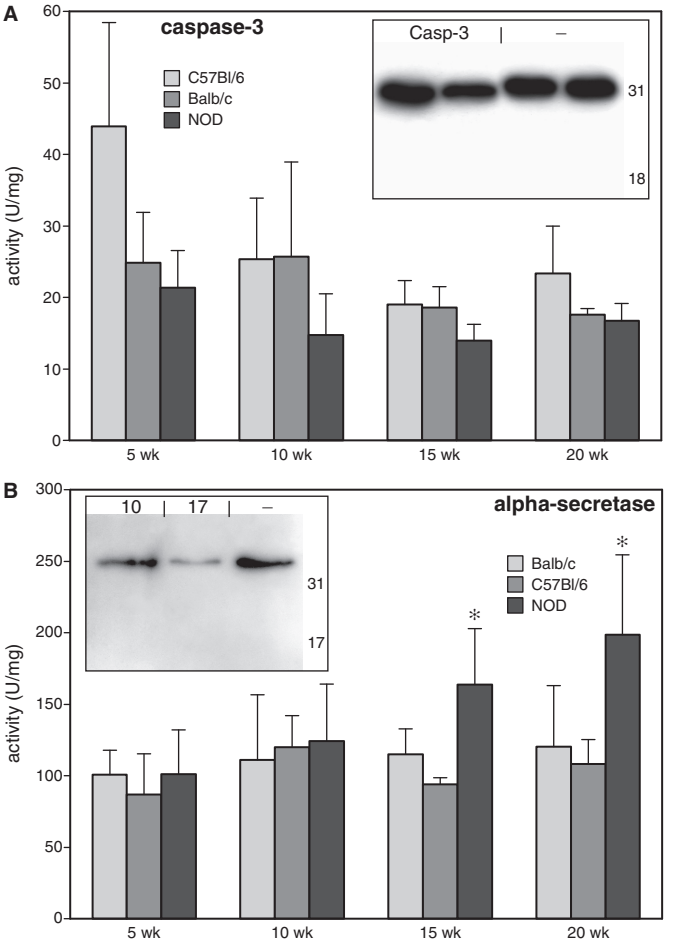

Figure 2. ADAM-17 but not caspase-3 cleaves 31 kDa fractalkine.
Caspase-3 (**A**) and alpha-secretase (**B**) activity was measured in tissue lysates prepared in PBS without protease inhibitors using a fluorimetric assay. Bars represent mean, error bars represent standard deviation (*n*=5), **p*<0.05. Recombinant mouse caspase-3 (**A**) or ADAM-10 or -17 (**B**) were incubated with brain lysate and analysed by Western Blotting (*n*=4). Numbers represent mass (kDa) as determined using a molecular weight marker.

Increased MMP-9 activity may play a role in fractalkine cleavage in salivary gland

Increased expression of matrix metalloprotease (MMP)-9 has been described in the salivary glands of SjS patients and RNA levels of both MMP-2 and MMP-9 are increased in the SMG of NOD mice (18-20). Since fractalkine has been shown to be a ligand for MMP-2 (21), increased activity of these metalloproteases may be involved in the altered proteolysis of fractalkine. When measured in whole salivary gland lysates, gelatinase

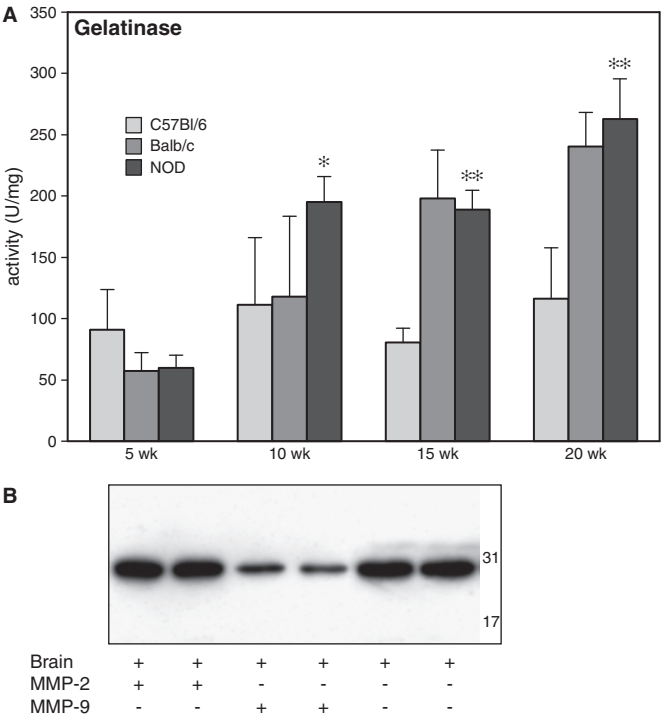

Figure 3. MMP-9 cleaves 31 kDa *in vitro*.
(**A**) Gelatinase activity was measured in tissue lysates prepared in PBS without protease inhibitors using a fluorimetric assay, bars represent mean, error bars represent standard deviation, *p<0.05 compared to C57BL/6 and Balc/c, **p<0.05 compared to C57BL/6 (n=5). (**B**) Recombinant mouse MMP-2 and MMP-9 were incubated with brain lysate and analysed by Western Blotting (n=3). Numbers represent mass (kDa) as determined using a molecular weight marker.

(combined MMP-2 and MMP-9) activity was increased in 10 week old NOD mice, the time point at which the increased cleavage of fractalkine in the NOD first becomes apparent (Figure 3A). Furthermore, incubation of brain lysates with recombinant MMP-9, but not with MMP-2, resulted in a clear reduction of the 31 kDa form of fractalkine (Figure 3B). However, the 19 and 17 kDa bands did not appear in this experiment, indicating that in addition to MMP-9 other proteases are likely to contribute to the fractalkine cleavage observed *in vivo*.

Presence of autoantibodies against fractalkine in NOD

Altered proteolysis of alpha-fodrin in NOD results in the generation of an autoantigen and the formation of autoantibodies (14). Therefore, the occurrence of autoantibodies against fractalkine was studied by testing the reactivity of mouse serum with blotted brain

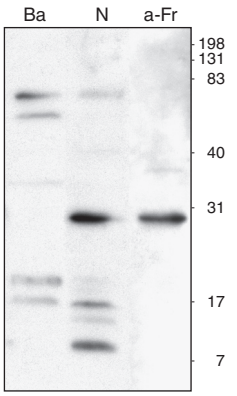

Figure 4. Autoantibodies against fractalkine are present in NOD. Tissue lysates prepared in PBS supplemented with protease inhibitors were run on an SDS gel and transferred to an Immobilon membrane. Membranes were then incubated with purified IgG from either NOD (N) or Balb/c (Ba) mice. Binding of autoantibodies was detected by anti-mouse-HRP and ECL analysis. Presence of specific fractalkine fragments in the lysate was detected using a monoclonal anti-fractalkine antibody (a-Fr). Numbers represent mass (kDa) as determined using a molecular weight marker.

lysate containing the 31 kDa form of fractalkine. In the serum of young (5 wk) animals, reactivity against the 31 kDa protein could not be detected. However, in the serum of animals over 15 weeks of age, antibodies against a protein running at 31 kDa were detected in 10/14 NOD (Figure 4). In controls, this was the case in significantly fewer animals (1/6, $p<0.05$). Similar results were obtained with purified IgG and total serum (data not shown). Fractalkine specificity of the anti-31 kDa band was confirmed by blotting against recombinant fractalkine (data not shown). These results indicate that fractalkine indeed becomes an autoantigen in the NOD mouse.

DISCUSSION

NOD mice display an abnormal breakdown of fractalkine in salivary glands resulting in the generation of a unique fragment. This breakdown did not occur in pancreas, indicating that the phenomenon is organ specific and not a result of local inflammation. Altered proteolytic cleavage in NOD salivary glands has been described previously for alpha-fodrin and parotid secretory protein (13, 14, 22, 23). In the case of alpha-fodrin, proteolysis is caused by the apoptotic enzyme caspase-3 (13). Although caspase-3 was detected in SMG of all ages, activity was not increased in NOD compared to controls. Furthermore, caspase-3 did not cleave 31 kDa fractalkine *in vitro*. Two proteases described to be involved in the physiological shedding of fractalkine are ADAM-10 and ADAM-17 (16, 17). However, although the joint activity of these enzymes did increase in NOD mice at older ages (>15 weeks) and ADAM-17 was capable of cleaving 31 kDa fractalkine *in vitro*, this did not results in the generation of 19 and 17 kDa bands.

The abnormal breakdown of extracellular matrix components has been described in SjS salivary glands, and this was linked to increased activity of MMPs (24). In particular, expression of MMP-9 has consistently been found to be increased in salivary glands of SjS patients. In NOD mice, increased expression of this metalloprotease in

SMG was reported in old (>20 weeks) animals (18-20, 24-27). Our study shows that MMP-9 activity increased already around 10 weeks of age, similar to the time frame where cleavage of fractalkine was first observed. Additionally, MMP-9 is shown to be capable of degrading the 31 kDa form of fractalkine. However, the characteristic 17 and 19 kDa forms did not appear. When fractalkine was incubated with MMP-9 and analyzed by total protein staining, neither the 19 and 17 kDa fragments nor smaller fragments were detected (data not shown), suggesting that at least *in vitro*, MMP-9 cleavage results in very small fragments. This suggests that although MMP-9 is involved in the degradation of fractalkine, other proteases are likely to be responsible for the generation of the 19 and 17 kDa fragments *in vivo*. Candidates for this function include members of the caspase family, such as caspase-1 and caspase-11, both of which are increased in the salivary glands under inflammatory conditions (28, 29). Also, granzyme B may be involved as this protease has been shown to generate antigenic fragments by cleaving alpha-fodrin, La protein and muscarinic receptor 3 (30, 31), all of which are known autoantigens in SjS.

The exact nature of the fractalkine fragments described in this study remains to be further elucidated. Attempts to isolate the fractalkine fragments from SMG lysates were unsuccessful due to the abundant presence of other proteins in the total gland lysates. However, the antibody used to detect fractalkine in this study recognizes the C-terminal end, indicating that both the 19 and 17 kDa fragments contain this part of the protein. One possibility is that the 19 and 17 kDa fragments result from the shedding of the N-terminal part of the protein, which includes the chemokine domain. Previous studies show that fractalkine is indeed a substrate for metalloproteinases, resulting in the generation of a highly active chemotactic fragment (32). If this process also takes place in the salivary glands, the local concentration of chemotactic soluble fractalkine would increase, and thus enhance the migration of responsive cells to the salivary glands. Interestingly, the fractalkine receptor positive population of Ly-6C^{low} monocytes is increased significantly in NOD mice (3). This population preferentially develops into tissue DC (33) and fractalkine receptor positive cells resembling DC were observed in murine salivary glands (Dr. H.C. Reinecker, personal communication). This supports the idea of chemotactic fractalkine contributing to the accumulation of DC in NOD salivary glands. To further investigate this contribution it would be interesting to cross CX3CR1 deficient mice (34) back to the NOD background, and see the effect on monocyte infiltration and DC accumulation.

In the case of alpha-fodrin, the altered cleavage in pSjS and NOD salivary glands results in an autoantigenic protein and the generation of autoantibodies as well as a specific T-cell response (14, 35). Importantly, when cleavage of alpha-fodrin is inhibited, pathology is prevented (36), indicating the significance of this process. In this study, we show that the abnormal cleavage of fractalkine is also accompanied by the occurrence of autoantibodies. In NOD mice, antibodies recognizing 31 kDa fractalkine were present from about 10 weeks and increased with age. In control mice, these autoantibodies were found in significantly fewer animals, and at lower concentrations. It is tempting to

speculate that the abnormal breakdown of fractalkine in NOD SMG contributes to a break of tolerance against this self protein, thus resulting in the formation of autoantibodies. The source of fractalkine in the NOD SMG remains to be elucidated, but studies in human salivary gland show that fractalkine is expressed in glandular epithelium as well as ductal structures (MEW, manuscript in preparation). An anti-fractalkine response would therefore be targeted directly against the salivary gland epithelium, and contribute to destruction of the salivary gland tissue.

CONCLUSIONS

Increased proteolytic activity in the salivary gland of NOD mice leads to the generation of aberrant fractalkine fragments. These fragments may enhance the ongoing inflammation both by their chemotactic properties, and by functioning as an autoantigen. This dual role for fractalkine in the local inflammation in SjS salivary glands indicates fractalkine may be an interesting target for future therapy.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

MEW designed the study, carried out the protease activity assays and was responsible for writing of the manuscript, CvHM carried out the Western Blot experiments and revised the method section of the manuscript, HAD provided critical revisions of the manuscript, MAV directed the project and provided critical revisions of the manuscript. All authors read and approved the final manuscript.

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6

GENERAL DISCUSSION

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Considering all findings in this thesis, a common theme of increased immune activation becomes apparent. At first sight this may seem obvious, given that SjS is an autoimmune phenomenon and thus defined by abnormal immunological responses. However, many of the findings in the NOD animal model were present prior to the onset of disease, suggesting they are not the result of ongoing inflammation. In the human studies, aberrancies were not directly related to the most obvious parameters of inflammation (i.e. cytokine levels and disease duration), again indicating that the defects are likely to be intrinsic rather than the result of the inflammatory process. This chapter will discuss the implications of the findings in this thesis in the light of pathogenesis, treatment and future experiments.

Role of plasmacytoid dendritic cells and type I interferons in SjS

Large scale gene expression profiling of SjS salivary glands revealed increased expression of type I IFN associated genes and increased levels of type I IFN proteins (1, 2). The best known type I IFN producing cell type is the natural killer (NK) cell, but given the fact that few NK cells are found in the infiltrates in these patients, a different source of type I IFN had to be present. In systemic lupus erythematosus (SLE), a disorder with a similar IFN signature, pDC were found to migrate to the affected tissues including the skin, and to produce high levels of type I IFN, putting the spotlight on this cell type (3). Increased numbers of pDC are also present in salivary glands of SjS patients, and localize to sites of IFN activity (4). Since levels of type I IFN in peripheral blood were low as detected by ELISA (2), this pDC-type I IFN activity was thought to be a strictly local phenomenon. The results described in this thesis, however, show that type I IFN associated genes are upregulated in peripheral blood monocytes, indicating a more systemic effect. A similar systemic type I IFN signature was described in SLE and also in rheumatoid arthritis (RA), both frequently associated with SjS. In SLE, induction of type I IFN genes was associated with increased IFN-α serum activity (5-7). Similarly, the results presented here show increased type I IFN activity in the serum of SjS patients. The discrepancy between our findings and the reported low levels of IFN-α in SjS may be explained by technical difficulties detecting type I IFN protein levels due to the large number of subtypes. Whereas only a limited number of subtypes are detected by ELISA, in this thesis we used a bio-assay to measure IFN activtity rather than protein level.

Peripheral pDC in SjS show signs of increased activation, and thus may be a source of type I IFN. These findings expand the role of pDC and type I IFN in the pathogenesis of SjS as these cytokines may well be involved in the systemic symptoms as well. Subjects treated with exogenous IFN-α exhibit extreme fatigue and arthalgia, complaints which also occur in a large proportion of SjS patients (8). In this light, it does not come as a surprise that treatment with anti-TNF-α was not successful in SjS (9). TNF and IFN-α are subject to cross regulation with TNF inhibiting both the generation of pDC from bone marrow and the secretion of IFN-α by this cell type (10). Anti-TNF treatment in other

conditions has been shown to induce increased expression of type I IFN and in some cases even lupus like symptoms (10). In SjS, while inhibiting one side of the inflammatory response, this treatment would further enhance pDC and type I IFN activity and lead to increased systemic complaints. In a recent study, treatment of SjS with a monoclonal anti-TNF antibody indeed resulted in increased levels of type I IFN as well as increased levels of BAFF (11).

The increased level of type I IFN activity appears to support the idea of a viral cause for SjS. The type I IFN signature described in SjS patients in this thesis is indeed similar to the gene signature induced by for example HIV infection (12). However, despite numerous studies, no consistent viral infection has been found in SjS patients thus far. SjS serum has been shown to contain immune complexes capable of activating pDC (2). These immune complexes contain autoantibodies and RNA/ribonucleoproteins and are thought to result from increased apoptosis combined with B-cell hyperactivity rather than viral infection. In this model, the systemic activation of pDC would be a consequence of the initial inflammation rather than a causal factor. The increased type I activity itself might contribute to the perpetuation of the disease, both locally and systemically (Figure 1).

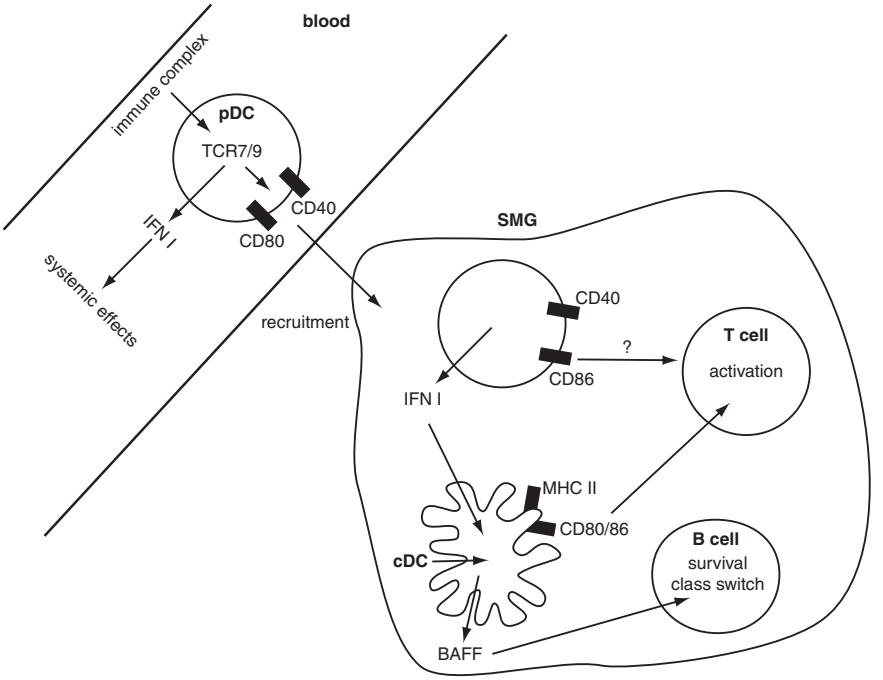

Figure 1.
Activated blood pDC produce type I IFN in response to binding of immune complexes. This results in an increase in systemic type I IFN activity. Activated pDC expressing CD40 and CD86 may also be recruited to the salivary glands. Here they can participate in the activation and survival of T- and B-cells either directly or through IFN stimulation of cDC, resulting in increased BAFF production and costimulation.

Given the putative role of type I IFN in our and other recent models for SjS, the logical next step in the development of a therapy for SjS would be the inhibition of these cytokines. However, a complete block of type I IFN leads to severely compromised immunity with high vulnerability for viral infections and aggressive course of disease as shown by patients with NK cell deficiencies (13, 14). Another option is the depletion of pDC, as this would leave NK cell activity intact. Depletion of pDC may reduce the local and systemic activity of type I IFN, but may also abrogate any regulatory effects exerted by this cell type. Both in the NOD diabetes model and a mouse model for asthma, depletion of pDC resulted in exacerbation of the disease, indicating a regulatory role in both Th1 and Th2 responses (15, 16).Plasmacytoid DC have been shown to express the regulatory molecule IDO, which depletes available levels of tryptophan and leads to inhibition of T-cell proliferation (17, 18). Expression of IDO is increased in SjS salivary glands, and may partly counteract the ongoing inflammation (M. Meeuwese, personal communication). Furthermore, pDC can induce potent $CD4^+CD25^+$ regulatory T-cells as well as IL-10 producing regulatory $CD8^+$ cells (19, 20). Whether pDC serve a regulatory function in SjS remains to be elucidated.

An interesting approach would be to target only the type I IFN activity in pDC. A number of recent studies reveal an important role for B-cell receptor signaling molecules (the BCR 'signalosome') in the modulation of type I IFN production (21). Since some membrane receptors involved in this pathway, including ILT-7 and BDCA-2, are only expressed by pDC (22, 23), this would enable specific targeting of this cell type. Thus far, the effects of this signaling on the tolerogenic properties of pDC is not known, but studies are highly warranted, as they may lead to therapeutic interventions aimed at decreasing type I IFN activity, while leaving regulatory properties intact.

Monocyte subsets and dendritic cell development

The subset of mature $CD16^+$ monocytes is considered as precursors for DC trafficking through tissues under non-inflammatory conditions. Dendritic cells generated from this subset *in vitro* have been shown to express relatively high levels of MHC class II, induce more T-cell proliferation in autologous mixed lymphocyte reactions and are highly potent stimulators of memory T-cells (24-26). The results presented in this thesis further delineate the phenotype of $CD16^+$ monocyte derived DC as relatively mature, expressing more CD83, DC-LAMP and CD86 than their $CD16^-$ monocyte derived counterpart. These markers of DC activation were also found in the SjS salivary glands, suggesting a role for $CD16^+$ monocytes in the accumulation of DC seen in these patients (Figure 2). In a recent study in mice, mature monocytes were described to crawl rather than roll along blood vessels *in vivo*, serving a patrolling function (27). Upon encounter of an immunogenic stimulus, these mature monocytes rapidly extravasate and produce TNF-α, thus playing an important role in the initiation of the immune response.

Renal transplant patients who exhibit high levels of $CD16^+$ monocytes are at increased risk of developing atherosclerosis, probably due to recruitment of these cells

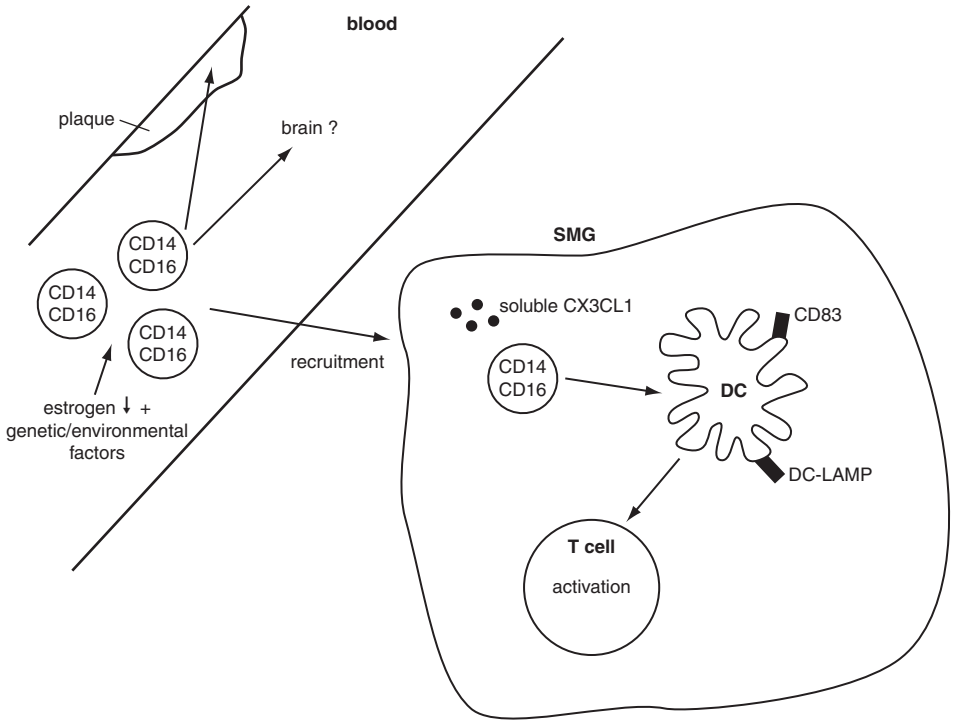

Figure 2.
Increased levels of CD16$^+$ monocytes arise possibly as the result of estrogen withdrawal and genetic factors. These monocytes can be recruited to the salivary glands in response to fractalkine and develop into activated DC. These DC express CD83 and DC-LAMP and may play a role in the local activation of T-cells.

into arterial lesions (28). Interestingly, signs of early atherosclerosis were found in SjS patients, indicating that CD16$^+$ monocytes may also be involved in the extraglandular manifestations of the disease (29). This may be even more relevant in the context of neurological involvement. A subset of SjS patients suffers from neurological complaints ranging from mild sensory neuropathies to seizures and dementia, altough the incidence of these symptoms remains under debate (30-32).

CD16$^+$ monocytes have been shown to preferentially adhere to and cross the brain endothelium, resulting in damage to the vascular system as well as parenchymal accumulation of inflammatory cells (33, 34). In HIV patients, the frequency of CD16$^+$ monocytes is increased dramatically, and an accumulation of CD16$^+$ cells is seen in the brain of patients suffering from HIV associated dementia (35, 36). In those SjS patients suffering from neurological involvement, in particular of the central nervous system, this may also be mediated by CD16$^+$ monocytes and DC derived from this subset (Figure 2). Whether levels of CD16$^+$ monocytes are directly related to the occurrence or severity

of neurological involvement requires further study in a larger group of patients carefully characterized for both immunological and neurological parameters. Such a study would be of interest because of the possible predictive value of this measurement.

Neurological and in particular vascular involvement is an important risk factor for SjS-related morbidity and mortality (37). As $CD16^+$ monocytes appear to play a role in the pathogenesis of these extraglandular manifestations, treatment aimed at reducing the frequency of this subset may be of clinical benefit. Deoxyspergualin reduces numbers of both $CD16^-$ and $CD16^+$ monocytes, but effects on $CD16^+$ monocytes are much stronger and lead to a reduction in proteinuria in patients suffering from glomerulonephritis (38). The glucocorticoid methyl-prednisolone appears to rapidly decrease levels of $CD16^+$ monocytes, while leaving the $CD16^-$ subset intact, probably due to differential expression of the glucocorticoid receptor (39). Although treatment with either of these compounds may be beneficial in patients suffering from acute severe complications, the relatively strong side effects of these drugs make them less attractive for life-long therapy. As relative levels of both subsets are highly stable and return to pre-set values even after strong pharmacological intervention, discontinuation of treatment will likely lead to the recurrence of the $CD16^+$ monocytes (40). Therefore, treatment aimed at inhibiting the adhesion and migration of $CD16^+$ monocytes by blocking the fractalkine receptor pathway may be an interesting alternative. In animal models, anti-fractalkine treatment was effective in inhibiting clinical and histological symptoms of both collagen-induced arthritis and experimental myositis (41, 42). Furthermore, mice deficient in expression of the fractalkine receptor appeared healthy and did not show abnormalities in response to many inflammatory stimuli, suggesting that blocking fractalkine signaling is not likely to generate strong immunological defects (43).

The strong effect glucocorticoids exert on $CD16^+$ monocytes may also shed more light on the gender bias observed in SjS patients. The female predominance among SjS patients points to a role for sex steroids in the pathogenesis. Since in many patients clinical symptoms of the disease start around menopause, it may be the withdrawal of steroids such as estrogen and progesterone rather than their presence that is involved in the disease. Indeed estrogen deficiency has been shown to result in increased apoptosis in salivary gland epithelium as well as B-cell hyperactivity (44). Estrogen also negatively regulates occurrence of $CD16^+$ monocytes as well as secretion of TNF-α *in vitro* (45). Therefore, it can be hypothesized that estrogen normally reduces levels of $CD16^+$ monocytes and that the perimenopausal drop in estrogen levels releases monocytes from this control (Figure 2).

Regulatory function of CCR5

Expression of CCR5 has been clearly established on immature DC as well as primed effector T-cells. As the ligands for this receptor (CCL3, CCL4 and CCL5) are expressed at sites of inflammation, CCR5 was considered to be an ‘inflammatory chemokine receptor’. The results presented here show that this is only part of the picture, as CCR5 also has

tolerogenic properties in regulating the secretion of IL-12 and limiting the bio-availability of inflammatory chemokines.

While bone marrow and lung derived DC in NOD mice showed a decrease in surface expression of CCR5, DC isolated from the salivary gland did not display any surface expression. This coincided with an increased intracellular presence of this chemokine receptor, suggesting the presence of a local stimulus leading to receptor internalization. Obvious candidates for this function are CCL3, -4 and -5, which are present in NOD salivary glands at increased levels. However, prolonged exposure of DC to these ligands did not result in a decrease in CCR5 expression, except for CCL3 at extremely high levels. Another possibility may be the presence of a viral agent. HIV does not only use CCR5 as a co-receptor for cell entry, but also signals through this receptor in macrophages (46). Furthermore, CMV infection of immature DC leads to internalization of CCR5 without leading to obvious maturation (47). Although this probably does not explain the decreased CCR5 expression in all SjS patients, it may contribute to the internalization in a subset of patients, thus enhancing the pro-inflammatory environment.

In human pSjS patients, expression of CCR5 was also decreased, and was correlated to increased levels of IL-12. Of note, these experiments were performed using monocytes, and CCR5 expression on patient DC remains to be determined. T-cell expression of CCR5 though, was also decreased in pSjS patients and correlated closely with CCR5 expression on monocytes (data not shown). This suggests a genetic background for the decreased expression. If DC would show the same phenomenon, this might contribute to a more pro-inflammatory environment in the salivary glands.

A role for CCR5 in dampening immune responses was suggested earlier by reports showing that $CCR5^{-/-}$ mice have exaggerated immune responses in the context of influenza A infection, dental infections and bone marrow and cardiac transplantation (48-51). Furthermore, oral tolerance could not be established in these mice (52). In this model, lack of CCR5 led to increased levels of IL-12, probably through a lack of CCL2 signaling (Figure 3). Besides decreasing levels of IL-12, CCL2 also increases levels of the regulatory cytokine TGF-β (53). Interestingly, TGF-β itself has been shown to induce expression of CCR5, possibly providing a positive feedback loop for regulation (54).

As the CCR5 deficiency in both NOD mice and pSjS patients might have a genetic background, the lack of CCR5 may also affect other cell types. Regulatory T-cells have been shown to express high levels of CCR5, indicating a role for this receptor in their homing (55). In an allogeneic bone marrow transplant model, lack of CCR5 in the T-cell compartment resulted in deficient homing of regulatory T-cells to secondary lymph nodes and a stronger graft-versus-host disease (56). This may be another aspect of immune regulation through CCR5, but its role in pSjS and the NOD mouse remains to be determined.

In contrast to the regulatory role of CCR5 described above, blockade of this molecule has been proposed as a treatment for various inflammatory diseases, including HIV infection, transplant rejection and autoimmunity. CCR5 was initially described as a co-receptor

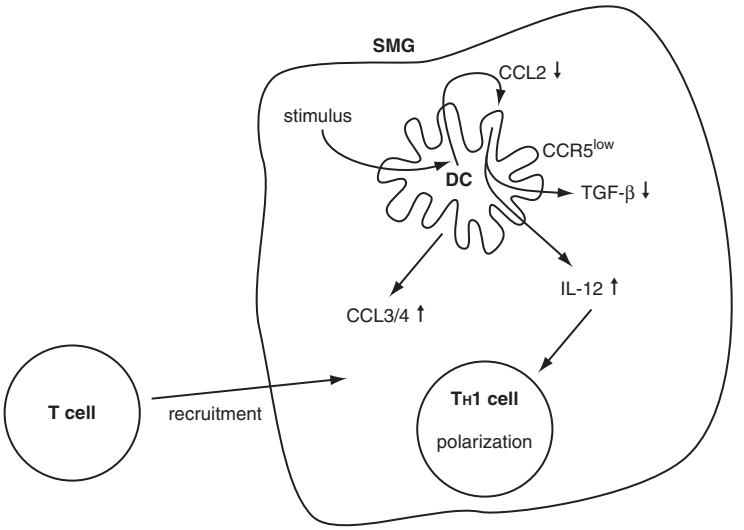

Figure 3.
Local salivary gland DC lack surface expression of CCR5, resulting in increased bio-availability of CCL3 and CCL4 and increased secretion of IL-12. This may lead to increased recruitment of peripheral T-cells, as well as induction of Th1 responses through the actions of IL-12.

for M-trophic HIV, enabling viral particles to enter monocytes and macrophages (57). Individuals homozygous for the delta-32 mutation of CCR5, which leads to undetectable surface expression, are resistant to HIV infection, suggesting that this receptor may be an interesting therapeutic target (58). A number of CCR5 antagonists have now been developed and are being tested in clinical trials, showing promising results in reduction of viral loads (59). This success led to studies focusing on CCR5 inhibition in other inflammatory disorders. Animals treated with anti-CCR5 antibodies or CCR5 antagonists show decreased leukocyte infiltration in kidney and lung transplants (60-62). In RA and multiple sclerosis (MS), high numbers of $CCR5^{+}$ cells and their ligands are found in lesions in synovial tissue and the brain respectively (63, 64), suggesting a role in these diseases. Indeed, antagonizing CCR5 in an animal model resulted in amelioration of collagen induced arthritis (65). In contrast, $CCR5^{-/-}$ mice developed experimental autoimmune encephalopathy similar to wild type animals (66), while inhibiting CCR5 signaling resulted in both inhibition and acceleration of diabetes in various studies (67, 68). These findings indicate that inhibition of CCR5 does not always have a therapeutic effect, and may even aggravate disease, depending on the specific model. Re-evaluation of the transplant models confirmed these findings, since leukocytic infiltration was decreased, but humoral rejection and tissue damage were increased (51, 69).

The data in this thesis may give a partial explanation for these findings by showing a DC specific immune-regulatory role for CCR5. These results indicate that great care

should be taken in using CCR5 inhibition in human disease, as the effects can vary greatly depending on the disorder in question, as well as the immunological state of the patient. Under steady state condition, $CCR5^{-/-}$ mice do no exhibit aberrancies (70), and humans carrying the delta-32 mutation are healthy. In HIV patients, who suffer from suppressed immunity, inhibition of CCR5 may be beneficial both in decreasing viral load and in boosting immune responses through the release from tolerance. However, in patients suffering from autoimmune disorders, inflammation is already increased and under these circumstances, inhibition of CCR5 signaling may lead to even more enhanced immune responses. Although in some patients the balance may shift towards a beneficial condition, a more detailed analysis of the effects of CCR5 blockade in animal models is warranted prior to the use in patients. Furthermore, patients will have to be monitored closely for signs of exaggerated immunity.

In summary, CCR5 has been well established as a receptor involved in the recruitment of pro-inflammatory leukocytes. Although this function is not under debate, it appears dispensable for recruitment of many cell types, as shown by $CCR5^{-/-}$ animals and human CCR5 polymorphisms. In immature DC, CCR5 was considered merely a molecule used for the recruitment to peripheral tissue. The current data suggest that the expression of CCR5 is linked to the tolerogenic phenotype of immature DC, and that its downregulation is not only required for proper migration to lymph nodes, but also for the induction of a true pro-inflammatory phenotype.

Proteolysis and generation of autoantigens

Increased activity of a number of proteolytic enzymes including granzyme B and caspase-3 has been described in the salivary glands of pSjS patients (71, 72). This activity results in a number of processes, including the induction of apoptosis and the generation of new protein variants (73). Especially the latter have received a lot of attention, as it was shown that in the case of α-fodrin, the generated 120 kD fragment functioned as an autoantigen (74). In this case, the enzyme responsible was caspase-3, as specific inhibition of this enzyme strongly reduced cleavage of the normal 240 kD α-fodrin protein (75). Another apoptosis inducing enzyme, granzyme B, has been shown to induce abnormal cleavage of the SS-B protein, also resulting in an autoantigen (76). A similar result was observed in dermatomyositis, where apoptosis resulted in the generation of the Mi-2 autoantigen in diseased tissue only (77).

Besides apoptotic molecules, other factors play a role in the altered protein composition in salivary gland. Matrix-metallo proteinases such as MMP-2 and 9 are expressed at higher levels in pSjS salivary gland than in controls, resulting in the breakdown of extracellular matrix components and the disturbance of the glandular architecture (78, 79). The results presented in this thesis show that MMP-9 and ADAM-17 in particular may play a role in the aberrant cleavage of fractalkine observed in the salivary glands of NOD mice. MMP-9 and ADAM-17 are capable of cleaving both recombinant and naïve fractalkine *in vitro*, but do not lead to the generation of the abnormal breakdown products.

Whether these enzymes are capable of generating this product *in vivo* remains to be established using specific inhibitors or MMP-9/ADAM-17 deficient animals.

The fragments generated by the altered fractalkine cleavage in NOD mice have not been fully characterized thus far. This will be an important step in determining the physiological consequences of the altered cleavage. One interesting possibility is the generation of an autoantigen, resulting in the production of autoantibodies. As fractalkine is expressed on acinar cells, induction of an immune response against this protein might lead to reactivity against the saliva producing cells. Furthermore, fractalkine is expressed on neurons, and is thought to be involved in the signaling and survival of this cell type (80, 81). Interference in this pathway by anti-fractalkine antibodies may inhibit proper neuronal function, and thus lead to reduced nervous signaling and saliva secretion. Experiments in mice show that increased levels of anti-fractalkine antibodies are indeed present in old (>15 weeks) NOD mice, correlating to the increased cleavage with age.

Another possibility is that the cleavage results in the release of a fragment containing the active chemokine domain, thus increasing chemotaxis of fractalkine receptor expressing cells, including monocytes and T-cells (82). Fractalkine induces increased avidity of ICAM-1 on endothelial cells, and may thus participate in retention of recruited cells (83). Soluble fractalkine has been shown to have dose dependent pro-inflammatory effect on macrophages (84). When stimulated with low dose fractalkine, macrophages were less sensitive to subsequent activation. However, when exposed to a high dose of fractalkine, subsequent stimulation by LPS resulted in increased secretion of both TNF-α and IL-23. This suggests that low levels of fractalkine may promote tolerance, while levels above a certain threshold may result in immunity. In normal mice, the baseline cleavage of membrane bound fractalkine results in a low local concentration, preventing immune responses. However, in the NOD mouse, this cleavage is increased, possibly raising the fractalkine concentration above the threshold for tolerance, which may induce an inflammatory response instead.

Of mice and men

Many studies using the NOD mouse attribute all findings to the development of diabetes, and implicitly assume the same mechanisms account for the development of SjS. This idea is not supported by the findings in this thesis, which suggest that although there may be a certain overlap, the pathogenic mechanisms of the two diseases are not identical. In the NOD mouse, elevated levels of Ly-6C^{low} monocytes have been found previously (40), but this was deemed of little relevance for the pathogenesis, as no increase in CD16^{+} monocytes was seen in diabetes patients (85). In pSjS, an increase in this homologous subset does occur, indicating that the increased levels in the NOD mouse may be relevant for the development of SjS. Altered proteolytic cleavage of fractalkine was observed in the salivary gland, but not in the NOD pancreas. In fact, fractalkine cleavage was completely absent from the pancreas, suggesting that this does not play a role in the pathogenesis of diabetes. Conversely, increased levels of CCL19

and CCL21 were present in NOD pancreas (86), but not salivary gland, indicating that these constitutive chemokines may be involved in the development of diabetes but not sialoadenitis. Finally, many studies have shown that particular genetic factors are not shared between the two diseases in the same animal. For example, the MHC I-Ag7 is crucial for the development of diabetes, but replacing it does not abrogate development of SjS (87). Similarly, replacement of a number of diabetes specific loci, including *idd1*, *idd6*, *idd9* and *idd13* did not hamper the development of sialoadenitis in NOD mice (88, 89).

Although this does not exclude the possibility that various abnormalities may contribute to the pathogenesis of both diseases, it does warrant caution when interpreting results, in particular those obtained systemically (i.e. from blood, bone marrow etc.). Studies including the specific target organs are required to determine the relevance of a particular finding for SjS and/or diabetes in the NOD model. Furthermore, the addition of patient studies is indicated for determination of disease specificity and clinical relevance.

Monocytes, dendritic cells and their ligands in SjS

Earlier studies have shown increased numbers of cDC as well as pDC in salivary glands of both SjS patients and NOD mice (4, 90, 91). Furthermore, in the NOD mouse, the accumulation of DC preceded the infiltration of lymphocytes, suggesting a role in the pathogenesis of this disease (90). Data on alterations in DC or monocytes themselves are scarce and mainly consist of one study describing a reduced number of blood DC in patients, possibly due to increased migration to the target organs (92). The results presented in this thesis provide new insights into the role of DC in the pathogenesis of SjS. The frequency of $CD16^{+}$ mature monocytes is increased in SjS patients. Since these monocytes preferentially develop into an activated type of DC, this may contribute to the accumulation of activated DC seen in SjS salivary gland. SjS monocytes and possibly also DC express lower levels of CCR5, which is linked to increased secretion of IL-12 as well as increased bio-availability of the inflammatory ligands CCL3 and CCL4. Expression of these ligand can induce recruitment of T-cells, which can be activated and skewed to a Th1 phenotype by the activated IL-12 secreting DC. Plasmacytoid DC show signs of increased activation in the peripheral blood, possibly as a result of activation by immune complexes. This may lead to an increase of systemic type I IFN activity, contributing to systemic complications such as fatigue and arthalgia.

In summary, a number of aberrancies in the monocyte-DC system occur in SjS involving both cDC, pDC and chemokines. Although none of these aberrancies in themselves are sufficient to induce SjS, they are likely to contribute to the complex immunological reactions leading to the clinical symptoms. Therefore, inhibition of one or more of these mechanisms may prove to be a viable strategy for treatment of this autoimmune disorder.

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SUMMARY

Sjögren's Syndrome (SjS) is an autoimmune disease affecting the salivary and lachrymal glands. Patients suffer from dryness of the mouth and eyes, as well as a number of systemic symptoms including fatigue and joint pain. Histologically, the disease is characterized by the formation of focal lymphocytic infiltrations in the affected glands. The underlying cause leading to this infiltration has not been elucidated, but clues have been obtained from the study of animal models such as the nonobese diabetic (NOD) mouse. Similar to human patients, this mouse develops lymphocytic infiltrates in the salivary glands and shows a decrease of salivary flow with increasing age. In the NOD mouse, the formation of lymphocytic infiltrates is preceded by an accumulation of dendritic cells (DC). As DC play a key role in the initiation of immune responses, this suggests a role in the early phase of the disease. This thesis focuses on various subsets of DC and their precursors in the pathogenesis of SjS.

In **chapter 2**, we focused on the role of plasmacytoid DC (pDC) in SjS. This subset of DC is known for its capacity to produce type I interferon (IFN). In SjS patients, both type I IFN and pDC have been shown to be present in the salivary glands, but this was thought to be a strictly local phenomenon. Our study shows that the increase in type I IFN activity is in fact systemic, as a type I IFN gene signature is found in peripheral blood cells. We show serum to be the source of this activity, as SjS serum is capable of inducing the type I IFN associated genes in an IFN-receptor dependent manner. In the peripheral blood from SjS patients, a decreased number of pDC is present. The pDC present have an increased expression of activation markers such as CD40. This expression is correlated to the type I IFN activity in the serum, pointing to the pDC as the source of this activity.

In **chapter 3**, the role of monocytes, which are considered to be precursors for DC, is studied. In other autoimmune diseases, a number of aberrancies in this cell population have been described, which in turn lead to abnormal DC. We found that in SjS patients, $CD16^+$ mature monocytes are more frequent. Since fractalkine, an important chemoattractant for these monocytes, is expressed in human salivary glands, $CD16^+$ monocytes may contribute to the DC accumulation observed in SjS patients. Indeed, in an *in vitro* system, $CD16^+$ mature monocytes developed into DC with an activated phenotype, expressing CD83 and DC-LAMP. These markers were also expressed in the salivary glands of SjS patients, suggesting that the local DC are activated and are likely to participate in driving the local inflammation.

In **chapter 4**, we studied the phenotype and function of the DC present in the salivary gland in the NOD mouse model. DC isolated from NOD salivary glands lack expression of the chemokine receptor CCR5, a marker which is found on DC from control strains. This defect appears to be largely intrinsic in the NOD mouse, as it is present prior to the onset of infiltration, as well as in DC generated from NOD bone marrow *in vitro*. We tested the consequences of the CCR5 defect using DC generated from bone marrow deficient

in CCR5 ($CCR5^{-/-}$). As LPS stimulation of $CCR5^{-/-}$ DC results in increased production of the pro-inflammatory cytokine IL-12 compared to wild type DC, these DC appear to lack a negative feedback loop. The relevance of this observation for human SjS patients is demonstrated by the finding that SjS monocytes also show a decreased expression of CCR5, which is inversely correlated to an increased level of serum IL-12.

Finally, in **chapter 5**, we focused on chemokines, the molecules attracting monocytes and dendritic cells to specific tissues. As described in chapter 3, SjS patients show increased levels of mature monocytes, and express the relevant chemokine fractalkine in their salivary gland. The NOD mouse also shows an increase of mature monocytes, but whether fractalkine was expressed in NOD salivary glands had not been elucidated. In this chapter we show that fractalkine indeed is expressed in mouse salivary glands. Although the molecule is expressed in both young and old animals, cleavage occurs with increasing age resulting in smaller fragments of the protein. In the NOD mouse, this cleavage started at a younger age than in the control strains. Furthermore, a unique fragment was found in NOD mice, that was never seen in controls. This aberrant cleavage is probably the result of increased proteolytic activity of MMP-9, ADAM-17 as well as other proteases. The enhanced cleavage may result in an increased release of the chemotactic domain of fractalkine, thus enhancing the influx of mature monocytes and boosting the accumulation of activated DC. In addition, the abnormal fractalkine fragment appears to result in an anti-fractalkine immune response, as anti-fractalkine antibodies were found in the NOD mouse.

In summary, our results show that systemic pDC are relatively activated, leading to increased systemic type I IFN activity and systemic complaints. Furthermore, both the NOD mouse and SjS patients have abnormal levels of mature monocytes, which can be attracted to the salivary glands, possibly through the increased chemotaxis by fractalkine. Once in the tissue, these monocytes develop into DC which themselves are abnormal, since they lack expression of CCR5. This results in an increased production of IL-12, contributing to a pro-inflammatory environment. The involvement of aberrancies in both monocytes and DC subsets locally as well as systemically suggests that these cell types are interesting targets for future therapies.

SAMENVATTING VOOR NIET-IMMUNOLOGEN

Het syndroom van Sjögren (SjS) is een auto-immuunziekte, waarbij het afweersysteem zich richt tegen het eigen lichaam. Patiënten met SjS hebben last van een droge mond en droge ogen, maar ook van klachten als extreme vermoeidheid en gewrichtspijn. De ziekte wordt gekenmerkt door het optreden van infiltraten (ophopingen van ontstekingscellen) in de speekselklieren. De oorzaak hiervan is nog niet bekend, maar onderzoek in het nonobese diabetic (NOD) muismodel heeft enige aanwijzingen opgeleverd. Net als SjS patiënten ontwikkelen deze muizen infiltraten in de speekselklieren. Ook neemt de productie van speeksel af naarmate de dieren ouder worden. In de NOD muis is ontdekt dat het ontstaan van de infiltraten voorafgegaan wordt door een toename van het aantal dendritische cellen (DC). Deze cellen spelen een belangrijke rol in het begin van een afweerreactie en daarom is het waarschijnlijk dat ze een rol spelen in de vroege fase van de ziekte. Het onderzoek dat wordt beschreven in dit proefschrift richt zich op afwijkingen in de verschillende soorten DC en in de voorlopercellen voor DC, de monocyten in het perifeer bloed.

In **hoofdstuk 2** ligt de nadruk op de rol van plasmacytoide DC (pDC), een type DC dat vooral bekend is om zijn vermogen om type I interferonen (IFN) te produceren. Type I IFN zijn eiwitten die een groot aantal ontstekingscellen kunnen activeren. Het was al bekend dat zowel type I IFN als de pDC in verhoogde mate aanwezig zijn in de speekselklieren van SjS patiënten. Men dacht echter dat dit een strikt lokaal fenomeen was, dat alleen in de speekselklieren optrad. Ons onderzoek laat zien dat de verhoging van type I IFN activiteit systemisch is, dat wil zeggen, in het hele lichaam voorkomt. Dit blijkt uit het feit dat genen die door type I IFN aangestuurd worden, verhoogd tot expressie komen in bloedcellen van SjS patiënten. Het aantal pDC in het bloed van SjS patiënten is verlaagd. De aanwezige pDC hebben een verhoogde expressie van het CD40 molecuul, hetgeen een aanwijzing is voor een verhoogde activiteit. De expressie van CD40 is direct gecorreleerd aan de type I IFN activiteit in het serum. Dit geeft aan dat de pDC de bron van de verhoogde activiteit kunnen zijn.

In **hoofdstuk 3** is de rol van monocyten bestudeerd. In een aantal andere auto-immuunziekten is al bekend dat er afwijkingen zijn in de monocyten, en dat deze tot afwijkende DC kunnen leiden. Wij hebben ontdekt dat in het bloed van SjS patiënten relatief meer $CD16^+$ rijpe monocyten aanwezig zijn. Verder hebben we in de speekselklier van de mens een molecuul gevonden dat deze monocyten aantrekt, het zogenaamde fraktalkine. Dit betekent dat de rijpe monocyten bij patiënten met SjS zouden kunnen bijdragen aan de ophoping van DC in de speekselklieren. In een modelsysteem ontwikkelden de rijpe monocyten zich tot geactiveerde DC, die de moleculen CD83 en DC-LAMP op hun oppervlakte hadden. Deze moleculen werden ook gevonden op DC in de speekselklieren van SjS patiënten, hetgeen er op wijst dat de lokale DC geactiveerd zijn en dus mogelijk een rol spelen in het stimuleren van de lokale ontsteking.

In **hoofdstuk 4** hebben we NOD muizen onderzocht om het uiterlijk en de functie van DC in de speekselklieren te bestuderen. DC uit de speekselklieren van NOD muizen misten

het molecuul CCR5 op hun oppervlakte, terwijl DC uit controle muizen dit wel hadden. Dit defect was al aanwezig vóór het ontstaan van de infiltraten en werd ook gevonden in DC die gekweekt werden uit het beenmerg van NOD muizen. Daarom is dit defect waarschijnlijk eigen aan de NOD muis. Om de gevolgen van een gebrek aan CCR5 te testen, maakten we gebruik van DC die werden gekweekt uit beenmerg van muizen die geen CCR5 hebben. Deze DC reageerden sterker op het bacteriële product LPS dan normale DC. In de DC zonder CCR5 was de productie van het ontsteking-stimulerende eiwit IL-12 hoger dan in normale DC. Dit wijst er op dat DC zonder CCR5 een regulerend mechanisme missen. Dat deze waarnemingen niet alleen relevant zijn voor muizen, maar ook voor SjS patiënten, blijkt uit het feit dat deze patiënten ook een abnormaal lage hoeveelheid CCR5 op hun bloedcellen hebben. Dit is gecorreleerd aan een verhoogde concentratie van IL-12 in het bloed.

Tenslotte hebben we gekeken naar moleculen die belangrijk zijn voor het aantrekken van monocyten en dendritische cellen, de chemokinen. Zoals beschreven in hoofdstuk 3, hebben SjS patiënten een verhoogde frequentie van rijpe monocyten in het bloed, en komt het relevante chemokine fraktalkine voor in de speekselklier. NOD muizen hebben ook meer rijpe monocyten in hun bloed, maar het was onbekend of fractalkine in hun speekselklier aanwezig was. In **hoofdstuk 5** laten wij zien dat fraktalkine inderdaad voorkomt in de speekselklieren van muizen. Het molecuul komt zowel bij jonge als bij oude muizen voor, en wordt afgebroken naarmate een muis ouder wordt. In de NOD muis begon deze afbraak op jongere leeftijd dan in controlemuizen. Bovendien ontstond in de NOD muizen een uniek eiwitfragment dat niet voorkwam in de controlemuizen. De afwijkende afbraak van fraktalkine trad tegelijk op met een verhoogde activiteit van de enzymen MMP-9 en ADAM-17. De afbraak leidt mogelijk tot de vorming van een fragment met een versterkte chemokine activiteit, waardoor er meer monocyten aangetrokken worden en de DC zich in de speekselklieren kunnen ophopen. Daarnaast kan het ontstaan van een abnormaal fragment leiden tot een reactie van het afweersysteem tegen het molecuul. Bij de NOD muis werden inderdaad antistoffen tegen fraktalkine gevonden.

Samengevat laten onze resultaten zien dat bij SjS patiënten de pDC ook buiten de speekselklieren geactiveerd zijn, hetgeen mogelijk resulteert in een verhoogde activiteit van type I IFN en systemische klachten. Bovendien bevat het bloed van zowel SjS patiënten als NOD muizen relatief veel rijpe monocyten welke kunnen worden aangetrokken door de speekselklier. Hierbij speelt mogelijk de afbraak van fraktalkine een rol. Eenmaal in het weefsel aangekomen, ontwikkelen de monocyten zich tot DC die ook abnormaal zijn, aangezien ze geen CCR5 op hun membraan tot expressie brengen. Als gevolg hiervan produceren ze meer IL-12, wat bijdraagt aan het induceren en in stand houden van de afweerreactie. Het vóórkomen van afwijkingen in zowel monocyten als verschillende DC subsets in SjS patiënten wijst er op dat deze cellen een interessant doelwit zijn voor het ontwikkelen van nieuwe behandelingen.

DANSK REFERAT

Sjøgrens Syndromet (SjS) er en autoimmun sygdom. Det betyder at det naturlige afværgesystem (immunsystemet) angriber kroppen selv i stedet for bakterier eller virus. I tilfælde af SjS angribes spytkirtler og tårekirtler, som resulterer i en reduktion af spyt og tårevæske. Patienter klager over mundtørhed og øjetørhed, men også over ekstrem træthed og smerter i led. På det mikroskopiske niveau karakteriseres sygdommen af en akkumulation af immunceller i spytkirtler. Årsagen til denne akkumulation er ikke kendt, men eksperimenter i en muse-model (den nonobese diabetic eller NOD mus) har genereret brugbare oplysninger. I denne mus opstår akkumulationer i spytkirtler ligesom i SjS patienter. På samme tid findes en reduktion af spytproduktion. I NOD mus har man opdaget at en forøgelse af antallet af dendritiske celler (DC) går forud for den akkumulation af immunceller. For disse celler er vigtige for at begynde en immunreaktion, kan man tænke sig at de er vigtige i den tidlige fase af sygdommen. Forskingen som beskrives i denne bog koncentrerer sig på fejl i forskellige former af DC og deres forløberceller, monocytterne.

I **kapitel 2** fokuseres på plasmacytoide DC (pDC), en form af DC som er kendt for sin evne til at producere type I interferoner (IFN). Type I IFN er proteiner som aktiverer mange forskelige immunceller. Man vidste allerede at begge type I IFN og pDC er mere aktive i SjS patienternes spytkirtler, men man troede at dette kun var en lokal aktivitet. Resultater i denne bog viser at forøgelse af aktiviteten faktisk er systemisk, det vil sige findes i hele kroppen. Gener som aktiveres af type I IFN findes i SjS patienternes blodlegemer, og kilden til aktiviteten er serum. Det ved vi, fordi patienternes serum også aktiveres 'type I IFN' gener i en model til blodlegemer, THP-1 cellen. I patienternes blod findes færre pDC, men de pDC som er til stede er meget aktive, som påvises af fremstilling af molekylet CD40. Denne fremstilling korreleres direkte til type I IFN aktivitet i serum som peger på pDC som aktiviteternes kilde.

Kapitel 3 handler om monocytter. Monocytter er forløberceller som kan udvikle sig til DC og findes i blodet. Monocytter har fejl i andre autoimmunsydomme, og disse fejl resulterer i abnormale DC. Vi har fundet at SjS patienter har flere $CD16^+$, modne monocytter. I et *in vitro* system udviklede de modne monocytter sig til aktive DC, som har molekylerne CD83 of DC-LAMP på deres yderside. Disse molecyler findes også i SjS spytkirtler, som viser at de lokale DC er aktive og er vigtige for at stimulere immunsystemet i spytkirtlerne.

NOD musen blev brugt i **kapitel 4** for at forske fenotypet (udseende) og funktion af spytkirtlernes DC. DC som blev isoleret fra NOD spytkirtler manglede molecylet CCR5, som findes i almindelige mus. Denne fejl er sandsynligvis NOD musens egen, for manglen var til stede allerede før akkumulationen af immunceller og fandtes også i DC som blev dyrket fra knoglemarv. For at undersøge følgerne af manglen af CCR5 blev DC dyrket fra knoglemarv som manglede genen til dette molekyl. Det virkede som om denne DC manglede en regulerende mekanisme, for de viste en ualmindelig stærk reaktion på det bakterielle produkt LPS. I DC uden CCR5 var produktionen af den immun stimulerende

protein IL-12 højere end i almindelige DC. Monocytter i blodet af SjS patienter har også mindre CCR5 molekyler på deres yderside. Dette var korrelativt til koncentration af IL-12 i blodet, så CCR5 er sandsynligvis også vitgtig i menneskers immunregulation.

Til sidst blev chemokiner studeret. Chemokiner er molekyler som spiller en rolle i organisation af DCernes bevægelse. Som beskrevet i kapitel 3 har SjS flere modne monocytter i deres blod og den vigtige chemokine fraktalkine findes i spytkirtler i mennesker. NOD mus også har flere modne monocytter, men vi vidste ikke om fraktalkine også findes i deres spytkirtler. I **kapitel 5** viser vi at fraktalkine er tilstæde i både unge og gamle mus, men i gamle mus fandtes kløvning af fraktalkine. Dette skete tidligere i NOD mus end i almindelig mus, og i NOD mus fandtes et unikt fragment. Den ualmindelige kløvning er sandsynligvis delvis resultatet af forøget aktivitet af enzymer MMP-9 og ADAM-17. Kløvningen fører muligvis til et fragment med styrket funktion, som kan tiltrække monocytter og DC til spytkirtlerne. Desuden kan det unikke fragment fremkalde en autoimmun reaktion mod fraktalkine for i NOD musenes blod fandtes autoantistoffer som reagerede mod fraktalkine.

Sammenfattende viser vores resultater at pDC også er aktiveret uden for spytkirtler, som muligvis fører til en forøget type I IFN aktivitet og systemiske symtomer. Desuden har SjS patienter og NOD mus mange modne monocytter, som kan tiltrækkes til spytkirtlen. Her må findes en rolle for den ualmindelig kløvning af fraktalkine. Når de en gang er til stede i vævet, udvikler monocytterne sig til DC som også har fejl, da de ikke har CCR5 på deres yderside. Dette resulterer i en forøget producktion af IL-12, som fremkalder og opretholder autoimmunreaktionen. Da vi finder fejl i både monocytter og forskelige former af DC i SjS er disse celler et interessant mål i udviklingen af nye behandlinger.

ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
APC	antigen presenting cell(s)
BAFF	B cell activating factor belonging to the tumor necrosis factor family
BCR	B cell receptor(s)
BDCA	blood dendritic cell antigen
cDC	conventional dendritic cell(s)
CFSE	5(6)-carboxyfluorescein diacetate N-succinimidyl ester
CL2MDP	(dichloro-phosphono-methyl)phosphonic acid
CMV	cytomegalovirus
CRISP	cystein-rich secretory protein
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DC	dendritic cell(s)
DC-LAMP	dendritic cell lysosomal-associated membrane protein
DC-SIGN	dendritic cell-specific ICAM3-grabbing non-integrin
DHEA	dehydroepiandosterone
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
Foxp3	forkhead/winged helix protein 3
GM-CSF	granulocyte macrophage colony-stimulating factor
HCV	hepatitis C virus
HEV	high endothelial venules
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV	human T-cell leukemia virus
HUVEC	human umbilical vein endothelial cell(s)
ICAM	intercellular adhesion molecule
ICOS	inducible costimulator
Idd	insulin dependent diabetes locus
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IKK	inhibitor of kappa B kinase
IL	interleukin
kDa	kilodalton
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MMP	matrix metalloprotease
MS	multiple sclerosis
NFkB	nuclear factor kappa B
NK	natural killer

NOD	non-obese diabetic
PBMC	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cell(s)
PDCA	plasmacytoid dendritic cell antigen
RA	rheumatoid arthritis
RQ-PCR	real time quantitative polymerase chain reaction
SCID	severe combined immunodeficiency
SjS	Sjögren's Syndrome
SLE	systemic lupus erythematosus
SMG	submandibular gland
SOCS	suppressor of cytokine signaling
SSDAI	Sjögren's Syndrome disease activity index
TAP	transporter associated with antigen processing
TGF	transforming growth factor
TLR	Toll like receptor
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
YG	yellow green dye

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Zoals iedereen weet, is het doen van onderzoek iets dat je niet alleen doet. Daarom wil ik op deze plaats de mensen bedanken die het mogelijk gemaakt hebben dat dit boekje er nu ligt.

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Manon

CURRICULUM VITAE

19 november 1980 **Geboren te Hilversum**

1992 - 1998 **Gymnasium, Gemeentelijk Gymnasium, Hilversum**

1998 - 2002 **Biomedische Wetenschappen, Universiteit Leiden**
oktober 2000 - mei 2001: stage 'The role of $CD25^+$ T-cells in the immunotherapy of melanoma' (Afdeling Immunohematologie en Bloedbank, LUMC; dr. R.P.M. Sutmuller, dr. R. Offringa).

januari 2002 - september 2002: stage 'The role of MBL polymorphisms in chronic obstructive pulmonary disease' (Afdeling Longziekten, LUMC; dr. J. Stolk, prof. dr. P.S. Hiemstra)

september 2002 - oktober 2002: scriptie 'The role of surfactant protein A in innate immunity'

2001 - 2003 **Scandinavische Talen en Culturen, Universiteit van Amsterdam**
Hoofdvak Deens

2003 - 2008 **Promotieonderzoek 'Monocytes and dendritic cells in Sjögren's Syndrome'.** Afdeling Immunologie, Erasmus MC, Rotterdam. Promotoren: prof. dr. R. Benner en prof. dr. H.A. Drexhage, co-promotor: dr. M.A. Versnel

Onderwijstaken
september 2004 - augustus 2007: assistent practica Immunologie in het thema 2.1 'Infectie- en immuunziekten'

Gevolgde cursussen
februari 2004: 'Molecular Immunology' (Postgraduate School of Molecular Medicine)
september - december 2004: 'English Biomedical Writing and Communication (NIHES)
september 2007: 'Management voor promovendi en post-docs' (NIBI)

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