Local gene therapy and the identification of therapeutic targets in Sjögren’s syndrome

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LOCAL GENE THERAPY AND THE IDENTIFICATION OF THERAPEUTIC TARGETS IN SJÖGREN’S SYNDROME

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“Local gene therapy and the identification of therapeutic targets in Sjögren’s syndrome”

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LOCAL GENE THERAPY AND THE IDENTIFICATION OF THERAPEUTIC TARGETS IN SJÖGREN’S SYNDROME

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Ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus prof. dr. D.C. van den Boom ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Agnietenkapel op vrijdag 10-2-2012, te 12 uur

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If we knew what it was we were doing, it wouldn’t be called research, would it?

- *Albert Einstein*
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SJÖGREN’S SYNDROME, TARGETS AND THERAPY

Sjögren’s Syndrome (SS) is a chronic inflammatory exocrinopathy that affects an estimated 0.1-1.0% of the general population, most commonly women in their 4th or 5th decade. Patients diagnosed with SS complain of dry eyes and dry mouth, leading to pain, dyscomfort, frequent infections and poor dental health. This dryness is the result of a poorly understood autoimmune process that mainly affects the secretory glands, such as the lacrimal and salivary glands (SG). In addition, patients can suffer from extra-glandular manifestations including peripheral neuropathy and vasculitis and often experience disabling fatigue. A potential life threatening complication of SS is the development of lymphomas, mostly of the mucosa-associated-lymphoid type (MALT); SS patients have an estimated 16 times higher risk compared to the general population. Despite the prevalence of the disease, limited knowledge exists on its aetiology. Genetic (reviewed in 1), infectious, hormonal, neurological and immunopathological factors may all play a role in the development of the disease.

There is a large unmet need for effective therapy in SS; curative treatment for SS is not available. Immune based therapies such as tumor necrosis factor (TNF) antagonists, which are successful in the treatment of other autoimmune diseases such as rheumatoid arthritis, have failed in SS. Most SS patients today are using artificial tears and sympathomimetics to stimulate salivary flow with unsatisfactory results.

One of the major problems in finding a therapy for Sjögren’s syndrome is that the right therapeutic target has not been identified, mostly due to the poor understanding of the disease pathogenesis. This thesis addresses a few questions in this regard:

1. Based on current knowledge, which novel immunological targets can be identified for the treatment of SS?
2. Can additional targets be identified studying a spontaneous mouse model for SS; the NOD mouse?
3. Is it possible to alter auto-immune SG disease in this mouse model by inhibiting or altering the previously identified targets with local gene therapy?
4. Are there additional targets to be identified in patients with SS?

Chapter 2 and chapter 3 introduce the experimental studies. In chapter 2 the immunological disbalance in cytokines observed in human SS patients is summarized. In addition, experiences with cytokine directed therapies in SS are discussed. In chapter 3, the possibilities to address this disbalance with (local) gene therapy are discussed and potential immunological targets for the treatment of SS are proposed.

A reason for the poor understanding of SS is that most patients are diagnosed when inflammation is clearly present and therefore little is known about the early onset and progression of the disease. Mouse strains that spontaneously develop a SS-like disease offer an insight into early disease processes that may be extrapolated to SS in humans. In addition, studying a mouse model makes it possible to closely follow pathogenesis over time. Currently there are multiple of such animal models available for human SS. We have chosen to study the (most commonly used) non-obese diabetic (NOD) mouse. These mice spontaneously develop autoimmunity resulting in
diabetes and thyroiditis. They also show progressive inflammation of the salivary and lacrimal glands, closely resembling the human inflammatory characteristics in SS disease. Over time these mice may develop salivary flow impairment. In chapter 4, the development of this SS-like disease in NOD mouse is followed and described in detail. The inflammatory and physiological changes in the salivary gland are specifically addressed.

Besides a model to study the pathogenesis of a SS-like disease, the NOD mouse also offers a model to test a variety of therapeutics. Based on the observations that were made in the NOD mouse and the current knowledge on pathological pathways in SS, we choose to target intercellular adhesion molecule type 1 (ICAM-1) (chapter 5), CD40 (chapter 6) and the B-cell activating factor (BAFF)/a proliferation inducing ligand (APRIL)-transmembrane activator and CAML interactor (TACI)-axis (chapter 7). These molecules and related pathways were found to be upregulated in the NOD mouse and are known to be aberrantly expressed in humans with SS. Immunomodulatory molecules interfering with the proposed targets were expressed in the salivary glands by local gene therapy in which the gene of interest is transferred into the SG with the aid of an adeno-associated viral vector (AAV). This ensures delivery of the gene into the epithelial cells (forming the ducts in the SG) and leads to a stable expression and secretion of the immunomodulating proteins. In chapter 8, two of these immunological targets identified in mice, the B cell related cytokines APRIL and BAFF and their receptors, are analyzed for their presence in human salivary gland biopsies and blood. In the last chapter, the findings described in this thesis are summarized, discussed and recommendations for the future are made.

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CYTOKINES IN SJÖGREN’S SYNDROME: POTENTIAL THERAPEUTIC TARGETS

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Sjögren’s syndrome (SS) is a systemic chronic inflammatory autoimmune disorder that affects secretory organs such as the salivary and lachrymal glands. Patients complain of dry eyes and dry mouth (sicca symptoms) and often have systemic manifestations, such as Raynaud’s phenomenon, arthritis, fatigue and vasculitis. Its estimated prevalence is 0.5% with a female to male ratio of 9:1. The pathogenesis of the disease is largely unknown and to date, no universally effective therapy is available. The histological hallmark of SS is the presence of focal infiltration of T and, to a lesser degree, B lymphocytes in the salivary glands. The chronic inflammation in SS patients is further reflected by an imbalance in cytokines both locally in the glands and systemically in the blood. The scope of this review is to summarize the current data on cytokine abnormalities described in patients with SS.

Cytokines

Cytokines are powerful regulators of the innate and adaptive immune system. They play a central role in controlling the direction, amplitude, and duration of the inflammatory response. Aberrations in their expression may lead to immune deficiency, allergy or autoimmunity. Cytokines are pleiotropic and can be secreted by hematopoietic cells and numerous other cell types. Most cytokines have a predominantly pro- or anti-inflammatory effect, but many can exert both functions, depending on their environment.

Immune responses have been traditionally divided into two groups, Th1 and Th2, although it has recently become clear that many immune responses have features of both. A Th1 response is characterized by activation of effector T cells and the production of interferon γ (IFNγ). A Th1 response clears intracellular organisms, but is also involved in many autoimmune diseases. The Th2 response is characterized by a humoral immune response and results in the activation of B cells and the production of antibodies. The main cytokine involved in Th2 responses is interleukin 4 (IL-4). An imbalanced Th2 response results in allergies but is also linked to autoimmunity (reviewed in4). SS is thought to be a Th1 dominated disease primarily because IFNγ and its related cytokines are consistently found to be highly expressed in SS patients. Moreover, salivary gland derived T cells from patients produce the Th1 cytokines IFNγ and IL-2 ex vivo, but no or low levels of the Th2 cytokine IL-4. However, the significant hypergammaglobulinaemia and high levels of autoantibodies together with high expression of IL-10, another Th2 cytokine, demonstrate a simultaneous activation of the Th2 response. These data reflect that although the Th1/Th2 concept is useful in understanding cytokine networks and responses, its direct applicability in systemic human autoimmune diseases, such as SS, is limited.
CYTOKINES AND SALIVARY GLAND DYSFUNCTION IN SJÖGREN’S SYNDROME

Cytokines may contribute to the pathogenesis of SS in various ways. They play a central role in the initiation and perpetuation of inflammation in the excretory glands. The imbalance of pro-over anti-inflammatory cytokines results in cumulative damage in the glands leading to decreased secretory function. However, although infiltration of the gland by lymphocytes is a hallmark of SS, some patients suffer from significant secretory dysfunction without major glandular destruction\textsuperscript{4,7}. Since some cytokines are upregulated even in the absence of lymphocytic infiltrates they may have a direct effect on epithelial cells independent from damage caused by inflammation. Moreover, SS patients are at a higher risk than the normal population for developing non-Hodgkin’s lymphoma in the exocrine glands\textsuperscript{8-9}. The mechanisms responsible for lymphomagenesis include cytokine-driven chronic stimulation of B and/or T cells and formation of ectopic germinal centers\textsuperscript{10}. In addition, since cytokines are key molecules in systemic inflammation, they may contribute to systemic complications of SS as well. Many cytokines that are found to be highly expressed in the glands and serum have been related to episodes of vasculitis or other systemic features.

MAJOR PRO-INFLAMMATORY CYTOKINES IN SJÖGREN’S SYNDROME

The major pro-inflammatory cytokines found to be important in SS are the interferons, IL-12, IL-18, tumor necrosis factor α (TNFα), IL-1β, IL-6 and B-cell activating factor (BAFF). Whether the recently discovered IL-17, IL-23 and the related T cell subset Th17 play an important role in SS is still unknown.

Interferons

Interferons were the first cytokines discovered. They were found to play a crucial role in the innate immune response against viruses\textsuperscript{11}, over the past decades their function has turned out to be much broader. Interferons activate T cells and macrophages, affect class switching of B cells, enhance antigen presentation and upregulation of inter-cellular adhesion molecules, all leading to an activated immune state ready to fight off pathogens. IFNs also play a crucial role in autoimmunity\textsuperscript{12-14}. There are two groups of interferons; Type I interferon (IFNα and IFNβ) is secreted by virus-infected cells and plasmacytoid dendritic cells (pDC) while type II, or IFNγ is mainly secreted by T cells, natural killer (NK) cells and macrophages\textsuperscript{15}.

IFNs play a central role in the pathogenesis of SS. They are aberrantly expressed in patients and most cytokines and transcription factors that are overexpressed in patients are IFN inducible\textsuperscript{14,16-19}. Moreover, activated pathways that are related to IFN signalling were significantly different between SS patients and healthy volunteers\textsuperscript{18}. This profile has been referred to as ‘the interferon signature’\textsuperscript{14}.
**IFNα**

Low levels of IFNα are constitutively present in a variety of cells and are found circulating in the blood, whereas high titres are rapidly produced in the presence of danger signals such as a viral infection. IFNα is also upregulated in autoimmune diseases like systemic lupus erythematosus (SLE). In salivary gland biopsies from SS patients IFNα is detected at higher levels in acini and endothelial cells compared to controls. Moreover, IFNα is mainly secreted by pDC, these cells are found in the salivary glands of SS patients but not in healthy controls. Serum levels of IFNα were found to be high in SS patients compared to healthy individuals by some groups, but not by others. On the contrary, some groups observed low circulating levels of IFNα and argued that this could lead to reduced beneficial NK cell activity and reduced anti-viral defense mechanisms in patients.

**IFNγ**

IFNγ is the major cytokine involved in a Th1 response, a response designed to clear intracellular pathogens. However, overexpression of IFNγ and an exaggerated Th1 response are also involved in many autoimmune diseases, such as RA and multiple sclerosis (MS). The production of IFNγ is directly stimulated, amongst other cytokines, by IFNα and IFNβ. The presence of IFNγ creates a pro-inflammatory environment in the salivary gland as illustrated by the observation that treatment of human salivary gland (HSG) cells with IFNγ, in the presence or absence of TNFα, results in increased levels of adhesion molecules and upregulation of antigen presenting molecules on the cell surface.

IFNγ mRNA is overexpressed by infiltrating cells in the salivary gland of patients with primary SS, but has normal systemic levels in the same patients. Contradicting results have been published on whether healthy individuals express IFNγ in the salivary gland at constitutive levels. IFNγ inducible proteins, however, were clearly found in salivary glands of SS patients but not in healthy controls, supporting the notion of increased IFNγ expression in SS.

Interestingly, IFNγ is also highly expressed in individuals with sicca symptoms who do not have any histological signs of inflammation in the gland. It is possible that, in addition to maintaining an inflammatory response with recruitment of T and B cells, IFNγ also has a direct effect on the secretory function of the gland. In vitro data support this: prolonged treatment of HSG with IFNγ in the presence or absence of TNFα leads to a persistent depletion of intracellular Ca²⁺ stores and thus to an exhausted response system. Moreover, IFNγ reduces the growth of HSG in a concentration dependent way, suggesting that IFNγ may impair damage repair in the salivary gland.

**IL-12 and IL-18**

IL-12 and IL-18 are pro-inflammatory cytokines, closely related to IFNγ, that work synergistically to drive a Th1 response. They are both predominantly secreted by monocytes and macrophages and promote IFNγ secretion. IL-12 and IL-18 were found to be overexpressed in SS. Expression of IL-12 was primarily observed in the infiltrating cells. IL-18 was detected in acinar cells, ductal cells and macrophages.
in salivary glands of SS patients, but not in healthy subjects, patients with chronic
graft-versus-host disease or chronic non-SS sialoadenitis. Some groups, but not
all, found that IL-18 is particularly high in those patients with anti-Ro and anti-La
autoantibodies37,40-41. IL-18 may be involved in the lymphomagenesis of SS patients,
since high levels of IL-18 correlated with low levels of circulating complement, salivary
gland enlargement and germinal center formation all of which are thought to be risk
factors for lymphoma development37,41.

**TNFα**

TNFα is produced by monocytes, CD4+ T cells and epithelial cells. It upregulates
the apoptotic receptor Fas on many cells including HSG42 and in combination with
IFNγ sensitizes cells to apoptosis43-44. Moreover, it also plays a role in the presentation
of autoantigens since the nuclear antigens Ro, La and alpha fodrin, recognized by
autoantibodies in many SS patients, are only transported to the membrane surface of
salivary gland cells which undergo apoptosis in the presence of TNFα45.

High levels of TNFα and TNFα secreting cells have been found in peripheral blood
and in lymphocytic infiltrates in salivary gland biopsies from patients with SS22,46-47.
TNFα, and its two receptors; TNFR-p55 and TNFR-p75 are present in biopsies of
healthy controls but are expressed at higher levels in SS patients47. The expression
level of TNFα did not correlate with the focus score48, but serum levels are especially
high in patients positive for rheumatoid factor (RF)49 suggesting a correlation of TNFα
expression and severity of systemic involvement.

**IL-1β**

IL-1β activates vascular endothelium and lymphocytes. Together with TNFα it is
considered to be the key inflammatory cytokine in chronic inflammation. However,
surprisingly little is known about its role in SS. IL-1β secreting circulating lymphocytes
are significantly upregulated in SS patients compared with healthy controls and
non-SS sicca patients, and its level correlates with disease duration and RF levels46,50.
Immunohistochemical staining of salivary glands of SS patients showed expression of
IL-1β whereas biopsies from controls did not22.

**IL-6**

IL-6 is important for B cell growth and differentiation. It is thought to induce the
production of autoreactive antibodies by infiltrating B cells via upregulation of specific
cytokines and through its effect on the terminal differentiation of the immunoglobulin
producing plasma B cell51. IL-6 has an active role in T cell stimulation and recruitment
since it promotes the transition of naïve T cells to cytotoxic T cells. It also upregulates
intercellular adhesion molecule 1 (ICAM-1) which functions as a receptor for activated
T cells and a co-stimulatory molecule for B cells, on many cells52.

IL-6 was found highly expressed in serum and in peripheral circulating lymphocytes
of SS patients, and was absent in most of the healthy controls. High levels of IL-6
correlated with the degree of infiltration in the gland and the number of extraglandular
symptoms49,53-57. IL-6 in saliva of SS patients was found to be consistently high.
Moreover, it was found in labial gland biopsies of SS, but was not detected or was
detected at lower levels in healthy controls\textsuperscript{52,55,57-59}. IL-6 together with TNF\textalpha\ seems to be directly associated with inflammation of the gland since these two cytokines are overexpressed in saliva of SS patients but not in patients with drug-induced xerostomia\textsuperscript{56}.

**BAFF**

One of the newest growth factors implicated in SS is BAFF which belongs to the superfamily of TNF related cytokines and promotes B cell survival\textsuperscript{60}. BAFF exists in a membrane bound and a secreted form. BAFF induces major lymphoproliferative disorders in transgenic mice with B cell hyperplasia and hypergammaglobulinaemia resembling the autoimmune phenotype of SLE\textsuperscript{61}. At a later age these mice develop a SS like disease with infiltrates in the salivary gland and a reduced salivary flow\textsuperscript{62}.

BAFF is equally expressed in ex vivo cultured epithelial cells of the salivary gland of healthy individuals and SS patients, however patients also express BAFF at low levels in infiltrating T cells in the salivary gland whereas healthy people do not\textsuperscript{63}. SS patients also have high serum and salivary levels compared to healthy individuals whereas the expression levels of membrane bound BAFF does not differ between patients’ and healthy controls’ epithelial cells\textsuperscript{64-65}. Plasma and salivary secreted BAFF levels are especially higher in patients with hypergammaglobulinaemia, higher focus scores and in patients positive for anti-Ro and anti-La antibodies\textsuperscript{66-68}. BAFF induces a significant anti-apoptotic effect in peripheral B cells of SS patients; this effect is even more evident in B cells from SS patients with high levels of gammaglobulin\textsuperscript{69}. This may indicate that BAFF is important in germinal center formation and may contribute to lymphomagenesis, but data on this are still inconclusive\textsuperscript{66-68}.

**IL-17 and IL-23**

A recently discovered subset of T lymphocytes involved in inflammation and autoimmunity, Th17 cells, was originally discovered in mice and is characterized by the secretion of the powerful pro-inflammatory cytokines IL-17 and IL-23\textsuperscript{70,71} and IFN\gamma when cells are stimulated with IL-12\textsuperscript{72}. In humans, Th17 cells are derived from memory T cells under the influence of IL-1\beta, IL-6 and/or IL-23. IL-4 inhibits the development of Th17\textsuperscript{73-76}. Th17 cells are very effective in clearing extracellular pathogens. They are also believed to have a pivotal role in the initiation and perpetuation of autoimmunity. IL-17 induces the expression of a variety of pro-inflammatory cytokines such as IL-6, TNF, and intercellular adhesion molecules in a variety of cells\textsuperscript{77}.

IL-17 may be an important player in the pathogenesis of SS, but data are lacking to support this to date. IL-17 could be detected in serum and saliva of about fifty percent of a small group of SS patients, but also in a similar percentage in healthy control subjects. In biopsies of patients, the lymphocytic foci stained positive for both IL-17 and IL-23, especially in the CD4\textsuperscript{+} T cells, and showed diffuse staining on epithelial cells. Healthy individuals and sicca patients also showed low expression of IL17 but this was confined to ductal epithelium only\textsuperscript{39,57}. 
ANTI-INFLAMMATORY CYTOKINES

Many pro-inflammatory cytokines are overexpressed in SS. Concomitantly, some, but not all of the anti-inflammatory cytokines are lacking or are expressed at relatively low levels.

**TGFβ**
Transforming growth factor β (TGFβ) is a bipolar cytokine. TGFβ is crucial to development of immunity. It is often associated with exaggerated immune excitability and overexpression is associated with increased fibrosis. Conversely, TGFβ is key in limiting innate and adaptive immune responses, particularly self-reactive T cells, to restore immune homeostasis and to prevent autoimmunity (reviewed in78-79).

TGFβ mRNA is found in normal and SS salivary glands40, but is reduced in SS patients with a high focus score80. TGFβ is immunohistochemically detected in the ductal epithelial cells of normal and inflamed salivary gland tissues but is absent in ductal epithelial cells surrounded by infiltrated activated T cells in the diseased gland81.

**IL-4**
IL-4 is another classical anti-inflammatory cytokine and the main cytokine in a Th2 type immune response. This cytokine is absent or low in mucosal biopsies of SS patients40. Moreover, the ratio of the pro-inflammatory cytokine IFNγ to IL-4 is higher in the salivary gland and lower in the peripheral blood of patients29 reflecting a skewed immune pattern towards a Th1 response locally in the gland.

**IL-10**
IL-10 is an anti-inflammatory cytokine involved in Th2 type responses. IL-10 produced by regulatory T cells suppresses the effector immune responses. However, in the presence of IFNγ it can exert a pro-inflammatory effect82. IL-10 is an important B cell activating factor and prolonged stimulation of naïve B cells with IL-10 leads to plasma cell formation83-84.

High plasma levels of IL-10 correlate with a higher susceptibility for SS24,85. High serum levels of IL-10 in SS patients are associated with higher titers of IgA rheumatoid factor, anti-Ro, and anti-La antibodies, and with the severity of lymphocytic infiltration in the salivary gland. Moreover, patients who have high levels of IL-10 had significantly more episodes of cutaneous vasculitis24. T cells isolated from salivary glands from SS patients produce significantly higher levels of IL-10 in contrast to the circulating T cells of the same patients5. A significant elevation of IL-10 was found in saliva of SS patients compared with healthy controls. In patients, these elevated IL-10 levels significantly correlated with the severity of dryness of the mouth and eyes and with the erythrocyte sedimentation rate84. These data indicate that higher levels of circulating IL-10 are associated with more systemic involvement and also play a role in the local inflammatory process. Since high IL-10 levels are related to more severe disease, it is possible that the increased secretion of IL-10 represents an anti-inflammatory control mechanism while it contributes at the same time to B cell activation.

In table 1 the cytokines we previously discussed and their role and expression levels are summarized. In short, the cytokine imbalance in SS is characterized by the
overexpression of pro-inflammatory cytokines, such as IFNγ, IL-12 and IL-18. Two other cytokines, IL-6 and BAFF, which are important in T and B cell activation and autoantibody production, are also upregulated. The presence of other cytokines like IL-1β and IL-17 may also play a role in the pathogenesis of SS, but data on these are incomplete. Concomitantly, IL-4 and TGFβ, two important anti-inflammatory cytokines are downregulated. In contrast, the anti-inflammatory Th2 cytokine IL-10 is highly expressed in SS patients compared to healthy controls and may contribute to B cell activation and autoantibody production. Figure 1 depicts (in a simplified way) the cytokines, the processes they are involved in and their relationship to each other.

Figure 1. The effect of key cytokines on the different aspects of Sjögren's syndrome (SS). An imbalance in the local expression of pro-inflammatory and anti-inflammatory cytokines leads to chronic inflammation and salivary gland dysfunction. Pro-inflammatory cytokines are shown in dark gray boxes, anti-inflammatory in green. IL-10, a bipolar cytokine with known pro- and anti-inflammatory characteristics, is shown in green and gray. The effect of cytokines on the most important pathological processes (white ovals) in SS are shown by blue arrows. The effect on cytokines on each other is shown in orange arrows. IL-4 and TGFβ are expressed at low levels or not detectable in SS. IL-17 and IL-23 (in orange) may play a role in SS (dotted lines) but conclusive data on this is not yet available. Cytokines in the red framed boxes depict cytokines which may provide a good target for therapy. DC, dendritic cells, IL, interleukin, TGF, transforming growth factor, BAFF, B cell activating factor, TNF, tumor necrosis factor, IFN, interferon.
Table 1. Cytokines involved in patients with Sjögren’s syndrome (SS), their function, their relative expression levels in salivary gland (SG) and serum compared to healthy controls and their proposed effect in SS. Natural killer, NK; major histocompatibility complex, MHC; human salivary gland cells, HSG; germinal centers, GC.

| Cytokine | General effect | Expression SG | Serum levels | Proposed effect in SS |
|----------|----------------|---------------|--------------|-----------------------|
| IFNα     | Anti viral response, activation of NK cells, induction of MHC I and cellular adhesion molecules, induces IFNγ | ↑18,22 | ↑23-24, =14, ↓20 | Upregulation of numerous pro-inflammatory pathways\(^{18}\), reduced NK cell activity/ reduced anti viral response\(^{25}\)? |
| IFNγ     | Central Th1 cytokine, antiviral and anti bacterial response. Induction of numerous pro-inflammatory cytokines, up-regulation of class I and II MHC antigens and leukocyte adhesion molecules, modulation of macrophage effector functions | ↑21-22,29,31,33 | =21-22,29 | Depletion of Ca\(^{2+}\) stores in HSG\(^{34}\), reduced growth of HSG\(^{28,35}\), induction of adhesion molecules and antigen presenting molecules on HSG\(^{28}\) |
| IL-12/IL-18 | Induction of IFNγ secretion, CD4\(^+\) T cell differentiation to Th1 cells | ↑37-41 | ↑37-38 | GC formation and lymphomagenesis/ auto-antibody antibody formation\(^{37,40-41}\) |
| TNFα     | Induction of apoptosis, activation of vascular endothelial cells and macrophages, clearing of pathogens | ↑22,47 | ↑46,49,87 | Auto-antigen presentation of Ro and La\(^{49}\), correlates with number of systemic complications\(^{49}\) |
| IL-1β    | Activation of vascular endothelium, activation of lymphocytes and NK cells, major component of acute inflammatory response | ↑22 | ↑46,50 | ? |
| IL-6     | B cell proliferation and terminal differentiation into plasma cells, T cell stimulation and recruitment, acute phase responses | ↑(↑ in saliva)\(^{22,55,57-59}\) | ↑49,53-57 | Correlates with degree of inflammation and systemic features\(^{49,53-57}\) |
| BAFF     | B cell development, maturation and survival | =/↑ in infiltrating T cells\(^{63-65}\),↑ in saliva\(^{39}\) | ↑64,68 | Resistance to apoptosis in B cells\(^{54}\), autoantibody formation/ GC formation and lymphoma genesis?\(^{66-68}\) |
| IL-17/IL-23 | Clearing of extracellular pathogens, essential for many auto immune diseases in animal models, induction of many pro-inflammatory cytokines | ↑ in infiltrating cells\(^{39,55}\), = in saliva\(^{37}\) | =57 | ? |
| TGFβ     | Anti-inflammatory effect, overexpression is related with fibrosis | =40/↓ high focus score\(^{60,61}\) | ? | Reduced protection from autoimmunity, inflammation? |
CURRENT EXPERIENCE WITH CYTOKINE DIRECTED THERAPIES

IFNα was the first cytokine used in a therapy for SS patients based on the observation that SS patients have low levels of circulating IFNα (a view which has since been challenged) and reduced sensitivity of NK cells indicating a potentially reduced antiviral response. Early small studies used high dose parenteral IFNα with overall positive results on salivary gland function and focus score. At the same time, several groups showed that oromucosal administration of low dose IFNα was biologically and clinically effective in animal models of autoimmune diseases. These observations and the concern about potential toxicities associated with high dose IFNα led to studies evaluating low dose oral IFNα. Initial studies showed improvement in some but not the same outcome measure of salivary gland function and subjective oral dryness. A Phase III study of 497 patients treated with placebo or IFNα lozenges chose stimulated salivary flow and subjective oral dryness as the co-primary outcomes. This study was negative for these primary endpoints because both the placebo and the interferon treated groups showed significant but similar improvement in stimulated saliva. Interestingly, compared to placebo recipients those treated with IFNα had a significantly higher improvement in unstimulated salivary flow, and showed improvement in several other subjective secondary endpoints.

Preliminary studies with TNF-blocking agents were also encouraging with positive outcome in both objective and subjective parameters after infliximab treatment. However, a larger randomized, double-blind, placebo-controlled study of infliximab with 103 SS patients showed no difference in response between the placebo versus the infliximab treated. Similarly, etanercept was also not more effective than placebo in a 12 week placebo-controlled.

A major limitation of these studies is that they do not provide an explanation for the disappointing results. The negative results could be due to suboptimal study design, ineffective dosing, insensitive outcome measures, biologic inefficacy or the combination of these factors. Patient selection is important and should be tailored to the goal of the therapy. If improving salivary flow is the goal, including only patients...
who have some salivary function will improve the chance to show an effect, since patients who may not have any functional tissue left due to fibrosis or atrophy will be excluded. The importance of this consideration was already shown in a study with rituximab. Alternatives to traditional outcome measures of saliva and tear flow are much needed, since our current measures are both insensitive and non specific and do not distinguish between disease activity and damage. Most importantly, early clinical studies should address the biologic effects of the treatments. For example, it is not known whether the lack of efficacy of TNF-inhibitors was due to suboptimal doses, which were insufficient to suppress TNF in the target organs or if effective suppression of TNF was achieved but other mechanisms of the underlying pathophysiology negated the benefit of TNF-blockade. This latter mechanism is supported by recent observations showing paradoxical elevation of TNF, IFNα and BAFF after etanercept treatment.

FUTURE TARGETS

Despite the disappointing clinical results to date cytokines remain attractive albeit challenging therapeutic targets. The pharmaceutical industry shows little interest to develop biologic therapies primarily for SS; therefore we will focus on cytokines which could be targeted with biologics that are available or are in clinical testing for other indications (Table 2).

IFN-regulated genes are overexpressed in both the exocrine glands and peripheral blood in SS suggesting an exaggerated interferon response. Suppressing IFNα represents an appealing therapeutic target but the clinical significance of the IFN signature and the role of IFNα in SS, in general is not yet understood. First, the IFN signature showed no association with any clinical manifestations other than the presence of anti-SSa and SSb antibodies in SS and, as discussed above, therapeutic trials with IFNα showed no harmful effects and may have had some benefit. So, on the one hand, although conventional wisdom overwhelmingly supports the blockade of IFNα as a treatment option in SS, based on the available clinical data of IFNα treatment of patients with established SS, it should not be dismissed as a potential therapy. On the other hand, the majority of experts support the idea of blocking IFNα to treat SS. Several IFN blocking agents are under development. Single administration of an anti-IFNα monoclonal antibody in SLE successfully suppressed the IFN signature. Further studies are required to test whether this translates into clinical benefit. Multiple dosing studies are underway and if they show a reasonable safety profile interferon blocking agents should be tested in SS.

A more recently described cytokine implicated in SS is BAFF a major promoter of B cell survival. In patients with SS BAFF levels are elevated in the serum, saliva and exocrine glands. BAFF is upregulated in salivary gland epithelial cells after viral infection and after treatment with IFNα suggesting that it may represent a link between innate and adaptive immunity. Moreover, BAFF seems to be involved in the formation of ectopic germinal centers in the salivary glands which may be an important step in lymphomagenesis. Several anti-BAFF agents are currently tested
IL-6, a potent pro-inflammatory cytokine, is involved in acute phase reactions and both B and T cell responses. It was found to be consistently high in saliva and serum, and is highly expressed in the salivary glands of SS patients but not in subjects with xerostomia only\textsuperscript{22,55,57-59}. A monoclonal antibody against the IL-6 receptor exhibited efficacy and a good safety profile in rheumatoid arthritis (RA)\textsuperscript{104}. The same antibody led to normalization of the abnormal peripheral B lymphocyte repertoire in a pilot study in patients with SLE\textsuperscript{105}. B cell abnormalities are similar between SS and SLE, and are characterized by a shift to increased plasma cell and memory B cell populations. Therefore, blocking IL-6 or its receptor may have a beneficial effect on both the local inflammatory process and systemic autoimmunity in SS.

Overexpression of IL-12 and IL-18 is associated with inflammation and decreased function in the gland as well as lymphomagenesis. Limited, preliminary studies with an IL-18 binding protein were performed in rheumatoid arthritis and psoriasis\textsuperscript{106}. A monoclonal antibody against the p40 subunit of IL-12 showed beneficial effects in Crohn's disease and psoriasis. Since the p40 subunit is shared with the recently discovered IL-23\textsuperscript{107}, it is likely that, at least some of, the beneficial effects are due to blocking IL-23\textsuperscript{108}. IL-23 was found at higher levels in the salivary gland in SS\textsuperscript{57,109} and if its role in chronic inflammation were confirmed, blocking the shared p40 subunit of IL-12 and IL-23 would be appealing. IL-17 secreting CD4\textsuperscript{+} T cells have recently been identified as a specific subset with an important role in inflammation and autoimmunity.
SS patients have increased expression of IL-17 in the salivary glands\textsuperscript{39,57,109} and higher levels in the serum\textsuperscript{109}. Further studies are needed to establish the role of IL-17 in humans but it may represent an exciting future target.

Successful cytokine-based therapies must have a reasonable safety profile, should reduce inflammation systemically and locally and should restore the secretory function. Because of the redundancy of the cytokine network targeting a single candidate may not achieve all criteria. Therefore, for an effective therapeutic response it may be necessary to use a combination of cytokine targets concomitantly or sequentially or target downstream effector molecules shared by several cytokines. A major limitation of these approaches is the increased risk of potentially severe side effects, which is not justified for many SS patients. Because the salivary glands are relatively easily accessible, an alternative to systemic treatment, which would greatly improve the risk benefit ratio of cytokine based therapy for SS patients, would be the local delivery of cytokines or their inhibitors, for example by gene therapy, successfully applied to animal models of SS\textsuperscript{110-111}. The increasing availability of biologics and the potential of gene therapy are exciting, but identifying the right target remains a challenge that can only be overcome by a better understanding of the pathogenesis of SS. Well designed proof of concept studies addressing the biologic effects of cytokine directed therapies will facilitate the identification of targets which can be tested for clinical efficacy. Since SLE and SS share many pathophysiological similarities and a large proportion of SLE patients have coexisting SS, SLE patients enrolled in studies with biologics that may work in SS should be evaluated for SS, as well. This could be done relatively easily and would provide significant information about the potential value of various cytokines as potential targets in SS.

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Gene Therapy: Sjögren’s Syndrome

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ABSTRACT

Sjögren’s syndrome (SS) is characterized by inflammation and dysfunction of the secretory organs. In the majority of patients the salivary and lacrimal glands are predominantly affected, although systemic symptoms are common. The pathogenesis of the disease is not well understood and to date there is no universally effective therapy available. The development of gene therapy and in particular local gene therapy applied to the salivary glands may be effective in the disease. Animal studies have shown that treatment with immunomodulatory molecules such as interleukin 10 or vasoactive intestinal peptide can influence salivary function positively while changing the local inflammatory environment. Future research will have to show whether this approach is feasible in humans.
SJÖGREN’S SYNDROME

Sjögren’s syndrome (SS) is a systemic chronic inflammatory autoimmune disease predominantly affecting the lachrymal and salivary glands. Patients complain of dry eyes and dry mouth (sicca symptoms), the latter leading to pain, discomfort, dental caries and infection of the mouth with opportunistic pathogens such as *Candida albicans*. SS is often accompanied by systemic symptoms, such as Raynaud’s phenomenon, arthritis, fatigue and vasculitis. Women are nine times more likely to be affected than men and the estimated prevalence is 0.5% for the general population¹. The diagnosis is based on objective and subjective criteria of dryness of the secretory glands, inflammation of the salivary gland and the presence of auto-antibodies in the serum. It is termed primary SS (pSS) in the absence of an underlying disease, and secondary SS (sSS) when related to other autoimmune disease such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)².

The pathogenesis and pathophysiology of SS are poorly understood. A number of human leukocyte antigen (HLA) subtypes are associated with SS³ and viral and other environmental factors together with these genetics are all thought to play a role in the initiation and progression of the disease⁴. Chronic inflammation is central to SS and this is reflected by an imbalance of pro-inflammatory cytokines over anti-inflammatory cytokines. Overexpression of numerous pro-inflammatory cytokines in the blood, salivary glands and the saliva has been shown in several studies. The most consistently upregulated cytokines are interferon γ (IFNγ), IFNα, interleukin (IL)-12, IL-18, IL-6, and B-Cell activating factor (BAFF). The presence of other cytokines such as IL-1β and IL-17 may also play a role in the pathogenesis of SS, but data on these are incomplete. Concomitantly, IL-4, an important anti-inflammatory cytokine and transforming growth factor β (TGFβ) are downregulated. In contrast, the anti-inflammatory cytokine IL-10 is highly expressed in SS patients compared to healthy controls and may contribute to B cell activation and auto-antibody production (reviewed in⁵). Histologically, SS is characterized by localized infiltrates (foci) of mononuclear cells in the salivary glands. The degree of infiltration observed ranges from mild to severe and can be accompanied by glandular atrophy and fibrosis. Foci are generally comprised of T cells (80%) and B cells (20%) and in a subgroup of patients these foci are organized to resemble germinal centers⁶. SS patients are at a higher risk than the normal population of developing non-Hodgkin’s lymphoma in the exocrine glands⁷-⁸. A possible mechanism for this may be chronic cytokine-driven stimulation of the infiltrating B and/or T cells in these germinal centers leading to lymphomagenesis⁹.

THE SALIVARY GLAND IN SS

The salivary gland is primarily composed of two cell types: Epithelial (ductal) cells and acinar (secretory) cells. Acinar cells are the only cells in the gland that produce saliva, they also produce more than 80% of the proteins found in saliva. The fluid secreted by acinar cells is modified by the ducts during its passage to the mouth. NaCl is reabsorbed from the secreted fluid whereas K⁺ and HCO₃⁻ are added by the ductal cells in addition to the secretion of proteins such as growth factors and
immunoglobulin (Ig) A (reviewed in 10). In SS both acinar and ductal cells are thought to be involved in the pathogenesis of the disease. Acinar cells loose their ability to secrete fluid, due to destruction by inflammation, altered cell signaling or aberrations in the neurological signal that drives secretion. The ductal epithelial cells also play an important role in SS. Ductal cells can function as antigen presenting cells in the gland and express co-stimulatory molecules such as CD40, CD80 and CD86 indicating an active role in inflammation. Moreover, histologically most infiltrates are found around the ducts, thus SS is also known as autoimmune epithelitis. Figure 1 shows a simplified image of the major processes involved in SS. The cause of sicca symptoms experienced by SS patients can be directly due to the destruction or dysfunction of parenchymal tissue of the secretory organs by inflammation in some patients. Recently recognized dysfunction without overt infiltration in a significant number of patients has lead to the hypothesis that the dryness of the gland may be caused by a mechanism independent of, or in addition to destruction. The type 3 muscarinic receptor (M3R), which is part of the parasympathetic nervous system, is the main neurological receptor involved in saliva secretion in murine models as shown by knockout studies. It is also thought to be the main receptor involved in human saliva secretion. There is cumulating evidence that serum of a subgroup of patients interferes with the function of the M3R in vitro. The details of this interaction are still unclear. One mechanism could be that autoantibodies against the M3R are involved, but data on the detection of these antibodies are conflicting.

CURRENT TREATMENT FOR SS

To date there is no universally effective therapy for SS. Therapy is based on symptom relief and the prevention and treatment of complications. Patients are advised to take extra oral care and undergo regular dental check ups. For the sicca symptoms, artificial tears, punctal plugs and artificial saliva can be applied. Some patients receive symptomatic relief from secretagogues such as cimeveline and pilocarpine. The use of immunosuppressants has been unsatisfactory; these drugs can improve systemic conditions associated with the disease but the drugs have little effect on the sicca symptoms. Most cytokine-directed therapies have also proved ineffective in SS, even though these therapies have been applied successfully in the treatment of other rheumatoid diseases. For instance, blocking the pro-inflammatory cytokine tumor necrosis factor α (TNFα) in RA, an autoimmune disease with many similarities to SS, has lead to greatly improved disease control with an acceptable safety profile. However, unlike RA, the use of TNFα blockers did not lead to improved saliva or tear production in clinical trials with SS patients. Another recently investigated therapy targets B cells. Rituximab (RTX), an anti-CD20 antibody that depletes CD20+ B cells, was first approved for treatment of patients with relapsed or refractory lymphoma and has quickly revolutionized the treatment of B cell lymphomas. To date RTX has been used not only in malignancies but also has been successfully used in the treatment of autoimmune diseases like RA and in small pilot studies for the treatment of SLE. RTX in patients with SS resulted in depletion of peripheral B cell without having an effect.
on the numbers of natural killer cells, T helper cells and cytotoxic T cells. Furthermore, it was shown to have some beneficial effect on parameters like fatigue, vasculitis, and arthralgias with minimal side effects. Some studies also reported beneficial effects on objective and subjective sicca symptoms\textsuperscript{33-35}. Since most studies were open label and the overall patient numbers were low, it is unclear to date whether RTX is a good therapy for SS.

GENE THERAPY FOR SS

In summary, several treatments that are effective in other autoimmune inflammatory diseases failed in SS. There are many reasons for this failure: First, it is possible that these drugs are aimed at the wrong target. The inhibition of TNF\textsubscript{α} in SS patients for instance was shown to lead to paradoxical upregulation of TNF\textsubscript{α}, and also IFN\textsubscript{α} and BAFF were shown to be upregulated after treatment\textsuperscript{36}. Second, it is possible that these drugs reached suboptimal dose levels in the affected organs due to poor penetration. Third, it is possible that the drug was not given for a sufficient period of time or too late in the course of the disease to elicit a response. SS patients often suffer from symptoms long before they are diagnosed\textsuperscript{1}. A therapy therefore may need to be applied long term to reverse damage that has accumulated over several years. As a therapeutic approach, gene therapy may offer the possibility to address some of these concerns.

THE USE OF LOCAL GENE THERAPY

Since the salivary gland is heavily involved in the pathogenesis of SS, locally applied therapy to the salivary gland is very attractive and has a number of advantages over systemic therapy. First, the salivary glands can be easily reached by retrograde cannulation of the orifices of the salivary ducts in the mouth (as is routinely performed in scintigraphy of the salivary glands). This technique can be used not only to introduce anti-inflammatory small molecules directly to the gland in a non-invasive manner but can also be used to introduce vectors able to direct the expression of immunomodulatory proteins or the gland physiology. Second, the entire salivary gland can be targeted since the luminal membrane of both the acinar and ductal cells within the gland is exposed. Third, because of its natural secretory activity, the salivary gland is an excellent organ to locally achieve a high level of the drugs compared to systemic administration. Fourth, long-term expression is possible from the salivary gland due to the relatively low turnover of the epithelial cells in the gland. Fifth, systemic exposure to the drugs can be limited by treating the salivary glands only, possibly leading to fewer side effects.

EXPERIENCE WITH LOCAL GENE THERAPY

Several studies have recently been conducted to address the utility of gene therapy in treating salivary gland dysfunction and inflammation in animal models of SS (Table
1). The non-obese diabetic (NOD) mouse model is the most widely used model to study the spontaneous development and treatment of SS. Classically, this mouse is studied for diabetes. From the age of 10 weeks these mice spontaneously develop insulin-dependent diabetes preceded by autoimmune insulitis of the pancreas. Independently of this disease and a few weeks later than the onset of diabetes the mice can also spontaneously develop autoimmune exocrinopathy of the salivary glands with gender-dependent loss in gland activity and lymphocytic infiltration of the salivary and lachrymal glands. With age, NOD mice also may develop auto-antibodies against the nuclear antigens Ro and La. However this SS-like phenotype is unstable and the factors that contribute to the disease are still largely unknown. One of the first studies to test the local application of gene therapy to the salivary gland used a viral vector encoding the anti-inflammatory cytokine IL-10. IL-10 was shown to be

![Simplified schematic of fluid secretion in the salivary gland and the processes involved in Sjögren's syndrome](image)

**Figure 1. Simplified schematic of fluid secretion in the salivary gland and the processes involved in Sjögren's syndrome.** Upon stimulation of the Muscarinic type 3 receptor (M3R) and via $\text{Ca}^{2+}$ signaling, fluid is secreted by the acinar cells through the aquaporins (AQP). The fluid (blue arrows) passes through the ducts into the oral cavity while epithelial cells modify its content. In SS reduced saliva can be the result of many processes interfering with the secretory capacity of the gland (thick black arrows). The infiltrating B and T cells interacting with each other through their respective receptors in interaction with the MHC-peptide complex and the epithelial cells and dendritic cells (DC) are shown in the enlarged area (the co-stimulatory molecules are depicted as black T's). Each of the processes involved in SS can potentially be targeted with gene therapy. (IgA, immunoglobulin type A; B, B cell; T, T cell).
beneficial in a number of preclinical models of autoimmune diseases\textsuperscript{40-41}. IL-10 was administered locally to the salivary glands of NOD mice by retrograde cannulation of the salivary gland ducts, or intramuscular injection in the quadriceps muscle using a recombinant adeno associated viral type 2 (AAV2) vector. The vector was given at 8 weeks, before onset of the disease, and at 16 weeks, at a more progressive stage of the disease. At 20 weeks, the mice treated with IL-10 directly into the salivary gland showed significantly higher salivary flow in the early and late treatment group compared with the intramuscular or control vector treatment groups\textsuperscript{42}.

Similarly, positive results have also been observed using an AAV vector encoding the neuropeptide vasoactive intestinal peptide (VIP). This peptide has a broad range of functions. It is a neurotransmitter causing vasodilatation, but is also known to be involved in immune tolerance and is a potent suppressor of a variety of pro-inflammatory cytokines\textsuperscript{43}. NOD mice were treated with a single dose of $1\times10^{11}$ viral particles containing the VIP gene per salivary gland. At the age of 16 weeks, 8 weeks after the treatment, VIP treated mice showed no loss of salivary flow compared to the control group. There was no difference in focus score or apoptotic index between the different groups, but there was significantly less IL-2, IL-12, TNF\textsubscript{α}, and IL-10 present in glandular extracts, in contrast to serum levels, where no difference was observed\textsuperscript{44}. Treatment of NOD mice with both the IL-10 and the VIP constructs did not lead to noticeable side effects; the mice appeared to be healthy during treatment. In contrast, local application of TNF inhibitors did not improve inflammation and dysfunction of the salivary glands in NOD mice. This supports the lack of efficacy seen in previous studies in humans when systemic treatment was studied. Salivary gland-directed gene therapy with a soluble TNF receptor (sTNFR1:IgG) in NOD mice decreased salivary gland activity. Treatment decreased some pro-inflammatory cytokines locally in the gland, while TGF\textsubscript{β} was upregulated. In plasma however, the opposite was observed, with upregulated pro-inflammatory cytokines and downregulated TGF\textsubscript{β} levels\textsuperscript{45}. The reason for the failure of this treatment in mice is not completely understood, but could be due to circulating receptor-complex formation\textsuperscript{28} or underlying mutations in the TNF pathway as have been described in humans and mice\textsuperscript{46-47}. This observation further emphasizes the negative effect of this class of drug on salivary gland function and suggests that other molecules should be explored as targets for therapeutic intervention in SS.

**FUTURE THERAPEUTIC TARGETS**

Although the studies with IL-10 and VIP are promising, other potential therapeutics should be explored not only as possible future drugs but also to better understand the underlying pathophysiology associated with SS. Novel therapeutics in SS can be divided in an immunological and a non-immunological group. Examples of immunological targets include molecules that can interfere with the proliferation and activation of B and T cells or affect chemotaxis of lymphocytes, macrophages and dendritic cells. Non-immunological therapies comprise of molecules that enhance fluid secretion, for instance the introduction of water channels such as aquaporins
Similarly, therapies that enhance the sensitivity of salivary and lachrymal glands to neuro-stimulatory signals could be explored as a therapeutic approach for the treatment of SS.

**IMMUNOLOGICAL TARGETS**

**Cytokines: IFNα, IFNγ and IL-12**

Many pro-inflammatory cytokines are upregulated in SS and blocking one or more of these cytokines may result in reduced inflammation. Some of these cytokines are also involved in secretory dysfunction making them even more interesting candidates for treatment. IFNα and IFNγ together with IL-12, are all closely related in function and are all overexpressed in SS patients. In addition, the majority of cytokines and transcription factors that are overexpressed in patients are IFN inducible, a profile that has been referred to as ‘the interferon signature’. IFNγ is also highly expressed in individuals with sicca symptoms who do not have any histological inflammatory markers in the salivary gland. Moreover, prolonged treatment of the human salivary gland cell line (HSG) with IFNγ in the presence or absence of TNFα leads to a persistent depletion of intracellular Ca2+ stores (important for signal transduction leading to fluid secretion), and thus to an exhausted response system. These two observations suggest that IFNs can affect the secretory capacity of the glands. Since IFNγ also reduces the growth of HSG in a concentration dependent way in vitro, interfering with the interferon system, for instance by introducing a soluble receptor, may reduce sicca symptoms, local damage and inflammation in the salivary glands of SS patients.

**Chemokines**

Chemokines are chemotactic cytokines that are very important in orchestrating mobilizing and, to a lesser degree, regulating homeostasis of a wide range of hematopoietic cells. The role of chemokines in many autoimmune disease such as RA and autoimmune thyroiditis is well established and there is evidence they play an important role in SS as well. Chemokines are important for the homing of T and B cells to the gland and for the survival of malignant B cells. The chemokines CXCL9 and CXCL10 have been shown to induce the expression of MHC class II and co-stimulatory molecules on the surface of HSG, thus facilitating the recruitment of immune cells to the gland. CXCL9, in particular, has been shown to be upregulated in the salivary glands of SS patients and to have a role in the recruitment of immune cells to the gland. Therefore, targeting chemokines may be a promising therapeutic approach for the treatment of SS. The following table summarizes the results of salivary gland gene therapy in NOD mice:

| Gene and vector | Results |
|-----------------|---------|
| IL10 AAV2       | Prevented loss of salivary flow, reduced number of infiltrates in the salivary gland |
| VIP AAV2        | Prevented loss of salivary flow, no effect on infiltrates in the salivary gland |
| sTNFR1:IgG1 AAV2| Reduced salivary flow over time, reduction in pro-inflammatory cytokines, upregulation of systemic pro-inflammatory cytokines |

NOD, non-obese diabetic mouse; IL10, interleukin 10; AAV, adeno-associated virus; VIP, vasoactive intestinal peptide; sTNFR1:IgG1, soluble tumor necrosis factor receptor type 1.
and CXCL10 for instance, were found to be significantly upregulated in SS salivary glands, but not in normal salivary glands. These chemokines were predominantly expressed in ductal epithelium in close proximity to lymphoid infiltrates. Moreover high titers of CXCL9 and CXCL10 were produced after patient-derived SG cells were treated with IFNg \textit{in vitro}\textsuperscript{63}. Upregulation of these chemokines is known to attract the IFN\textgamma\ producing plasmacytoid DC\textsuperscript{54} and B cells\textsuperscript{64}. Interfering with such a system may be useful in reducing inflammation in the gland by blocking chemotaxis of lymphocytes and dendritic cells, and may also reduce the risk of developing lymphoma.

The co-stimulatory pathway

The co-stimulatory pathway has been shown to play a role in many autoimmune diseases. In studies on RA\textsuperscript{65-66} and psoriasis\textsuperscript{67-68} the principle of blocking co-stimulatory pathways as a treatment has been shown to be safe and effective. In short, to activate B and T cells, simultaneous binding of co-stimulatory molecules is required in addition to the binding of the T or B cell receptor with the peptide-major histocompatibility complex (MHC). In the absence of the co-stimulatory signal, binding of the T or B cell receptor to the peptide-MHC can lead to tolerance and anergy\textsuperscript{69}. Co-stimulatory signals are bidirectional and can lead to activation of B cells, upregulation of adhesion molecules, class switching and enhancement of CD4\textsuperscript{+} and CD8\textsuperscript{+} effector functions as well as many other pro-immune activities. One of the co-stimulatory pathways known to be involved in SS is the CD40 interaction with CD40 ligand (CD40L/CD154)\textsuperscript{12,70-71}. In NOD mice, blocking the CD40–CD40L interaction around 4 weeks of age prevents the onset of insulitis and diabetes by inhibiting the development and chemotaxis of pathogenic Th1 cells to the islets of Langerhans in the pancreas\textsuperscript{72}, the effect on the SS phenotype has not been studied yet. Blocking these pathways in SS may promote tolerance over autoimmunity by reducing auto-reactive T cells, moreover it may affect the activation of B cells and the differentiation of B cells into plasma cells.

NON-IMMUNOLOGICAL TARGETS

Aquaporins

In order to treat the main symptoms of SS, dry mouth and dry eyes, gene therapy could be used to enhance fluid secretion by introducing water channels (AQP) into the ducts of the salivary glands. This re-engineering of the salivary gland has been successfully performed in rats, mini pigs and non-human primates for the treatment of radiation-induced xerostomia. In these studies, vectors encoding water channels were used weeks to months after radiation induced loss of salivary flow. The treatment restored salivary gland activity without any significant side effects\textsuperscript{73}. Currently a Phase I trial is ongoing to evaluate the safety of this approach (clinicaltrials.gov, NCT00372320 \textsuperscript{74}). In this trial, patients with a history of damage of the salivary glands due to head and neck radiation and objective and subjective symptoms of dry mouth receive an adeno-viral vector encoding AQP type 1 locally in the parotid gland. The primary outcome of this study is to determine safety of this novel treatment. The secondary outcome will be to measure the effectiveness of gene transfer of AQP1
to increase parotid gland salivary output and improve symptoms associated with irradiation-induced parotid hypofunction. If this proves to be safe, further studies will provide us with more information on the efficacy of the therapy and may lead to studies on its applicability in SS.

Neuro-stimulatory pathway
Evidence is accumulating that the function of the muscarinic type 3 receptor (M3R) is impaired in SS. It is thought that antibodies blocking the M3R are responsible for the dysfunction of the receptor, leading to dryness of the secretory organs, but it has been challenging to prove this in a direct manner. Enhancing the sensitivity of the M3R by using soluble receptors as decoy for possible autoantibodies or introducing properly functioning receptors in excess to the gland may overcome the secretory dysfunction. However, at this moment the mechanism responsible for the malfunction of the receptor is not fully understood and further research will be necessary to identify its exact nature.

FUTURE DIRECTIONS AND LIMITATIONS
The most difficult challenge facing the treatment of SS to date is the identification of the proper target(s). Since we understand little of the pathogenesis of SS and we do not know the autoantigen that is recognized, choosing the correct target is largely empirical. However, by using local delivery of key immunomodulatory proteins or other potential therapeutic molecules directly to the gland in animal models we can begin to identify the critical pathways involved in the inflammatory process and the loss of secretory function.

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TEMPORAL CHANGES IN SALIVARY GLANDS OF NON-OBESE DIABETIC MICE AS A MODEL FOR SJÖGREN’S SYNDROME

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ABSTRACT

Objective: Non-obese diabetic (NOD) mice develop an autoimmune exocrinopathy that shows similarities with Sjögren’s syndrome. They provide an experimental model to study the pathoetiogenesis of this disease.

Materials and Methods: Salivary gland (SG) function and salivary sodium content were measured in 8-, 12-, 16- and 20-week-old NOD and age-matched CB6 mice. In NOD mice, SG expression of phenotypic cell markers, B cell-stimulating and co-stimulatory molecules were evaluated. Cytokine levels were measured in serum and SG homogenates.

Results: Microscopically evident SG inflammation in NOD mice was preceded by expression of intercellular adhesion molecule 1 on epithelial cells in the presence of macrophages and relatively high levels of cytokines. Next, an influx consisting of mainly T, B, natural killer, plasma and dendritic cells was seen. Most cytokines, except for interleukin (IL)-12/IL-23p40 and B cell-activating factor, decreased or remained stable over time, while glandular function deteriorated from 16 weeks of age onward compared with CB6 mice.

Conclusion: Sjögren’s syndrome-like disease in NOD mice occurs in multiple stages; immunological and physiological abnormalities can be detected before focal inflammation appears and salivary output declines. Extrapolating this knowledge to human subjects could help in understanding the pathogenesis and aide the identification of potential therapeutic targets.
INTRODUCTION

Sjögren’s syndrome (SS) is a chronic, systemic autoimmune disorder of unknown aetiology with a poorly understood pathogenesis. The disease is characterised by inflammation and dysfunction of secretory glands leading to ocular and oral dryness. There is currently no cure for SS and treatment options are mostly limited to palliation of symptoms. Immune cells, such as macrophages, dendritic cells and lymphocytes and cytokines, immunoglobulins and autoantibodies are all thought to play a role in the disease development and progression. One of the hallmarks of SS is the presence of typical focal, periductal and perivascular lymphocytic infiltrates in the secretory glands. In human salivary glands (SGs), T cells initially outnumber B cells in small inflammatory aggregates, but in more severe lesions, the B to T cell ratio is increased. Eventually, focal infiltrates may organise into germinal centre-like formations, which is regarded as an intermediate state that could lead to the potentially lethal development of B cell lymphomas. In addition, the infiltration of certain types of inflammatory cells is associated with several clinical features, including systemic disease manifestations, such as C4 hypocomplementaemia, cryoglobulinaemia and purpura.

Most patients are not diagnosed until a relatively late disease stage, when many glandular changes have already occurred, and disease parameters are often not followed longitudinally. Therefore, only limited data regarding the early stages and subsequent progression is available, making it difficult to discern the mechanisms of the disease and to develop appropriate treatment. The non-obese diabetic (NOD) mouse could provide more insight; NOD mice spontaneously develop an inflammatory syndrome affecting the exocrine glands, which shows many histological parallels with its human counterpart, coinciding with a progressive impairment of secretory function. Female (more so than male) NOD mice develop focal infiltrates in the SGs typically between 8 and 12 weeks of age and SG output declines between 12 and 24 weeks. The loss of salivary flow was associated with changes in cytokine levels in serum and saliva and to a lesser degree with the glandular influx of lymphocytes in NOD mice followed up to 24 weeks. In studies on SS-like disease in NOD mice, salivary sodium content, or SG cytokines, B cell-stimulating factors and co-stimulatory molecules were not assessed.

In this descriptive study, we examined the NOD mouse and followed several pathological and physiological processes during disease development, starting at a preclinical phase, and continuing up to late-stage inflammation and secretory dysfunction. The salivary flow rate (SFR) and salivary levels of sodium, expression of cytokines in SGs and serum, and additional local inflammatory biomarkers were assessed. Translating this knowledge to SS will help to unravel its pathogenesis and could potentially lead to identification of novel therapeutic targets.

MATERIAL AND METHODS

Mice

Female NOD (001976 NOD/ShiLtJ; Jackson Laboratories, ME, USA) and female CB6 mice (CB6F1/Cr, BALB/CAnNCr_ x C57BL/6NCr_) National Cancer Institute Mouse
Repository, Frederick, MD, USA) were obtained at 6 weeks of age and housed under specific pathogen-free conditions in our animal facility. Animal studies were approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. All procedures were conducted in compliance with the NIH guidelines on the use of animals in research. Strains were housed separately with a maximum of five mice per cage on a 12-hour light/12-hour dark cycle and had access to food and water ad libitum.

Blood glucose levels of NOD mice were measured weekly, starting at 12 weeks of age, using a OneTouch monitor (LifeScan, Milpitas, CA, USA). A subcutaneous injection of long-acting Humalin N (1 U/mouse, every 24 hours; Eli Lilly, Indianapolis, IN, USA) was administered to mice with blood glucose levels ≥250 mg/dL to treat hyperglycaemia and related dehydration. In our facility, the incidence of diabetes is normally 50-70% by 20 weeks of age using this cut-off value.

Saliva collection and assessment of sodium concentration

Five days prior to sacrificing, whole stimulated saliva of NOD mice was collected, as previously described. For CB6 mice, saliva was collected at the same age as NOD mice. Briefly, mild anaesthesia was induced with ketamine (100 mg/mL, 1 mL/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (20 mg/mL, 0.7 mL/kg body weight; Phoenix Scientific, St. Joseph, MO, USA) solution given intramuscularly, and saliva secretion was stimulated by a subcutaneous injection of pilocarpine (0.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). Next, whole saliva was collected from the oral cavity for 20 minutes with a haematocrit tube (Drummond Scientific Company, Broomall, PA, USA) placed in a pre-weighed 0.5 mL microcentrifuge tube and volume was determined gravimetrically. Saliva was stored at -80ºC until analysis. Saliva samples were diluted 1,000 times in a solution containing 5 g/L CsCl and sodium concentrations were measured by atomic absorption spectrometry using a K-Na lamp (AAnalyst 200 Atomic Absorption spectrophotometer; PerkinElmer, Waltham, MA, USA). Calibrations for sodium were performed by linear regression analysis using 1, 2 and 3 ppm (mg/L) as standards.

Histological assessment of salivary gland inflammation

NOD mice were sacrificed at 8, 12, 16 and 20 weeks of age and the submandibular SGs were removed, cleaned of any adjacent (possibly lymphoid) tissue and cut in three cross-sectional parts. The first part was collected in formalin, embedded in paraffin and cut in 5 µm sections. Three sections (each 50 µm apart from the previous) were mounted on glass slides and stained with haematoxylin and eosin. The number of foci (where one focus is defined as an aggregate of ≥50 infiltrated cells per 4 mm²) on each section was counted blindly by two different examiners, and the mean focus score (FS) was determined.

Immunohistochemical analysis of salivary gland tissue

The second submandibular SG part was emerged in Tissue-Tek optimal-cutting temperature (OCT) compound (Miles, Elkhart, IN, USA) and quickly frozen on dry ice.
Sections (7 µm) were cut with a cryostat, mounted on glass slides and stored at -80°C until further use. For staining, frozen sections were thawed and fixated in acetone for 10 minutes, except for anti-BAFF (B cell-activating factor belonging to the tumour necrosis factor [TNF] family) that was used on formalin-fixed, paraffin-embedded tissue, which had undergone heat-induced antigen retrieval in a citrate buffer (pH 6.0) after deparaffinisation with xylene and alcohol. Endogenous peroxidase was blocked, slides were washed in phosphate-buffered saline (PBS), and further blocked with 1% bovine serum albumin (BSA) and 10% normal goat serum (NGS; Dako, Glostrup, Denmark) in PBS overnight. The next day, slides were incubated with primary antibodies in 1% PBS and 5% NGS at room temperature for 1 hour. Monoclonal primary antibodies used were anti-CD4 (clone L3T4, eBioscience, San Diego, CA, USA) to detect T helper (Th) 1 and 2 cells; anti-CD8 (clone 53-6.7, eBioscience) for cytotoxic T cells; anti-CD11c (clone N418, Abcam, Cambridge, MA, USA) for plasmacytoid dendritic cells; anti-CD19 (clone 1D3, BD Biosciences, Franklin Lakes, NJ, USA) for B cells; anti-CD138 (clone 281-2, BD Biosciences) for plasma cells; anti-IgD (clone 11-26c [11-26], eBioscience) for mature, non-switched, peripheral B cells; anti-CD68 (clone FA11, Abcam) for monocytes/macrophages; anti-CD49b (BD Biosciences) for natural killer (NK) cells; and anti-CD40 (clone 3/23, BD Biosciences) and anti-ICAM-1 (intercellular adhesion molecule 1; clone YN 1/1.7.4, Abcam) for cell adhesion and co-stimulatory molecules. Anti-BAFF (clone buffy-2, Enzo Life Sciences, Farmingdale, NY, USA) antibody was used to detect the cellular B cell-stimulating factor BAFF. For anti-Foxp3 antibody (clone FJK-16s, eBioscience), to detect T regulatory cells (Tregs) in the SG tissue, a 1:50 dilution and an additional amplification step with biotin-labelled tyramide (PerkinElmer) followed by streptavidin-labelled horse-radish peroxidase (HRP; PerkinElmer) was performed. All primary antibodies used were of the rat IgG class of immunoglobulins, except for anti-CD11c and anti-CD49b (hamster IgG), and anti-BAFF (rat IgM) antibodies, and used at a 1:100 dilution, except for anti-CD3 (1:200), anti-CD4 (1:250) and anti-CD138 (1:50). Staining was visualised with HRP-labelled goat anti-rat secondary antibody (Southern Biotechnology, Birmingham, AL, USA) or, for anti-CD11c antibody only, goat anti-hamster secondary antibody (Jackson ImmunoResearch, Suffolk, UK) in a 1:100 dilution, and developed with aminoethylcarbazole (AEC) substrate (Dako). To be able to analyse SGs before focal inflammation had appeared, five 8-week-old mice without an FS were analysed in more detail. From week 12, when all mice showed inflammatory aggregation, five randomly selected mice were assessed. For control sections, immunoglobulin class- and species-matched control antibodies instead of the primary antibody were used.

For all markers, images of foci and adjacent tissue of in total 18 high-power fields were assessed using the Qwin digital image analysis system (Leica, Cambridge, UK), as previously described. For Foxp3, only staining in foci was examined, since amplification with tyramide resulted in nonspecific staining of surrounding epithelial tissue. For BAFF, CD40 and ICAM-1, the staining for foci and tissue were digitally separated after which they were analysed. Staining was expressed as internal optical density (IOD)/mm², an arbitrary unit representing the intensity of staining per mm².
Preparation of salivary gland tissue homogenates

The third submandibular SG part was snap-frozen on dry ice and stored at -80ºC until further use. Prior to homogenisation, samples were thawed and kept on ice. Samples were crushed, placed in 2 mL tubes containing 1 mL HEPES lysis buffer (20 mM HEPES, 0.5 M NaCl, 0.25% Triton X-100 and 1 mM EDTA) and complete protease inhibitor (Roche, Mannheim, Germany), and lysed by shaking at 4ºC overnight. The next day, samples were centrifuged at 1,500 x g at 4ºC for 10 minutes. Protein content of supernatants was measured using a bicinchoninic acid (BCA) protein detection kit (Pierce, Rockford, IL, USA) and stored at -80ºC until further use.

Cytokine detection in salivary gland homogenates and serum

Blood was collected by heart puncture immediately post-mortem, left to clot on ice for 3 hours and centrifuged at 2,500 x g for 25 minutes at 4ºC to obtain serum. Levels of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12/IL-23p40, IL-17, IL-18, IL-23p19, interferon (IFN)γ, TNFα and transforming growth factor (TGF)β were measured in serum and SG homogenates using a multiplex sandwich ELISA (Aushon Biosystems, Billerica, MA, USA). This assay does not distinguish between the p40 subunit of IL-12 and IL-23, or free p40. Results for SG homogenates were corrected for protein concentration. Duplicates for each sample were tested in three dilutions and the mean values of the duplicates from every optimal dilution were reported. Detection levels (pg/mL) were for IL-1β and TNFα >0.9, IL-2 and IL-4 >1.2, IL-6 >8.2, IL-10 and IL-17 >1.3, IL-12/IL-23p40 >0.2, IL-18 >2.4, IL-23p19 >33.7, IFNγ >4.3, TGFβ >8.0.

Statistical analysis

Data were described as mean ± standard deviation (SD), and displayed in dot plots with individual values for each mouse and a mean (line) or in bar graphs with mean ± SD. A one-way ANOVA was used to detect differences within the groups for the various ages followed by a post-hoc Tukey's test. Depending on the data distribution, an unpaired Student’s t test or a Mann-Whitney test was used to compare the mouse strains at each time point. The Pearson’s correlation coefficient was used to investigate the relationship between FS and SFR, between cytokines and SFR, between cytokines and FS, or between Th1 and Th2 cytokines. All analyses were performed with GraphPad Prism v5.01 statistical software (GraphPad Software, La Jolla, CA, USA). A p-value ≤0.05 was considered statistically significant. In figures, an * indicates a p-value ≤0.05 and >0.01, ** for 0.01 ≤ p > 0.001, *** for p ≤0.001 and p = ns was used for non-significant values.

RESULTS

Salivary flow rate of NOD mice is decreased after 16 weeks of age

The progressive loss of salivary function in NOD mice starts between 12 and 24 weeks. To determine when salivary function declines at our facility, stimulated SFR was measured at age 8, 12, 16 and 20 weeks. As shown in Figure 1A, at 8 weeks, SFR (mean ± SD)—corrected for body weight (μl/gram body weight*20 minutes)—for
NOD and CB6 mice was similar, and had significantly increased more than 50% by 12 weeks in both mouse strains (from $3.3 \pm 1.4$ to $5.3 \pm 1.2$ and from $2.6 \pm 1.1$ to $4.3 \pm 1.8$ for CB6 and NOD mice, respectively). At 16 and 20 weeks, NOD mice showed a significant decrease in SFR to a level which was comparable to 8-week-old mice ($2.5 \pm 1.4$ and $2.6 \pm 1.5$ respectively). In contrast, SFR was unaltered in CB6 mice after 12 weeks. As was previously described\textsuperscript{17}, the decline in SFR was independent of diabetic state (data not shown).

Altered salivary sodium levels in saliva of NOD mice

Saliva is initially produced as an isotonic solution by acinar cells. During transportation through the ducts, sodium is actively reabsorbed by the ductal epithelium, resulting in a hypotonic solution by the time the saliva reaches the oral cavity\textsuperscript{18}. In the healthy gland, a lower SFR results in a lower salivary sodium concentration\textsuperscript{54}, due to prolonged exposure of the saliva to the epithelial sodium channel ENaC in the apical membrane of ductal epithelium\textsuperscript{19}. In SGs of SS patients, sodium reabsorption is disturbed and

![Figure 1. Changes in salivary gland function and salivary sodium concentration of NOD and age-matched CB6 mice.](image-url)
saliva, despite the lower SFR, has a relatively high sodium content, indicating ductal cell dysfunction. This parameter can be used as an indirect indicator for inflammation of the ductal epithelium and can aid the diagnosis of SS\textsuperscript{20}. We tested saliva from NOD and CB6 mice for sodium content, corrected for SFR. Overall, sodium levels (mean ± SD, mmol/SFR) of CB6 mice slightly decreased over time, reaching significance at 20 weeks (27.4 ± 8.4, 21.6 ± 13.4, 17.5 ± 6.5 and 14.8 ± 5.1 for 8, 12, 16 and 20 weeks, respectively). The sodium levels of NOD mice showed a different pattern: the sodium concentration at 8 weeks was significantly higher in NOD than in CB6 mice, but at 12 weeks, had dropped to the same level as that of CB6 mice (27.4 ± 8.4 versus 67.6 ± 53.8 and 21.6 ± 13.4 versus 19.5 ± 3.4, respectively). Thereafter, it significantly increased again (17.5 ± 6.5 versus 47.3 ± 18.4 at 16 weeks). At 20 weeks, NOD mice still showed a trend towards higher sodium levels (14.8 ± 5.1 versus 36.6 ± 32.3, \( p = 0.06 \); Figure 1B).

The focus score of NOD mice increases with age, but does not correlate with salivary flow rate

The major histological hallmark of SS is the presence of clustered lymphocytic infiltrates in the SG, which are thought to progressively increase in number, size and organisation\textsuperscript{21}. Occasionally, focal infiltrates can be found in the major and minor SGs of healthy individuals\textsuperscript{22-23}. Non-autoimmune prone mice only sporadically develop small non-focal infiltrates regardless of age\textsuperscript{11-12,17} and the CB6 mice in our study did not show any other sign of disturbed SG physiology. Therefore, we continued with the analysis of SG inflammation and infiltrating cells of NOD mice only. The majority (5/8) of 8-week-old NOD mice did not have focal infiltrates, whereas at the age of 12

\begin{figure}[h]
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\caption{Inflammation in salivary glands from NOD mice. Salivary glands were obtained from non-obese diabetic (NOD) mice at 8, 12, 16 (each \( n = 8 \)) and 20 (\( n = 16 \)) weeks of age and focus score (FS) was determined in paraffin-embedded tissue sections by two independent examiners. Dot plots with distribution and mean values for individual animals, the group mean for each time point, and significant \( p \)-values are shown (A). Correlation between individual mean FS and pilocarpine-stimulated salivary flow rate (SFR) of mice from all ages (\( n = 8 \); B).}
\end{figure}
weeks, all mice did. The FS (mean ± SD) was 0.9 ± 1.3, 2.1 ± 1.0, 2.8 ± 0.9 and finally increased to 3.5 ± 1.5 at 8, 12, 16 and 20 weeks, respectively. Compared with 8-week-old mice, mice at 16 and 20 weeks displayed significantly more infiltrates (Figure 2A).

The relationship between SG inflammation and dysfunction in SS is complex. Although an FS of ≥1 and objective SG dysfunction in humans makes the diagnosis of SS very likely, severely reduced salivary output can be seen in patients with only minor infiltration of the gland and vice versa. Moreover, in NOD mice secretory function can abruptly deteriorate without obvious inflammatory progression. As in human subjects, the FS of the NOD mice also did not correlate with SFR, confirming no direct correlation with the extent of focal glandular inflammation and the volume of stimulated saliva (Figure 2B).

Focal inflammation of the salivary gland is preceded by the presence of macrophages and is quickly followed by a massive influx of T, B and other immune cells. Assessing the sequence of inflammatory processes in humans requires longitudinal follow-up with frequent, repetitive SG biopsies, which can lead to practical and/or ethical objections. In NOD mice, the order of cell influx and the composition of infiltrates at different disease stages can be studied. Immunohistochemical staining for different immune cell types showed that SGs of 8-week-old NOD mice without focal infiltrates contained a considerable amount of CD68+ monocytes/macrophages (Figure 3A and B). Staining for this cell marker was significantly elevated when infiltrates were present in all mice (12 weeks), but did not increase thereafter (Figure 3B). Other immune cells detectable at the age of 8 weeks were CD138+ plasma cells, found distributed throughout the tissue with a preference for periductual tissue, and CD49b+ NK cells that were found sparsely distributed throughout the tissue (Figure 3A). At week 12, CD4+ and CD8+ T cells, Foxp3+ Tregs, CD11c+ plasmacytoid dendritic cells, CD19+ B cells, and IgD+ mature, non-switched, peripheral B cells had also infiltrated into the SG, and were found concentrated in distinct foci. The IOD/mm² for some cell subsets, including CD4+ T cells, NK cells, IgD+ B cells and Foxp3+ Tregs (foci only), steadily increased with age. Expression for plasma and plasmacytoid dendritic cells was high in some mice at 16 and 20 weeks, whereas other mice showed levels comparable with 12 weeks. The staining intensity for CD8+ cells increased significantly up to 16 weeks, but at 20 weeks, showed a reduction trend to the 12-week level (p = 0.06; Figure 3B).

Increased BAFF, CD40 and ICAM-1 levels in salivary glands of NOD mice

The cytokine BAFF is aberrantly expressed in SGs of SS patients. It is thought that BAFF is important in ectopic germinal centre formation and may contribute to lymphomagenesis, but data are still inconclusive. In 8-week-old NOD mice, low levels of BAFF were detected, confined to the ductal epithelium. When foci had formed, BAFF was seen in the infiltrating cells and this expression was higher for 3 out of 4 mice at 20 weeks compared with 16 weeks. In the ductal epithelium, expression levels (mean ± SD, IOD/mm²) remained stable up to 16 weeks until an increase was

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Figure 3. Immunohistochemical staining of salivary glands from NOD mice. Frozen and paraffin-embedded sections of salivary glands obtained from non-obese diabetic (NOD) mice at age 8, 12, 16 and 20 weeks (n = 5) were used. Immunohistochemical staining with antibodies against cellular markers (A) or immunomodulatory molecules (C), including immunoglobulin class- and species-matched control antibodies (negative control), are shown (scale bar in mm). Images of in total 18 high-power fields were assessed for foci and adjacent tissue, except for Foxp3, using digital image analysis. For cellular markers, dot plots with distribution for individual mice, mean staining intensity per area (expressed as internal optical density [IOD]/mm²) of foci and adjacent tissue together (foci only for Foxp3), and significant p-values are shown (B); for BAFF, CD40 and ICAM-1, foci and adjacent tissue were digitally separated, analysed and displayed in separate graphs. Significant p-values are shown. (D). For reference, arrows indicate ductal epithelium in tissue stained for BAFF. IHC – immunohistochemical; BAFF – B cell-activating factor belonging to the tumour necrosis factor (TNF) family; ICAM-1 – intercellular adhesion molecule 1.
Figure 3 continued.

**B**

| Marker       | IHC Intensity (IOD/mm²) |
|--------------|-------------------------|
| CD4          |                         |
| CD8          |                         |
| Foxp3 [foci only] |                |
| CD19         |                         |
| CD138        |                         |
| IgD          |                         |
| CD68         |                         |
| CD49b        |                         |
| CD11c        |                         |

**C**

| Marker       | age (weeks) | isotype control |
|--------------|-------------|-----------------|
| BAFF         | 8 12 16 20  | rat IgM         |
| CD40         |             |                 |
| ICAM-1       |             |                 |
CD40 and ICAM-1 are also overexpressed in SS patients’ SGs\textsuperscript{30-32}. These integral membrane proteins function as co-stimulatory molecules, and ICAM-1 has an important role in adhesion and migration of lymphocytes into the inflammatory milieu as well. Both immunomodulatory molecules are involved in the autoimmune pathogenesis of many diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and SS (reviewed in\textsuperscript{33-34}). In NOD mice, ICAM-1 was present in ductal epithelial and endothelial cells at 8 weeks, before infiltrates were detected, and expression was significantly increased both in epithelium and in scattered infiltrating cells appearing throughout the tissue at 12 weeks (Figure 3C). Within foci, ICAM-1 was found highly and stably expressed on the infiltrating cells through 20 weeks. In contrast, specific CD40 staining could not be detected in adjacent tissue at the age of 8 weeks, but this marker was clearly expressed from 12 weeks and onward, mostly in infiltrates. Individual mice showed higher CD40 staining at 12 weeks compared with 8 weeks, but this increase was not significant ($p = 0.10$; Figure 3C and D).
Salivary gland cytokine levels indicate an early, local pro-inflammatory environment

Many pro-inflammatory cytokines are found to be upregulated in SGs of SS patients compared with healthy controls and anti-inflammatory cytokines are mostly absent or expressed at low levels (reviewed in\textsuperscript{35}). We measured SG levels of different classical and novel pro- and anti-inflammatory cytokines in NOD mice at age 8 (only mice without SG infiltrates), 12, 16 and 20 weeks. At 8 weeks, pro-and anti-inflammatory cytokines could be detected in the SG homogenates, but they revealed different expression patterns during follow-up. The pro-inflammatory cytokines IFN\textgamma, IL-1\beta, IL-2 and IL-23p19 showed an overall decrease over time. IL-12/IL-23p40 levels increased up to 16 weeks, but at 20 weeks, 4 out of 5 mice had levels comparable to 12 weeks. Mean levels of the other pro-inflammatory cytokines IL-6, IL-17, IL-18 and TNF\alpha, and anti-inflammatory TGF\beta remained stable (data not shown). Typical Th2 cytokines IL-4 and IL-10 also exhibited a constant mean expression level (Figure 4A).

In contrast to local SG expression, most cytokines could not be detected above threshold in serum of the same NOD mice. At 8 weeks, only serum levels (mean ± SD, pg/mL) of IL-12/IL-23p40 (46.4 ± 17.0), IL-18 (630.3 ± 133.0) and TGF\beta (623.9 ± 266.6) were measurable. The mean levels of these cytokines did not change significantly over time, although 4 out of 5 mice showed higher systemic IL-18 levels by week 20 (mean ± SD of all five mice, 2414.0 ± 2128.6). None of the cytokines, measured in SG or serum, was correlated with SFR or FS (data not shown).

Th1 and Th2 responses are not mutually exclusive in salivary glands of NOD mice

A Th1 type immune response, or cellular response, is characterised by high levels of IFN\textgamma, whereas high levels of IL-4 and IL-10 mark the Th2 type, or humoral, response. In SS, activation of B cells and elevated immunoglobulin production are seen concomitantly with high IFN\textgamma levels, indicating both Th1 and Th2 type responses (reviewed in\textsuperscript{35}). We analysed the correlation of IFN\textgamma with IL-10 or IL-4 in the SGs of NOD mice of all ages, and found simultaneous activation of Th1 and Th2 type responses (Figure 4B).

DISCUSSION

SS is a common, but poorly understood, heterogeneous autoimmune disease for which there is currently no adequate treatment. Several therapies that were effective in other autoimmune diseases have failed for SS, e.g., TNF inhibitors for RA. Identification of new therapeutic targets is essential and finding a successful treatment will benefit from a better understanding of the pathological process(es). Since patients are often diagnosed when loss of salivary function and focal infiltration is already present, little is known about the early stages of the disease, impeding the development of new treatments. Mouse strains that either spontaneously or after experimental induction develop an SS-like phenotype provide models to study the disease parameters longitudinally. The NOD mouse is an especially useful model to study SG inflammation, since its infiltrates resemble those in humans, and it also
Figure 4. Expression of cytokines in salivary gland tissue from NOD mice. Cytokine levels were analysed in homogenates of salivary gland (SG) tissue obtained from non-obese diabetic (NOD) mice at 8, 12, 16 and 20 weeks of age (n = 5) by multiplex sandwich ELISA. Values were normalised for total protein and final concentrations are displayed. Per cytokine, dot plots for individual mice and mean concentrations for individual animals are shown (A). Correlation between Th1 cytokine interferon (IFN)γ and Th2 cytokines interleukin (IL)-10 or IL-4 levels of mice from all ages (B). Significant p-values are shown.
develops SG dysfunction (reviewed in\(^9\)). We performed an extensive, descriptive study of the disease progression from before symptom onset to secretory dysfunction and focal SG inflammation in NOD mice, and focused on salivary output and content, SG focal infiltrates and their cellular composition, and expression of local cytokines and co-stimulatory molecules.

Between 8 and 12 weeks, both NOD and the healthy control mice showed a significant increase in stimulated salivary output. The reason for this rise in SFR was not studied herein, but is possibly due to not fully developed responsiveness to secretagogues, such as pilocarpine, at an early age. Interestingly, despite a similar SFR at 8 weeks, saliva of NOD mice contained significantly higher sodium levels, which normalised to age-matched CB6 mice levels at 12 weeks, but thereafter increased again. Elevated sodium levels were also found in submandibular and parotid saliva of SS patients compared with healthy controls\(^{36}\); treatment with rituximab normalised sodium levels concomitant with improved histology of the SGs\(^{37}\). Since saliva can be acquired non-invasively, the measurement of sodium levels in humans and mice may provide a useful tool in determining disease status and the effect of treatment. The elevated sodium levels in 8-week-old NOD mice indicate epithelial dysfunction despite a normal SFR. At this age, focal infiltrates were not present in most mice, but we found markedly raised levels of the pro-inflammatory cytokines IFN\(_\gamma\), IL-1\(_\beta\), IL-2 and IL-23p19 in the gland. Additionally, we observed upregulation of ICAM-1 on SG ducts and endothelium at 8 weeks, suggesting an active role for epithelium in the early stages of inflammation. These events may have caused or contributed to the impaired Na\(^+\) reabsorption by the ducts. Support for this hypothesis can be found, for instance, in the fact that IL-2 treatment in cancer patients resulted in mucositis, reduced SFR and high salivary sodium levels\(^{38}\), and in vitro stimulation of human SG cells with IFN\(_\gamma\) and TNF\(_\alpha\) led to altered calcium signalling\(^{39}\), which illustrates the glandular epithelium's sensitivity to certain cytokines. The early presence of these inflammatory markers and high sodium levels could be the result of a (sub)clinical infection of the ductal epithelium that may be cleared by 12 weeks (based on the normalised sodium levels and reduction of many pro-inflammatory cytokines), followed by the triggering of an (auto)immune response. It could also be due to the emergence of autoantigens based on early developmental disorders of the SGs\(^{40}\) leading to the production of (a variety of) autoantibodies. In this study, we did not assess the autoantibodies anti-Ro and -La commonly found in SS, as the penetrance of this feature is highly variable in NOD mice. Previously, we (unpublished observations) and others\(^{41}\) found no or only low serum levels, making it unlikely that these autoantibodies contribute significantly to disease progression in this mouse model. However, it cannot be ruled out that other less commonly studied autoantibodies, such as anti-muscarinic receptor and anti-carbonic anhydrase antibodies, play a role in the late(r) stage disease. They may provide an explanation for disease progression despite overall reduction of many pro-inflammatory cytokines. Studying these phenomena could yield clues about the development of SS in humans.

The aetiology and pathogenesis of the salivary hypofunction in SS are still elusive. It has become apparent that the observed immune cell infiltration cannot be the only
cause of dysfunction; the reduction in SFR does not always correlate with the degree of SG inflammation, as measured by FS, in SS patients\textsuperscript{21,25}. The same holds true for the NOD mouse model. NOD-SCID mice, for example, developed SG dysfunction despite the absence of focal infiltrates\textsuperscript{42}. Moreover, abnormalities in salivary control and signal transduction can be seen prior to focal inflammation and salivary volume decline\textsuperscript{11,42}, illustrating an uncoupling of focal infiltrates and decreased SFR. Comparable with a previous report on NOD mice\textsuperscript{12}, we observed an increase in inflammation at 12 weeks—based on FS and numbers of infiltrating immune cells—before an SFR decrease and salivary sodium concentration increase at 16 weeks took place without any further dramatic inflammatory changes; no relationship was found between FS and SFR. These data confirm the complexity of salivary flow and inflammation. Interestingly, although no correlation between SFR and SG cytokine levels was observed, expression of subunit p40, but not p19, peaked at 16 weeks, indicating that IL-12 may contribute to the decline in SFR. This is also illustrated by IL-12 transgenic mice, which showed reduced SFRs compared with wild-type mice\textsuperscript{43}. Taken together, the possible role of IL-12 and/or the p40 subunit in salivary dysfunction needs to be further explored.

Relatively high levels of pro- and anti-inflammatory cytokines were seen at 8 weeks, which, except for IL-12/IL-23p40 and BAFF, diminished with age and progression of microscopic inflammation. Since lymphocytes had not yet infiltrated the gland of these mice, two other possible cytokine producers emerge: the numerous CD68\textsuperscript{+} monocytes/macrophages, which we and others\textsuperscript{44} found, and/or the activated epithelial cells. Macrophages are potent cytokine secretors under inflammatory circumstances\textsuperscript{45}, and SG epithelial cells in SS are known as important contributors to and orchestrators of the local autoimmune process\textsuperscript{46}. Additional double staining for cytokines and cellular markers, or laser capture microscopy followed by microarray analysis could identify the responsible cell(s), and further studies regarding this research will be set up in our department.

The onset of the SS-like disease in the inbred NOD mouse strain varies, possibly due to environmental factors\textsuperscript{47}. We and other authors found some focal infiltrates from the age of 8 weeks onward\textsuperscript{12}, whereas others only detected sporadic infiltrates at 16 weeks\textsuperscript{11}. In addition, we did not detect CD11c\textsuperscript{+} dendritic cells at 8 weeks, which is in contrast to van Blokland et al. who observed these cells already at 5 weeks\textsuperscript{44}. This variation may be explained by the fact that the mice of van Blokland et al. were bred in their own facility, while our mice were purchased directly from a supplier. Differences in food, water content and/or the presence of pathogens in the housing facility may be involved as well. Exploring these diversities in disease presentation and the relation with the environment may give us interesting insights in SS, which also varies in its presentation.

B cell-targeting therapy has shown promising results in clinical trials with SS patients with some residual SG function\textsuperscript{48}, indicating a significant role for B cells in this disease and the importance of timing of therapy. At 12 weeks, B cells were already prominent in SG lesions of NOD mice. At the same age we also detected the expression of BAFF, a cytokine important in B cell survival and stimulation, in the foci. BAFF transgenic mice exhibit B cell hyperplasia and hyperglobulinaemia resembling
the autoimmune phenotype of SLE\textsuperscript{49}. At a later age, BAFF transgenic mice develop an SS-like disease with infiltrates in the SG\textsuperscript{50} and a loss of SFR\textsuperscript{51}. Local BAFF expression in our NOD mice showed a dichotomous progression: a steady expression of BAFF once the infiltrates were present versus a seemingly abrupt increase in ductal tissue at 20 weeks of age. This finding was rather unexpected, as BAFF is produced by epithelial cells in the initial (triggering) phase of innate immunity and forms a link with the adaptive immunity\textsuperscript{52} and this late change in epithelial BAFF has not been reported in SS patients; in fact, epithelial BAFF was found to be similar for healthy controls and SS patients\textsuperscript{26}. Interestingly, epithelial BAFF augmentation at 20 weeks coincided with increases in IgD and CD138 staining, but not with CD19, indicating epithelial BAFF might be involved in specific recruitment and/or local differentiation of mature B and plasma cells in NOD mice. We did not assess BAFF expression in CB6 mice and it can therefore not be ruled out that this increase is related to something else than an SS-like phenomenon, e.g., aging. Whether epithelial BAFF expression develops similarly in human subjects will also have to be further examined.

With immunohistochemistry and digital image analysis, it is technically difficult to identify and count individual cells in foci, where many cells are packed closely together. We therefore chose to assess and compare the staining intensity instead of the cell count, which would have led to an underestimation of the numbers of cells. Although staining intensity can differ depending on the used primary antibody and additional amplification, we believe that a temporal comparison for each of the cell type markers is valid. One of the other limitations of our study is the relatively small number of mice per group, which may have restricted the statistical power. In addition, we did not study the expression of SG inflammatory parameters in CB6 mice. However, based on the data presented herein, we suggest that the next step should be exploring the (timely) modulation of local expression of ICAM-1, BAFF, IL-12 and/or recruitment of macrophages in NOD mice. We are currently examining the use of local gene therapy to address some of these factors and have recently shown that interfering with the ICAM-1-ligand interaction early in the disease resulted in a lower FS, whereas later intervention led to increased CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell numbers in the gland\textsuperscript{53}. Translating results of animal models to the clinic requires careful considerations, but several therapeutic options are already available. Belimumab (an anti-BAFF fully human monoclonal antibody) is on the market for SLE and phase II clinical trials for SS are ongoing; atacicept (a soluble receptor that binds and neutralises BAFF and a proliferation inducing ligand (APRIL) is being investigated in phase II/III studies for SLE (http://clinicaltrials.gov, NCT00732940); and ustekinumab (an anti-IL-12/IL-23p40 human monoclonal antibody) has been approved for psoriasis, while phase III trials for psoriatic arthritis are currently being undertaken (http://clinicaltrials.gov, NCT01009086).

In conclusion, our observations confirm the notion that SS-like disease in NOD mice develops in multiple stages: initially, macrophages infiltrate the SG when epithelium is activated as indicated by ICAM-1 expression and high levels of salivary sodium and pro- and anti-inflammatory cytokines; second, there is an influx of other immune cells that seemingly quickly assemble into focal infiltrates; and in the last
phase, salivary secretion declines while local IL-12/IL-23p40 levels peak and epithelial BAFF increases.

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EFFECT OF SOLUBLE ICAM-1 ON A SJÖGREN’S SYNDROME-LIKE PHENOTYPE IN NOD MICE IS DISEASE STAGE DEPENDENT

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CHAPTER 5

ABSTRACT

Introduction: Intercellular adhesion molecule-1 (ICAM-1) is involved in migration and co-stimulation of T and B cells. Membrane bound ICAM-1 is over expressed in the salivary glands (SG) of Sjögren’s syndrome (SS) patients and has therefore been proposed as a potential therapeutic target. To test the utility of ICAM-1 as a therapeutic target, we used local gene therapy in non-obese diabetic (NOD) mice to express soluble (s) ICAM-1/Fc to compete with membrane bound sICAM-1 for binding with its receptor. Therapy was given prior to and just after the influx of immune cells into the SG.

Methods: A recombinant serotype 2 adeno associated virus (rAAV2) encoding sICAM-1/Fc was constructed and its efficacy tested in the female NOD mice after retrograde instillation in SG at eight (early treatment) and ten (late treatment) weeks of age. SG inflammation was evaluated by focus score and immunohistochemical quantification of infiltrating cell types. Serum and SG tissue were analyzed for immunoglobulins (Ig).

Results: Early treatment with sICAM-1/Fc resulted in decreased average number of inflammatory foci without changes in T and B cell composition. In contrast, late treated mice did not show any change in focus scores, but immunohistochemical staining showed an increase in the overall number of CD4+ and CD8+ T cells. Moreover, early treated mice showed decreased IgM within the SGs, whereas late treated mice had increased IgM levels, and on average higher IgG and IgA levels.

Conclusions: Blocking the ICAM-1/LFA-1 interaction with sICAM-1/Fc may result in worsening of a SS like phenotype when infiltrates have already formed within the SG. As a treatment for human SS, caution should be taken targeting the ICAM-1 axis since most patients are diagnosed when inflammation is clearly present within the SG.
INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1) binds to lymphocyte function-associated antigen-1 (LFA-1) and macrophage 1 antigen (Mac-1) on immune cells, and is involved in adhesion and migration of leucocytes into an inflammatory environment. ICAM-1 also plays an important role in the co-stimulatory pathway involved in T cell activation and clonal expansion, and T cell dependent B cell activation. ICAM-1 is upregulated in endothelial cells, lymphocytes, fibroblasts, and ductal epithelium of salivary glands (SG) from Sjögren’s syndrome (SS) patients. SS is a systemic autoimmune disorder affecting secretory tissue, including the lachrymal and salivary glands, resulting in keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). One of the pathological hallmarks of the disease is the focal infiltration of mononuclear cells into these secretory glands.

Currently, there is no effective treatment for SS. Since ICAM-1 is consistently found to be upregulated in SS, it has been suggested that targeting ICAM-1 and the interaction with its ligands may positively affect the disease outcome. In previous studies, blocking ICAM-1 interaction by systemic administration of sICAM-1 has proven to be an effective therapy for autoimmune diabetes in the non obese diabetic (NOD) mouse. Intraperitoneal (ip) injection with sICAM-1 before the clinical onset of disease in NOD mice, resulted in decreased monocytic infiltration into the pancreas, reduced Th1 cytokines levels and a lower diabetes incidence. Another study showed that treatment of NOD mice with sICAM-1 after the onset of diabetes resulted in long-term remission of diabetes in >50% of treated mice. Interestingly, remission was not accompanied by decreased diabetogenic T cells and did not result in overall immunosuppression, suggesting induction of tolerance by sICAM-1.

Independent of diabetes, the NOD mice also spontaneously develop a complex of features that resembles SS in humans. These mice spontaneously develop SG focal infiltrates, mainly consisting of B and T cells, and within the inflamed SG, membrane bound endothelial and epithelial ICAM-1 and LFA-1 are upregulated. These characteristics make the NOD mouse a reasonable model to study the potential therapeutic effect of ICAM-1 interference on the development of SS. Since ICAM-1 plays a role in the migration of immune cells into tissue, we tested the effect of sICAM-1/Fc overexpression and secretion by ductal cells in SG of NOD mice, before (early treatment) and after (late treatment) the influx of inflammatory cells, to see whether we can intervene with the formation of the first focal infiltrates. The ductal cells are thought to play a crucial role in the pathogenesis of SS since focal infiltrates in SS are mainly found surrounding the ductal epithelial cells. Moreover, ductal cells produce high levels of pro-inflammatory cytokines and can act as nonprofessional antigen presenting cells, making these cells an attractive target. In this study, we investigated whether sustained expression of sICAM-1/Fc by ductal epithelial cells via local gene therapy in the SG of NOD mice could affect the development of SS-like clinical and immunological symptoms.
MATERIAL AND METHODS

Vector design and in vitro expression
The plasmid for mouse ICAM-1 coupled to the Fc-part of mouse immunoglobulin G1 (IgG1) was kindly provided by Dr. Lemarchand (Université René Descartes, France). We previously reported the construction of recombinant (r)AAV-LacZ encoding β-galactosidase. The plasmid for rAAV-luciferase was a kind gift of Dr. Mizukami (Jichi Medical School, Japan). Each gene was cloned into an rAAV plasmid containing a cytomegalovirus (CMV) promoter and the inverted terminal repeat (ITR) sequences for AAV2. The resulting plasmid (pAAV2-CMV-mICAM-1-mlgG1) was transfected into HEK 293 cells and protein secretion into the supernatant was quantified by an ELISA kit for mouse ICAM-1 (R&D systems, Minneapolis, MN, USA). The size of the fusion protein (ICAM-1/Fc) was confirmed by western blotting of the supernatant after transfection under reduced and nonreduced conditions using a 10% SDS gel, rat anti-mouse ICAM-1 (R&D systems) as a primary antibody and a labeled (IRDye 800 CW) goat anti-rat IgG (Li-Cor, Lincoln, NE, USA) as a secondary antibody. A recombinant mouse ICAM-1/Fc chimera (R&D systems) was used as a positive control.

In vitro biological activity
The biological activity of the expressed sICAM-1/Fc was tested in vitro for the ability to bind LFA-1. A flat bottom 96-well plate was coated overnight at 4°C with supernatant containing sICAM-1/Fc or recombinant ICAM-1/Fc (R&D systems) as a positive control. Human T cell blastoma cells (HSB2, ATCC, Manassas, VA, USA) were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) at 50 nM final concentration for 30 minutes (min) at 37°C to increase LFA-1 expression on the membrane. Cells were labeled with fluorescent calcein-AM solution (Invitrogen, Carlsbad, CA, USA) at 25 μM final concentration for 30 min at 37°C. Stimulated and labeled HSB2 cells (5x10^4 cells/well) were incubated for 1.5 hr at 37°C in the pre-coated wells. The plate was read before and after washing at 485-538 nm in a Spectramax M2 plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The difference in optical density between before and after washing was expressed as percentage adhesion.

Mice
NOD mice (001976 NOD/ShiLtJ, Jackson, Bar Harbor, ME, USA) were ordered at 6 weeks of age and were kept under pathogen-free conditions. All procedures involving animals were performed in compliance with the National Institutes of Health (NIH) Guidelines on Use of Animals in Research. Animal protocols were approved by the National Institute of Dental and Craniofacial Research (NIDCR) Animal Care and Use Committee (ACUC) and the NIH Biosafety Committee. The approval identification number is 09-512.

In vivo vector delivery and detection
Prior to SG administration, all vectors were dialyzed for 3 hours (hrs) against saline. Eight (early treatment) or ten (late treatment) week old mice were anesthetized...
intramuscularly (im) with a combination of ketamine (100 mg/mL, 1 mL/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (20 mg/mL, 0.7 mL/kg body weight; Phoenix Scientific, St. Joseph, MO, USA). To each submandibular gland 50 µl vector solution, containing 1x10^11 vector (sICAM-1/Fc or control vector LacZ) particles plus 1x10^9 luciferase reporter vector, was administered by retrograde ductal instillation using a thin cannula (Intermedic PE10, Clay Adams, Parsippany, NJ, USA), as previously described. This way of administration is known to effectively and stably transduce the epithelial ductal cells in the SG. At the end of the treatment (20 weeks of age), localization of vector delivery was confirmed by luciferase activity after ip injection of 100 µl (40 mg/ml) luciferin (Gold Biotechnology, St. Louis, MO, USA) and luminescence was imaged by an in vivo luminescence imager (Xenogen IVIS® imager using living image software®, Alameda, CA, USA). Vector DNA delivery was also confirmed by homogenization of SG and total genomic DNA was isolated using DNeasy blood & tissue kit (Qiagen, Venlo, the Netherlands). The vector was detected using on an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using the following primers specific for the therapeutic gene region: forward 5’-TAGAATTCGCAATGGCTTCA-’3, reverse 5’-CTTCTCTGGGATGGATGGAT- and probe 5’-CACCAGGCCAAGGGATAC-3’.

Saliva collection
Mice were anesthetized as described above and saliva secretion was induced by subcutaneous injection of pilocarpine (0.5 mg/kg BW; Sigma-Aldrich, St. Louis, MO). Stimulated whole saliva was collected for 20 minutes from the oral cavity with a hematocrit tube (Drummond Scientific Company, Broomall, PA, USA) placed into a preweighed 0.5 ml microcentrifuge tube, and the volume was determined gravimetrically.

Histological assessment and immunohistochemistry
At the end of the study the mice were euthanized, and one cross sectional part of the SG was embedded in paraffin and sections were cut at 5 µm. Three non-consecutive sections were stained with hematoxylin and eosin (H&E). The FS was determined for each mouse, in which one focus is defined as an aggregate of 50 or more lymphocytes and the FS defined as the average foci per 4 mm^2 SG tissue. Slides were scored blindly by at least 2 different researchers. Other paraffin sections were stained with rat anti-mouse B220 (kindly provided by Dr. K. van Gisbergen, University of Amsterdam, the Netherlands) after heat-induced citrate antigen-retrieval. Another cross-sectional part of the SG was collected and frozen into OCT compound (Tissue-Tek, Sakura, Zoeterwoude, the Netherlands) and sections were cut at 5 µm. Sections were stained with rat anti-mouse CD4 (eBioscience, San Diego, CA), rat anti-mouse CD8 (eBioscience), rat anti-mouse CD68 (Abcam, Cambridge, MA, USA), and hamster anti-mouse CD11c (Abcam), each at a 1:100 dilution. Anti-Foxp3 (eBioscience) was used at a 1:50 dilution using an extra amplification step with biotin labeled tyramide (PerkinElmer, Waltham, MA, USA) and streptavidin labeled HRP (PerkinElmer). Foxp3 is considered a reasonable marker for the presence of Tregs in tissue. For endogenous ICAM-1 expression, salivary glands
of 8, 12, 16 and 20 week old non-treated were frozen in OCT compound, sections were cut at 7 μm and were stained with rat anti-mouse ICAM-1 (Abcam). As secondary antibodies goat anti-rat-HRP (Southern Biotechnology, Birmingham, AL, USA) and goat anti-hamster-HRP (Jackson Immunoresearch, Suffolk, UK) were used. Staining was developed with AEC substrate (Dako, Glostrup, Denmark). Images of the high-power fields taken of the foci were analyzed using the Qwin analysis system (Leica, Cambridge, UK), as described previously. Within each group of treated and control mice, absolute staining intensity values were divided by the average of controls and expressed as relative IOD. In addition, the percentage of total SG tissue surface area taken up by foci was quantified for each section.

Detection of IgA, IgG and IgM in serum and SGs
Serum was collected by heart puncture just after sacrifice, blood was left to clot for 3 hrs on ice and was spun down for 25 minutes at 2500*g at 4°C. SG homogenates were made by homogenizing a part of the frozen SG in HEPES lysis buffer containing protease inhibitors (Protease Mini, Roche, Indianapolis, IN, USA) overnight at 4°C and total protein was determined with BCA™ protein assay kit (Pierce, Rockford, IL, USA). IgA, IgG and IgM in serum and tissue homogenates were determined using commercially available ELISA kits (Bethyl Laboratories, Montgomery, TX, USA) and final concentrations were corrected for total protein concentration.

Statistical analysis
Differences between experimental groups in focus scores were assessed using Student’s t-tests. Differences in all other experiments were assessed using the non-parametric Wilcoxon’s ranksum test or the parametric Student’s t-test depending on the data distribution. All the analyses were performed with GraphPad Prism statistical software (GraphPad Software Inc. version 5.01, La Jolla, CA, USA). A p value ≤ 0.05 was considered to be statistically significant.

RESULTS
Natural expression of ICAM-1 in the SGs of NOD mice
ICAM-1 expression was determined in the SGs of NOD mice at the age of 8, 12, 16 and 20 weeks. Eight week old NOD mice do not have focal infiltrates, but already expressed ICAM-1 in the ductal and endothelial epithelium (Figure 1A). From 12 weeks to 20 weeks of age, when infiltrating lymphocytes are clearly present, ICAM-1 was still expressed within the epithelial and endothelial cells, but was also strongly expressed in the focal infiltrates (Figure 1B-D). Quantification of ICAM-1 showed a nearly 10-fold increase (p < 0.005) of expression levels between 8 and 12 weeks of age, and this stayed stable after 12 weeks (Figure 1E). This increase was based on infiltrating cells, since increase in expression of the ductal and endothelial cells did not change (data not shown).
Figure 1. Natural ICAM-1 expression in NOD SGs. Frozen sections from 8 (N = 5), 12 (N = 5), 16 (N = 4), and 20 (N = 5) week old non-treated NOD SGs were analyzed and quantified for natural ICAM-1 expression. Representative images (400x magnification) of ICAM-1 expression in NOD SGs are shown at 8 (A), 12 (B), 16 (C), and 20 (D) weeks of age. Arrows indicate ICAM-1 expression at ductal and endothelial epithelium. E) Quantification of ICAM-1 expression for all ages. Data shown are mean values +/- SD. The p-value was determined by the parametric Student’s t-test.

Figure 2. In vitro and in vivo expression and biological activity of ICAM-1/Fc. A) In vitro expression of ICAM-1/Fc was shown using a reducing and nonreducing SDS-PAGE gel. Under reducing conditions, ICAM-1/Fc migrated as a monomer with a molecular mass of approximately 120 kDa and under nonreducing conditions as a dimer with a molecular mass of approximately 240 kDa. Lane 1 is recombinant ICAM-1/Fc and lane 2 is ICAM-1/Fc-transfected supernatant. B) The biological activity of the ICAM-1/Fc was determined by adding stimulated and labeled T cell blastoma cells to plates coated with ICAM-1/Fc-transfected supernatant. The optical density between before and after washing the wells is expressed as percentage binding to LFA-1. Data shown are mean values +/- SD of 3 experiments. PBS = phosphate buffered saline coated wells, mock = mock-transfected supernatant coated cells. C) In vivo imaging of luciferase expression within the SGs. One representative mouse is shown.
**In vitro** sICAM-1/Fc is secreted as a dimer and is biologically active

sICAM-1/Fc was cloned into an AAV2 vector and the vector was tested for protein expression after transduction of human embryonic kidney (HEK 293) cells. Supernatant was harvested 48 hrs after transduction and a western blot was performed under reducing and non-reducing conditions. A expected, sICAM-1/Fc secreted in the supernatant migrated as a dimer with a molecular mass of approximately 240 kDa under non-reducing conditions and as a monomer at 120 kDa under reducing conditions, similar to commercially available recombinant sICAM-1/Fc (Figure 2A). Supernatant was tested in an ELISA and 0.62 μg/ml of fusion protein was detected, which was within the expected range for AAV-plasmid expression (data not shown). An **in vitro** biological activity assay showed a 52% binding of activated lymphocytes, over expressing LFA-1, to wells coated with supernatant containing sICAM-1/Fc, similarly to the commercially available recombinant ICAM-1/Fc (Figure 2B).

Confirmation of local transgene delivery and stable transduction of the SG **in vivo** after local administration

SGs of NOD mice of 8 or 10 weeks of age were cannulated with AAV2-vectors containing sICAM-1/Fc or LacZ together with a luciferase reporter vector. We used 1x10^{11} viral particles per SG since previous studies from our group have shown that the optimal dose for protein expression without causing a significant immune response lies between 1x10^{10} and 1x10^{11} particles per gland^{16,18}. Prior to sacrifice, mice were anesthetized and were given luciferin ip, after which luminescence was measured by an in vivo luminescence imager (IVIS) to ensure localized transfer of the vectors to the SGs. Figure 2C shows a representative mouse expressing luciferase in the SG, 10 weeks after a single administration. Vector delivery was also confirmed using quantitative polymerase chain reaction (qPCR) on total DNA extracted from treated SGs (data not shown). In agreement with previous studies by our group expression was localized to the salivary gland and not detected in other tissues of the animal (data not shown).

Early treatment decreased overall inflammation of the SGs

To determine the effect of treatment with sICAM-1/Fc on inflammation of the SGs, NOD mice treated at 8 weeks (early treatment) or at 10 weeks (late treatment) of age were euthanized at 20 weeks and the focus score (FS) was determined. FS in early sICAM-1/Fc-treated mice were significantly lower compared with LacZ-treated mice (2.3 ± 0.9 versus 3.3 ± 1.0, p < 0.005). In contrast, late treatment did not affect the FS (3.2 ± 1.4 versus 3.0 ± 1.1, p = 0.78; Figure 3).

Late sICAM-1/Fc treatment increased CD4\(^+\) and CD8\(^+\) T cells in the SG

To identify the cell types affected by the sICAM-1/Fc treatment, we performed immunohistochemistry (IHC) analysis and quantification of the foci of SG sections from early and late treated mice. Despite the reduced number of foci, no specific cell type was found to be reduced within these foci (Figure 4A). In contrast, late treated mice showed a significant number of mice with an increased relative integrated optical density (IOD) of positive CD4\(^+\) (p = 0.03) and CD8\(^+\) T cell staining (p < 0.01; Figure 4A).
No differences in the relative IOD of macrophages (CD68), dendritic cells (CD11c), B cells (B220) and Treg (Foxp3) were detected in both early (p = 0.97, p = 0.99, p = 0.40, and p = 0.94 respectively) and late (p = 0.45, p = 0.41, p = 0.61, and p = 0.78 respectively) treated mice (Figure 4B-C).

sICAM-1/Fc can affect local and systemic immunoglobulin levels and is depending on disease stage

ICAM-1 is involved in T cell activation and T cell dependent B cell activation. Therefore, we studied whether interrupting the ICAM-1/LFA-1 interaction would alter local and systemic immunoglobulin (Ig) levels. Early treatment showed decreased levels of IgM in the sICAM-1/Fc treated SGs compared to LacZ controls (0.42 ± 0.13 μg/ml versus 0.54 ± 0.17 μg/ml, p = 0.02); IgG (p = 0.47) and IgA (p = 0.44) levels were unchanged (Figure 5A). In contrast, late treatment with sICAM-1/Fc resulted in increased levels of IgM (0.93 ± 0.35 μg/ml versus 0.60 ± 0.29 μg/ml, p = 0.04) in the SGs, and tended to increase local IgG (5.36 ± 1.47 μg/ml versus 3.90 ± 1.82 μg/ml, p = 0.08) and IgA levels (5.78 ± 2.34 μg/ml versus 3.85 ± 2.19 μg/ml, p = 0.08; Figure 5B). Ig serum levels in the early treated mice were unaltered (Figure 5C). In contrast, late sICAM-1/Fc treated mice showed increased serum levels of IgM (0.52 ± 0.06 mg/ml versus 0.36 ± 0.11 mg/ml, p < 0.005), and IgA (0.78 ± 0.19 mg/ml versus 0.57 ± 0.19, p = 0.05), and tended to increase serum IgG levels (1.71 ± 0.44 mg/ml versus 1.34 ± 0.51 mg/ml, p = 0.19; Figure 5D).

Salivary flow was unaltered after sICAM-1/Fc delivery

NOD mice in our facility start to develop decreased salivary flow from 16 weeks of age. To study whether the reduced SG inflammation after sICAM-1/Fc treatment affected the salivary flow, we measured stimulated salivary flow at the end of study (age 20 weeks). Despite the observed reduction in focus score, stimulated salivary flow was not improved in early treated mice. In addition, to make sure we did not miss the window of effect for sICAM-1/Fc treatment, we also tested an additional group of early treated mice at 16 weeks of age and also in this group no differences in salivary
flow was observed. Also in the late treatment group salivary flow was unchanged flow compared with controls despite the increase observed in SG infiltrating T cells (data not shown).

Figure 4. Increased relative IOD of CD4+ and CD8+ T cells in late treated mice. SGs from early and late treated mice with an average of 7 mice per group were quantified for CD4+ T cells, CD8+ T cells (A), CD68+ macrophages, CD11c+ dendritic cells (B), and B220+ B cells and Foxp3+ regulatory T cells (C). Data shown are the mean relative IOD. The p-value was determined by the parametric Student's t-test or the non-parametric Wilcoxon's ranksum test depending on data distribution for each staining.
DISCUSSION

ICAM-1 is upregulated in many inflammatory diseases. It functions as an adhesion molecule and plays a role in the interaction and reciprocal activation of B and T cells by binding to LFA-1 and Mac-1. Targeting the interaction between ICAM-1 and LFA-1 as a treatment for (auto)immunity has been the subject of a number of studies (reviewed in\(^2\)), and was shown to have beneficial effects in animal models for autoimmune diseases\(^9\)-\(^10\). ICAM-1 and LFA-1 are upregulated in SG of SS patients\(^3\)-\(^6\) suggesting an important role for these molecules in this disease. This is further supported by the observation that systemic administration of antibodies to LFA-1 in NOD mice resulted in inhibition of infiltration of the lacrymal glands\(^21\). Moreover, \textit{in vitro} stimulation of human salivary gland (HSG) ductal cells with interferon (IFN)\(\gamma\), a cytokine central to SS\(^22\), in the presence or absence of tumor necrosis factor (TNF), increased the expression of ICAM-1 on the cell surface\(^23\). It has therefore been speculated that interfering with the ICAM-1/LFA-1 interaction may improve the clinical outcome in SS. To study this,

**Figure 5.** The timing of ICAM-1/Fc delivery affects local and systemic Ig levels differently. SG homogenates (A-B) and serum (C-D) from early (average of N = 8 per group) and late (average of N = 18 per group) treated mice were analyzed for IgG, IgA and IgM. Data shown are mean +/- SD. The p-value was determined by the parametric Student’s t-test or the non-parametric Wilcoxon’s ranksum test depending on data distribution for each Ig.
sICAM-1/Fc was expressed in the SG of NOD mice in an initiation phase and in a more advanced stage of a SS-like disease and the effect on inflammatory parameters was assessed.

NOD mice start to form SG infiltrates from 8 weeks of age. At this age, prior to the development of infiltrates, mice clearly expressed ICAM-1 in the ductal epithelium and endothelium. Four weeks later the overall ICAM-1 expression was increased 10 fold, based on the expression of newly infiltrated cells. These data confirm that endothelium and epithelium are already activated prior the influx of lymphocytes and suggest that ICAM-1 signaling is important in an early phase of the pathogenesis of SS in these mice. Based on these results and on existing literature indicating that timing of ICAM-1 interference can determine the outcome\textsuperscript{24}, we hypothesized that treatment with sICAM-1/Fc at an initiation phase of the disease may differ from and be more effective than treatment later in the disease course. Therefore we treated 8 week old mice, just before inflammatory cells are entering the SG, and at 10 weeks at an accelerated phase of the disease when distinct foci have formed in the majority of mice\textsuperscript{19}.

Mice were cannulated with a non-replicating AAV type 2 vector, known to stably transduce ductal epithelial cells in the SG\textsuperscript{25}. Indeed, at the age of 20 weeks, vector DNA of sICAM-1/Fc was still detected in the SG indicating stable transduction. Moreover, in vivo imaging of luciferase activity, which was co-transduced during administration of sICAM-1/Fc, showed localized expression in the SG. No luciferase activity was detected in the liver or in any other organ, indicating no systemic spread (data not shown).

Inflammation of the SG was reduced in the early treated mice, although no reduction of any specific cell type was found within the foci, suggesting a general block on the influx of cells into the SG. Interestingly, although the absolute FS was unchanged in the late treatment group, the relative IOD of CD8\textsuperscript{+} cells and CD4\textsuperscript{+}, but not of Treg (Foxp3\textsuperscript{+}) cells was increased compared with the control mice. We also noted a ~2 fold increase in the average size of the foci (data not shown), suggesting a specific recruitment to or local expansion of effector T cells in the SG foci. These data is consistent with previously reported T cell proliferation and IL-2 secretion after incubation with sICAM-1 in vitro, indicating direct or indirect T cell activation by sICAM-1\textsuperscript{10}. In addition, the contrasting effects observed in the different timing of treatment indicate a shift in the role for therapeutic sICAM-1/Fc over the course of disease progression. Although we did not study this in detail, this notion is also supported by literature. Recently, anti-ICAM-1 antibodies have been shown to positively affect the clinical course of experimental autoimmune encephalitis when given in an early phase of the disease, whereas treatment given in a later phase, worsened the clinical score of the treated mice\textsuperscript{24}. Based on these data and on the early expression of endogenous ICAM-1 in the SG of mice, it would be interesting to study sICAM-1/Fc delivery at an earlier age than 8 weeks. However, the SG of NOD mice are not fully developed until the age of 8 weeks and hence the orifices of the main ducts of the submandibular gland draining into the oral cavity are very small making it technically not feasible to cannulate the glands before this age.
In parallel to our animal study, a clinical study was undertaken at the National Institutes of Health (NIH) in patients with SS using a monoclonal antibody to the CD11a subunit of LFA-1 (efalizumab), a ligand of ICAM-1. Unexpectedly, this treatment increased inflammation in minor salivary glands and development of de novo autoantibodies was detected in some patients. Although this therapeutic approach is different from the approach we present in this paper (e.g., the use of a monoclonal antibody versus a soluble molecule), these results may further indicate that blocking the ICAM-1/LFA-1 interaction may cause adverse effects once inflammation is established.

Many SS patients have high levels of circulating immunoglobulins, indicating B cell activation. Since ICAM-1 is involved in T cell dependent B cell stimulation, directly or via the production of IL-2 by activated T cells, we measured the effect of sICAM-1/Fc on local immunoglobulin production. Immunoglobulin levels in the SG were differentially affected based on the timing of sICAM-1/Fc expression, with the most striking differences observed in IgM levels, suggesting again a dichotomous role for ICAM-1 in the course of murine SS. Little is known about the direct role of ICAM-1 on immunoglobulin synthesis, but the effects observed could be the result of direct B cell stimulation or indirect stimulation via activation of T cells by sICAM-1/Fc. Additional research is required to elucidate the exact mechanism.

Stimulated salivary flow was unchanged despite reduced inflammation in the early treatment group, indicating that infiltration of the SG by lymphocytes in the early treated group was not sufficiently suppressed. On the other hand, salivary flow was also unchanged in the late treatment group, despite the increase of CD4+ and CD8+ T cells in the SG, suggesting that T cells may not play a direct role in SG dysfunction of the inflamed glands. Most importantly, these data imply that the loss of salivary flow in SS is the end result of a complicated pathological process that is poorly understood.

In summary, the expression of sICAM-1/Fc in the SG of NOD mice led to a modest decrease in autoimmune sialadenitis when treatment was given at an early stage of the disease. However, late treatment increased the number of CD4+ and CD8+ T cells in the SG, and immunoglobulin levels in the SG and serum, indicating a dichotomous role for sICAM-1/Fc on the pathogenesis of murine SS depending on disease stage. These data also indicate that caution must be taken in treating human SS with therapies targeting the ICAM-1/LFA-1 interaction, since it is likely that most patients are diagnosed and request treatment in a more progressed stage of the disease.
CHAPTER 5

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LOCAL ADMINISTRATION OF SOLUBLE CD40:FC TO THE SALIVARY GLANDS OF NON-OBESE DIABETIC MICE DOES NOT AMELIORATE AUTOIMMUNE DISEASE

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ABSTRACT

Objective: CD40–CD154 (CD40 ligand) interaction in the co-stimulatory pathway is involved in many (auto)immune processes and both molecules are upregulated in salivary glands of Sjögren's syndrome (SS) patients. Interference within the CD40 pathway has ameliorated (auto)inflammation in a number of disease models. To test the potential role of the CD40 pathway in loss of gland function and inflammation in SS, an inhibitor of CD40-CD154 interaction was overexpressed in the salivary glands (SGs) of a spontaneous murine model of SS, the NOD mice.

Material and Methods: At different diseases stages an adeno associated viral vector encoding CD40 coupled to human Fc domain (CD40:Fc) was injected locally into the SGs of NOD mice. Delivery was confirmed by PCR. The overall effect on local inflammation was determined by assessment of the focus score (FS), quantification of infiltrating cell types, immunoglobulin levels, and microarray analysis. The effect on SG function was determined by measuring stimulated salivary flow.

Results: CD40:Fc was stably expressed in the SG of NOD mice, and the protein was secreted into the blood stream. Microarray analysis revealed that expression of CD40:Fc downregulated the expression of many genes involved in regulation of the immune response. However, FS, infiltrating cell types, immunoglobulin levels, and salivary gland output were similar for treated and control mice.

Discussion: Although endogenous CD40 is expressed in SG inflammatory foci in the SG of NOD mice, the expression of soluble CD40:Fc did not lead to reduced overall inflammation and/or improved salivary gland function. These data indicate possible redundancy of the CD40 pathway in the SG and suggests that targeting CD40 alone may not be sufficient to alter the disease phenotype.
INTRODUCTION

The inflammatory foci observed in the salivary gland (SG) of non-obese diabetic (NOD) mice resemble the foci comprised of mononuclear cells seen in SG of patients with Sjögren's syndrome (SS)\(^1\). The local autoimmune process is characterized by the influx of T cells and to a lesser degree B cells, and a variety of other immune cells that accumulate in the gland and reorganize over time\(^2\). It is unclear what initiates the inflammatory process, but the upregulation of co-stimulatory-, adhesion- and antigen-presenting molecules are thought to play a role in the recruitment and the organization of inflammatory cells in the secretory glands of both patients and mice. The engagement of the co-stimulatory molecules CD40, belonging to the tumor necrosis factor (TNF) receptor superfamily, and its ligand, CD154 is known to induce B cell activation and maturation, immunoglobulin isotype switching and the secretion of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12 and interferon (IFN) \(\gamma\)\(^3\). CD154 is expressed on CD4\(^+\) T cells, but can also be found on a variety of non-lymphoid cells. CD40 can also be found on many cell types such as B cells, endothelial cells, dendritic cells and monocytes\(^4\). In the SG of SS patients, CD40 is detected on epithelial cells, lymphocytes and endothelial cells\(^5\)\(^-\)\(^6\). CD40 is upregulated on epithelial cells \textit{in vitro} when stimulated with cytokines such as IFN\(\gamma\) and IL-1\(\beta\)\(^7\). In addition, stimulation through CD40 leads to activation of SG epithelial cells as indicated by upregulation of intercellular adhesion molecule type 1 (ICAM-1)\(^8\). CD154 can be found in the clustered infiltrating cells\(^5\)\(^-\)\(^6\).

The interaction of CD40 and CD154 has been implicated in a number of diseases such as arthritis, cancer, atherosclerosis, lupus nephritis, and acute or chronic graft-versus-host disease\(^4\). Blocking and/or interfering with this specific co-stimulatory pathway has been studied previously in animal models of transplant rejection\(^9\)\(^-\)\(^10\), diabetes\(^11\) and experimental autoimmune encephalomyelitis (EAE)\(^12\) with improved clinical outcome. The effect of altered CD40-CD154 interaction has not been studied in animal models of SS.

Therefore, we tested the effect of overexpression of soluble CD40 on the SG inflammation of NOD mice at 3 different stages of the disease; at 8 weeks of age, before focal inflammation is present and endogenous CD40 is not detected in SG of NOD mice. At 10 weeks when focal inflammation starts to appear and CD40 can be found in the early SG foci and at 16 weeks in a more advanced disease stage when CD40 is strongly upregulated within infiltrates\(^2\). Expression of CD40:Fc lead to changes in the SG transcriptome. However, it did not result in a reduction in inflammation nor in improved salivary gland function.

MATERIAL AND METHODS

Vector production

We used a plasmid for the murine CD40 coupled to the constant region of human immunoglobulin (Ig:Fc), previously used for adeno viral vector gene transfer\(^10\). This gene was cloned into a recombinant adeno associated virus (AAV) plasmid containing a Cytomegalovirus (CMV) promoter and the inverted terminal repeat (ITRs) sequences.
for AAV serotype 2 (AAV2). To generate recombinant AAV serotype 2 vectors (rAAV2), we used the adenoviral helper packaging plasmid pDG. Plates (15 cm) of ~40% confluent 293 T cells were cotransfected with either pAAV-LacZ or pAAV-CD40 according to standardized methods. Clarified cell lysates were adjusted to a refractive index of 1.372 by addition of CsCl and centrifuged at 38,000 rpm for 65 hr at 20°C. Equilibrium density gradients were fractionated and fractions with a refractive index of 1.369-1.375 were collected. The titer of DNA physical particles in rAAV stocks was determined by quantitative (Q)-PCR and the vectors were stored at -80°C. The generated plasmid (pAAV2-CMV-mCD40-hFc) was transfected into HEK293 cells and secretion of the protein in the supernatant was measured by western blotting and an ELISA-kit for murine CD40 (R&D systems, Minneapolis, MN, USA). On the day of vector administration to NOD mice, the vector was dialyzed for 3 hr against saline.

Animals, vector administration and detection
Female NOD mice (Jackson Laboratory, Bar Harbor, ME, USA) were kept under specific pathogen-free conditions in the animal facilities of the National Institute of Dental and Craniofacial Research (NIDCR). Animal protocols were approved by the NIDCR Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. Vectors were delivered into the submandibular glands at 8, 10 and 16 weeks by retrograde instillation as previously described. In short, female NOD mice were anesthetized with mild anesthesia (a combination of ketamine and xylazine) and 50 µl containing 1x10¹¹ vector particles was administered to each submandibular gland by retrograde ductal instillation using a thin cannula (Intermedic PE10, Clay Adams, Parsippany, NJ, USA). To compare local versus distal treatment, one group of mice was injected in the right thigh muscle with 1x10¹¹ vector particles at 10 weeks and was analyzed in a similar way as the locally treated mice. Mice were sacrificed at 16 or 20 weeks of age. At time of sacrifice, submandibular SGs were removed and cut in 4 equal parts. One part was homogenized and total genomic DNA was isolated using DNeasy blood & tissue kit (Qiagen, Venlo, the Netherlands). Vector was detected using Q-PCR and probes specific for promoter-gene sequences on an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Histological assessment and immunohistochemistry
Two other parts of the SGs were cleaned of any adjacent (possibly lymphoid) tissue and cut into three cross-sectional parts. The first part was collected in formalin, embedded in paraffin and cut in 5 µm sections. Three sections (each 50 µm apart from the previous) were mounted on glass slides and stained with haematoxylin and eosin. The number of foci (where one focus is defined as an aggregate of ≥50 infiltrated cells per 4 mm²) on each section was counted by two different examiners who were blind to the assigned treatment group, and the mean focus score (FS) was determined. Sections were cut (7µm) for staining with specific antibodies. The second SG part was emerged in Tissue-Tek optimal-cutting temperature (OCT) compound (Miles, Elkhart, IN, USA) and quickly frozen on dry ice. Sections (7 µm) were cut with a cryostat, mounted on glass slides and stored at -80°C until further use. For staining,
sections were thawed and fixed in acetone for 10 minutes (for frozen sections) and deparaffinised and hydrated (for paraffin embedded slides). Endogenous peroxidase was blocked, slides were washed in phosphate-buffered saline (PBS), and further blocked with 1% bovine serum albumin (BSA) and 10% normal goat serum (NGS; Dako, Glostrup, Denmark) in PBS overnight. The next day, slides were incubated with the following primary antibodies in 1% PBS and 5% serum of the host of the secondary antibody at room temperature for 1 hour: CD4, CD8, B220, CD19, CD11c, CD49b and CD68. Conditions used and information on the specific antibodies were previously published.

Preparation of salivary gland tissue homogenates

The fourth SG part was snap-frozen on dry ice and stored at -80°C until further use. Prior to homogenisation, samples were thawed and kept on ice. Samples were crushed, placed in 2 mL tubes containing 1 mL HEPES lysis buffer (20 mM HEPES, 0.5 M NaCl, 0.25% Triton X-100 and 1 mM EDTA) and complete protease inhibitor (Roche, Mannheim, Germany), and lysed by shaking at 4°C overnight. The next day, samples were centrifuged at 1,500 x g at 4°C for 10 minutes. Protein content of supernatants was measured using a bicinchoninic acid (BCA) protein detection kit (Pierce, Rockford, IL, USA) and stored at -80°C until further use.

Saliva and serum collection

Saliva was collected 5 days before sacrifice. Saliva secretion was induced in anesthetized mice (see cannulation procedure) by subcutaneous (sc) injection of pilocarpine (0.5 mg/kg BW; Sigma-Aldrich, St. Louis, MO) and stimulated whole saliva was collected for 20 minutes (min) from the oral cavity by gravity with a hematocrit tube (Drummond Scientific Company, Broomall, PA, USA) placed into a preweighed 0.5 ml microcentrifuge tube. Saliva volume was determined by weight and expressed as μl/20 min*gram body weight. Serum was collected during sacrifice by heart puncture. Blood was left to clot on ice for 3 hours and centrifuged to collect serum.

CD40:Fc detection in serum, saliva, and tissue homogenates in ELISA

A commercially available mouse CD40 detection kit (Mouse CD40/TNFRSF5 DuoSet DY097, R&D systems) was used for detection in supernatant, serum, saliva and tissue homogenates. An adapted protocol was used for detection of the human Fc domain in the CD40 construct as follows. Plates were coated with the CD40 capture antibody and incubated with the samples as described in the protocol of the DY097 kit. After incubation with the samples, plates were washed three times with wash buffer and incubated with 100 ul of 1:7500 anti human IgG-HRP (GTX 26759, Genetex, Irvine, CA, USA) for 1 hr. Plates were washed three times and incubated with substrate solution (R&D systems, Minneapolis, MN, USA) and stopped with 50 ul stop solution (R&D systems). The plates were read at 450 nm using a Spectramax M2 plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).
CD40:Fc detection in a western blot
Supernatant of CD40:Fc and mock transfected HEK293 cells was loaded (10ug/lane) onto a Bis-Tris gel, after boiling for 5 minutes at a 1:1 ratio with 2.5% beta mercapto/NU page LDS sample buffer (Invitrogen, Carlsbad, CA, USA), run and transferred to a nitrocellulose membrane. The blot was blocked with 5% milk powder in TBST buffer and incubated with biotin labeled anti-murine CD40 (R&D systems, MAB4401), followed by streptavidin-HRP (R&D systems) or anti human IgG-HRP (Genetex) and developed with ECL reagent (GE Healthcare, Buckinghamshire, UK). Anti-murine CD40 recognizes CD40 reagent (GE Healthcare) dimers well and monomers poorly (R&D product information).

RNA extraction, amplification and synthesis of fluorescent cRNA
Total RNA was extracted from frozen SG samples using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations. The quality of RNA was measured with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), only samples with a 28S/18S ribosomal RNA ratio of 1.7 and RIN score > 6.5 were used in the microarray study. The RNAs were amplified and labeled with a lower RNA input linear amplification kit (Agilent, Santa Clara, CA, USA). A total of 500 ng RNA was labeled with Cyanine 3-CTP according to the manufacturer’s instructions: Briefly, 500 ng of total RNA was first mixed with 2.0 µl of RNA spike (One-Color Spike, Agilent) previously diluted in a 1.5 ml tube including T7 primer and was the incubated at 65 0C for 10 min. The reaction temperature was changed to 40°C after adding 8.5 µl of cDNA Master Mix reagents (Agilent) for 2 hours, and the samples were moved to a 65 ºC circulating water bath for an additional incubating for 15 min, and quench on ice for 5 min. Sixty µl of transcription Master Mix was added to the reaction including Cyanine 3-CTP (Agilent) for each sample for additional 5 hours at 40°C. The labeled and amplified cRNA was purified using an RNeasy Mini Kit (Qiagen). The cRNA was quantified by using a NanoDrop ND-1000 UV-VIS Spectrophotometer (version 3.2.1). Only cRNA with a total yield >1.65 µg/reaction and specific avidity >9.0 pmol Cy3 per µg cRNA were used in the following hybridization step.

Microarray hybridization and data extraction
Labeled cRNA was used to probe a 4 x 44K microarrays (Agilent) containing 44K mouse oligo probes. Microarrays were hybridized according to the manufacturer’s recommendations from One-Color Microarray-Based Gene Expression Analysis (Agilent). Briefly, each tube contained the following reaction reagents: 1.65 µg of new Cy3-labelled cRNA, 11 µl of 10x blocking agent, and 2.2 µl of 25x Fragment buffer. The mixture was incubated at 60°C for exactly 30 min and then 55 µl of 2 x GE x Hybridization buffer was added to stop the fragmentation reaction. Following centrifugation 13.000 rpm for 1 min, 100 µl of the sample solution was loaded onto each array on the microarray slides that was then assembled in the hybridization chamber. The assembled slide chamber was then placed in a hybridization oven (Agilent) with rotating speed of 10 rpm at 65°C for 17 hours. After disassembling the array hybridization the chambers were washed. Following the washing procedures, the slides were immediately scanned using a Microarray Scanner (Model: Agilent G2565AA...
System) to minimize the environmental oxidation and loss of signal intensities. The microarray data (tif images) file was extracted using the Agilent Feature Extraction (FE) (software version 9.5.1) program, for One-color gene expression, the default gene expression is specified in the FE grid template properties with selection of “GE1_QCM_Feb07” in this protocol. Only those chips that meet 9/12 of the report criteria were using in subsequent analysis.

Pathway analysis

Microarray analysis was performed using GeneSpring (GX 11) and included pre-processing raw-data, normalize data, QC samples/entities, t-test plus Benjamin correction, clustering, annotation and access biological context. Gene changes greater than 2 fold were used in the analysis. The comparisons of biological functional pathway or biomarkers were run by an IPA program (Ingenuity® systems, Redwood city, CA, USA).

Determination of immunoglobulin levels

IgG, IgA and IgM were measured by commercially available ELISA kits (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer’s protocol. Values were corrected for total protein content in the SG protein homogenates.

Statistical analysis

Differences between experimental groups were assessed using the non-parametric Wilcoxon’s rank-sum test or parametric Student’s t-test depending on data distribution. Correlations between vector copy number and FS were assessed using Spearman’s Rho test (non-parametric). All analyses were performed with GraphPad Prism statistical software (GraphPad Software Inc. version 5.01, La Jolla, CA, USA). A p value ≤ 0.05 was considered to be statistically significant.

RESULTS

In vitro and in vivo expression of CD40:Fc

Murine CD40 coupled to the constant region of human immunoglobulin (Ig:Fc) under the regulation of a CMV promoter was cloned into a recombinant AAV vector plasmid and was transduced into HEK293 cells. After 48 hours (hrs), supernatant was collected and tested for expression by western blot and ELISA. The secreted CD40:Fc protein migrated as expected as a dimer under non-reduced conditions (90 kD) and as a monomer of approximately 45 kD under reduced conditions. Furthermore, the product could be detected by an anti-human-Ig and an anti-murine CD40 antibody (Figure 1). In an ELISA, 2.86 μg/ml protein was detected by anti-murine CD40 in the supernatant of transduced cells. The fusion protein could also be detected by anti-human Ig, suggesting full protein expression (data not shown).

NOD mice express CD40 in focal infiltrates in the SG that start to appear around 8 weeks of age and become prominent at 12 weeks of age². To study the effect of soluble CD40 on different stages of autoimmune sialadenitis, the CD40:Fc vector was infused
into the SG of NOD mice at the age of 8, 10 and 16 weeks (just prior to cell infiltration, during the accelerated phase of immune cell influx and at a more established disease phase respectively). AAV2 is known to stably transduce epithelial cells in the salivary gland and provokes by itself only a minimal immune responses\textsuperscript{17}. At the age of 16 or 20 weeks, mice were euthanized and Q-PCR was performed to confirm local delivery of the vector to the SG and vector was detected in SGs of the treated mice but not in liver (data not shown). Saliva, serum and protein homogenates from CD40:Fc treated and control mice was analyzed for CD40 expression and showed high expression in serum (~10 ng/ml) of treated mice while it was not detected in control mice (< 3.5 pg/ml). The fusion protein was not detected in saliva or protein homogenates. In the i.m. treated mice CD40:Fc was not detectable (data not shown).

Changes in NF-κB network

Microarray analysis was performed on the mRNA derived from the SGs of mice treated at 10 weeks and euthanized at 20 weeks and this revealed a number of changes in the transcriptome of the CD40 treated mice compared with the LacZ treated mice. Using either all statistically significant differentially expressed genes or just those over 2 fold identified the gene expression/immune response network as a key network for the differentially expressed genes. Central nodes in this network include the transcription factor nuclear factor (NF) κB complex and transforming growth factor (TGF) β. Many of the genes surrounding these nodes were downregulated in the CD40:Fc treated mice compared with the LacZ group suggesting decreased activation in the CD40:Fc group (Figure 2).

In total, 558 Genes were identified as statistically significantly differentially expressed in CD40 mice compared with controls with 137 genes that were up- and 421 genes that were down-regulated. Table 1 shows genes that were found to be more than 10 times down or more than 5 times upregulated.

Microarray analysis on salivary gland (SG) RNA from non-obese diabetic (NOD) mice treated with CD40:Fc showed 558 down- or up regulated genes. The table shows 18 downregulated (>10 fold) and 2 upregulated genes (>5 fold) when compared with control vector treated mice.
Figure 2. Network based analysis of genes affected by CD40:Fc treatment. Microarray analysis was performed on SG of CD40:Fc treated and control mice (n=4 each, treatment at 10 weeks, end of study at 20 weeks) and gene changes greater than 2 fold were used in the analysis and compared for biological functional pathways or biomarkers by an Ingenuity Pathway analysis (IPA). The pathway of NF-κB and TGFβ was affected in CD40:Fc treated mice. Green colored shapes indicates upregulated genes, red colored shapes downregulated; gray colored shapes genes in the pathway that were not differentially expressed. Color intensity reflects the degree of differential expression. Triangles represent phosphatases; horizontal diamond, peptidases; vertical diamond, enzymes; horizontal oval, transcription factors; vertical oval, transmembrane receptors; trapezoid, transporters; circle, other type of protein. Dotted lines indicate an indirect interaction; solid line, direct interaction, solid arrow head indicates “acts on”.

The function of many of the most significantly differentially expressed genes are unknown. However, amongst the list of downregulated genes in the CD40:Fc treated group compared with control mice were a few genes of particular interest. Dio1 (downregulated 18.2 times) is the gene for type 1 deiodinase (D1) which provides the major portion of the circulating thyroid hormone T3 in vertebrates. T3 is known to be downregulated under (chronic) inflammation, possibly related to the expression
CHAPTER 6

of pro-inflammatory cytokines such as IL-6 and IL-1\(^{19}\). Another gene found to be downregulated in the CD40:Fc treated mice was Ltf, encoding for lactoferrin (Ltf), a iron-binding glycoprotein which has anti-microbial properties and is involved in inflammation, mainly in innate immunity\(^{20}\). It can activate macrophages and induce IL-6\(^{21}\). Dmbt1 (deleted in malignant brain tumors 1) is downregulated 12.9 fold and is involved in the mucosal immune defense\(^{22}\). The transcription factor GATA4, which is involved in TGF\(\beta\) signaling and is upregulated in patients with inflammatory bowel disease, was also downregulated. Together these results suggest that overexpression of CD40:Fc is able to decrease the SG pro-inflammatory/autoimmune environment associated with the NOD mouse.

Focus scores and composition of leukocyte infiltrates is not affected after CD40:Fc treatment

In order to test if changes in the gene profile in CD40:Fc treated mice had an effect on the SS-like phenotype in the NOD mice, the SG associated FS was quantified in the CD40:Fc treated and control mice. Infiltrates were detected in both the CD40:Fc and LacZ treated groups, however no differences in scores were measured between the two groups (Figure 3). In addition, we quantified a number of different immune cells that

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Table 1. Up or down-regulated genes in CD40:Fc treated NOD mice salivary gland tissue.

| Downregulated | Upregulated |
|---------------|-------------|
| Gene          | Fold change | Gene          | Fold change |
| AK086138      | 24.9        | A430089I19Rik | 8.2         |
| AK052096      | 22.3        | 0610040A22Rik | 5.5         |
| ENSMUST0000103537 | 20.1    |               |             |
| Zfp59         | 20.0        |               |             |
| Dio1          | 18.2        |               |             |
| 2310035C23Rik | 17.8        |               |             |
| BB473918      | 16.4        |               |             |
| AB070552      | 16.0        |               |             |
| Ltf           | 15.7        |               |             |
| Slc38a5       | 13.9        |               |             |
| Dmbt1         | 12.9        |               |             |
| Gata4         | 12.4        |               |             |
| Tmc5          | 12.2        |               |             |
| Xkr8          | 12.1        |               |             |
| Gsta3         | 12.0        |               |             |
| Elavl4        | 11.9        |               |             |
| Nkx2-3        | 11.4        |               |             |
| Kira5         | 10.9        |               |             |
Figure 3. Focus score (FS) of salivary glands (SG) of non-obese diabetic (NOD) mice treated at different ages. FS was determined for each group of CD40:Fc treated mice and compared with control mice (n=8-10 mice per group. Shown is the average FS per group, error bar = standard deviation (SD), i.m. = intramuscular).

normally form the focal infiltrates CD4+ and CD8+ T cells, CD19+ and B220+ B cells, CD11c+ dendritic cells and CD49b+ natural killer (NK) cells or diffusely infiltrate the SG (CD68+ macrophages) and found no statistically significant differences in the presence of these subtypes of cells (shown are B (B220) and T (CD4 and CD8) cells; Figure 4).

Salivary flow does not change after CD40:Fc treatment

One of the clinical hallmarks of SS is the loss of salivary flow. NOD mice show reduced salivary flow rates (SFR) with increasing age; in our facility the SFR starts to be reduced after the age of 16 weeks. This reduction in SFR often coincides with focal inflammation, but focal inflammation and SFR are not directly correlated. To study the effect of CD40:Fc on SFR, we measured the pilocarpine stimulated salivary flow 5 days prior to the end of the study for all the mice treated at the different time intervals. As shown in figure 5, stimulated SFR was unaffected by CD40:Fc treatment when compared with controls.

Immunoglobulin levels were not affected by CD40:Fc treatment

CD40 stimulation leads to isotype switching of B cells and antibody production by plasma cells. Despite the changes in the expression of genes related to the immune response and the known role of CD40 in B cell function, the number of B and plasma cells in the SG was unaffected after treatment. Immunoglobulin (Ig) A, G and M levels were measured in salivary gland homogenates and serum and no change was observed for the different Ig-types in either serum or SG homogenates for CD40:Fc compared to LacZ treated mice (data not shown).
Figure 4. T and B cell presence in SG of 20 week old CD40:Fc or control treated NOD. Salivary gland (SG) tissue was cut in sections and stained with anti-CD4, anti-CD8 and anti-B220 antibodies. Representative photos (group n = 8 mice each) of mice treated at 10 weeks are shown at the end of the study at 20 weeks. B220⁺; B cells, CD4⁺; Th cells and CD8⁺; cytotoxic T cells.

Figure 5. Stimulated salivary flow of CD40:Fc treated and control non-obese diabetic mice. At the indicated age, mice were anesthetized and given pilocarpine to stimulate salivary flow and saliva was collected. Salivary flows corrected for body weight (SFR) of the different treatment groups are shown as a mean + standard deviation (SD; n=8-10 per group, i.m. = intramuscular, n/a = not available).
DISCUSSION

CD40 and CD154 are upregulated in SG of SS patients and in NOD mice developing an SS-like phenotype. Interfering with the CD40-CD154 interaction by monoclonal antibodies, soluble ligands or antisense oligonucleotides has been successfully applied to mouse models of autoimmune disease, such as diabetes, transplant rejection, and EAE, but has not been studied before in animal models of SS. Therefore we tested whether the expression of soluble murine CD40 coupled to an Fc domain of human IgG locally in the SG of NOD mice could affect the SS-like phenotype that develops in these mice. We show that local overexpression of CD40:Fc in the SG of NOD mice led to changes in the expression of genes involved in inflammation and immunity, but this did not result in reduced microscopic inflammation or improved salivary gland function.

We administered CD40:Fc to the SG of NOD mice of different ages/disease stages with the aid of an AAV2 vector. Local delivery of the vector was confirmed by PCR of the SG tissue. We detected the CD40:Fc fusion protein in serum, but not in saliva. This indicates that the CD40:Fc protein was properly processed for secretion via the constitutive secretory pathway of the SG. CD40:Fc was not detected in the SG protein homogenates from the treated mice. This was possibly due to high background and cross-reactivity with endogenous (murine)CD40 and immunoglobulins which may have masked proper detection in our tests. After i.m. administration, the fusion protein could not be detected in serum. This is in agreement with data showing that AAV2 transduced muscle cells are poor producers of protein and supports the use of the SG as a bio-producer of immunomodulatory proteins.

After CD40:Fc treatment, we found a number of differentially expressed SG mRNAs in the CD40:Fc treated mice compared with control (LacZ) treated mice. Analysis identified the gene expression/immune response network as a key network for the differentially expressed genes with NF-κB and TGFβ at the center. NF-κB is a major transcription factor regulating genes responsible for both the innate and adaptive immune response. Upon activation of either the T- or B-cell receptor in combination with CD40, NF-κB becomes activated through specific signaling pathways. TGFβ is involved in this pathway and can regulate NF-κB and CD40 under different circumstances. In addition, microarray analysis revealed that several down-regulated genes in the CD40:Fc treated mice were involved in inflammation. Finding alterations in the NF-κB network and these gene changes supported the notion that CD40:Fc is functional and able to affect the local immunological environment.

In NOD mice, endogenous CD40 expression can be found in SG in and around aggregates which start to form around 10 weeks, but are most prominent from 12 weeks of age onwards. Unlike ICAM-1, which is also involved in the co-stimulatory pathway, CD40 is not expressed prior to the influx of immune cells. Previously we studied the effect of soluble (s)ICAM-1:Fc on inflammation of the SG in NOD. ICAM-1 was found to be expressed in epithelial and endothelial structures before infiltrates formed. We found that treatment with sICAM-1:Fc at 8 weeks, but not at 10 weeks, reduced overall inflammation based on FS. In contrast, late treatment led to a higher number of infiltrated CD4+ and CD8+ T cells. sICAM-1:Fc expression did not prevent the loss of salivary function in either treatment group.
There is a number of possible reasons for the lack of therapeutic effect. First CD40, although present in SG of patients and mice with autoimmune sialadenitis, may not be crucial for immune cell infiltration. ICAM-1 is a molecule involved in co-stimulation but also in cell-adherence and this may explain why sICAM-1:Fc administration resulted in reduced inflammatory foci when applied to NOD mice whereas CD40:Fc did not. Second, the CD40 inflammatory pathway in the SG may be redundant and blocking the interaction of CD40 with its ligand may be overtaken by other co-stimulatory pathways such as the CD80-CD28 interaction\textsuperscript{33}. Combination therapies may therefore prove more successful. This has previously been shown in transplantation rejection experiments in which CD40 was used in combination with CTLA4 blockade. In this study, the combination of blocking both co-stimulatory pathways was 100 times more efficient than blockade of CD40 or CTLA4 alone\textsuperscript{34}. This hypothesis is supported by the observation in our study that local CD40:Fc gene transfer did result in changes in the genes expression profile. Third, we cannot completely exclude the possibility that soluble CD40 may have led to stimulation of CD154 on cells expressing this ligand, counteracting the effect of blockade of the cell-cell interaction between CD40 and CD154. Agonistic monoclonal antibodies to CD40 reduced collagen-induced arthritis, possibly through upregulation of IL10 and to lesser extent IL4\textsuperscript{35}, indicating an anti-inflammatory role in addition to the pro-inflammatory role of CD40. In our study, although the immune response genes were mainly down-regulated, Phospholipase A2, group x (PLA2G10) was higher (4.3 fold) in CD40:Fc treated mice compared to controls. PLA2G10 upregulation was recently described in patients with SS\textsuperscript{36}. Future studies using anergic monoclonal blocking antibodies could address whether this mechanism influences the effectiveness of treatment with CD40:Fc.

In conclusion, despite the rather extensive literature supporting CD40 as a therapeutic target in autoimmune diseases, our study shows no clinical benefit of interfering with the CD40-CD154 interaction by expression of soluble CD40 in the SG in an animal model of SS. We observed changes in the gene profile after treatment, but this did not translate into reduced inflammation or improved SG function. This may suggest that the CD40-CD154 interaction alone is not as important in SS as previously suggested.

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TACI-FC GENE THERAPY IMPROVES AUTOIMMUNE SIALADENITIS BUT NOT SALIVARY GLAND FUNCTION IN NOD MICE

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ABSTRACT

Objective: Sjögren’s syndrome (SS) patients show aberrant expression of the B cell-related mediators B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) in serum and salivary glands (SGs). We studied the biological effect of neutralizing these cytokines by local gene transfer of the common receptor transmembrane activator and CAML interactor (TACI) in an animal model of SS.

Material and Methods: A recombinant serotype 2 adeno-associated virus (rAAV2) encoding TACI-Fc was constructed and its efficacy was tested in the SGs of non-obese diabetic (NOD) mice. Ten weeks later, SG inflammation was evaluated and serum and SG tissue were analyzed for inflammatory markers including immunoglobulins (Ig) and cytokines.

Results: AAV2-TACI-Fc gene therapy significantly reduced the number of inflammatory foci in the SG, due to a decrease in IgD⁺ cells and CD138⁺ cells. Moreover, IgG and IgM levels, but not IgA levels were reduced in the SG. Overall expression of mainly pro-inflammatory cytokines tended to be lower in AAV2-TACI-Fc treated mice. Salivary flow was unaffected.

Conclusion: Although local expression of soluble TACI-Fc reduced inflammation and immunoglobulin levels in the SG, further research will have to prove whether dual blockade of APRIL and BAFF by TACI-Fc can provide a satisfying treatment for the clinical symptoms of patients.
INTRODUCTION

Sjögren’s syndrome (SS) is a common systemic autoimmune disorder characterized by lymphocytic infiltration of the exocrine glands. Patients, mostly females in their 4th and 5th decade, suffer from dry eyes and dry mouth and may also experience systemic symptoms associated with vasculitis, arthralgias and peripheral neuropathy. Current treatment options are very limited and therapeutic approaches effective in other autoimmune diseases such as rheumatoid arthritis (RA) have been largely ineffective in SS patients. Rituximab, a B cell depleting agent, has had modest but promising effects in clinical trials, indicating an important role for B cells in the disease. This role is further supported by the recruitment of activated and memory B cells in salivary gland (SG) infiltrates, the presence of circulating autoantibodies to the nuclear antigens Ro and La, germinal center (GC) formation, and an increased risk for SS patients to develop B cell non-Hodgkin’s lymphoma.

The cytokines B cell activating factor (BAFF or BLyS) and a proliferation-inducing ligand (APRIL), exert different effects, but are both involved in B cell activation and survival. Both cytokines are aberrantly expressed in SS patients, which might explain the activation and survival of pathogenic B cells in this condition. The potential pathogenic role of BAFF in SS is further supported by animal models. BAFF transgenic (Tg) mice develop a systemic lupus erythematosus (SLE)-like disease, a condition with many similarities to SS. These mice show elevated numbers of mature B cells and effector T cells, and high levels of rheumatoid factor, anti-DNA autoantibodies as well as immunoglobulin deposition in the kidneys. In addition, with age these mice show increased inflammation of the SGs and decreased salivary flow, resembling SS in humans. APRIL transgenic mice display a different phenotype and suggest a different and not yet elucidated role for APRIL, compared to BAFF, in the pathogenesis of SS; young APRIL Tg mice have no signs of B cell hyperplasia, but they show enhanced survival of CD4+ T cells without an increase in T cell number, enhanced IL-2 production of CD8+ T cells ex vivo and enhanced T cell dependent IgM and T cell independent IgM and IgG responses. At a later age, these mice develop progressive hyperplasia and prolonged survival of B1 B cells in mesenteric lymph nodes and Peyer’s patches and disorganization of affected lymphoid tissue.

Blockade of APRIL and BAFF by intraperitoneal (ip) injection of soluble human transmembrane activator and CAML interactor (TACI), the common receptor for BAFF and APRIL, coupled to an immunoglobulin heavy chain (TACI-Fc), has been shown to delay disease onset in SLE-prone NZB/W F1 mice. Also, in several SLE-prone mouse strains, a single injection of adenovirus serotype 5 (Ad5) encoding murine TACI-Fc resulted in prolonged survival, depletion of plasma cells, marginal zone (MZ) and follicular B cells, and decreased IgM and IgG serum levels. These findings have led to the development of clinical studies with human TACI-Fc (atacicept) in patients with SLE, multiple sclerosis (MS) and RA. In contrast to RA and SLE, TACI-Fc has never been tested in mice and humans with SS. Therefore, we decided to evaluate the biological effects of adeno-associated virus (AAV)-based transfer of the gene encoding TACI-Fc into the SG in an animal model of SS.
MATERIAL AND METHODS

Construction, expression and biological activity of plasmid

The murine extra-cellular domain (ED) of TACI was synthetically designed using 20 partially overlapping 40-mer oligonucleotides spanning the TACI-ED sequence (Phe5-Thr129) and synthesized using regular polymerase chain reaction (PCR) under general conditions. This gene was cloned into the rAAV plasmid containing a cytomegalovirus (CMV) promoter, the Fc-region of mouse IgG1 and the inverted terminal repeat (ITR) sequences for AAV serotype 2 (AAV2; the shuttle vector is previously described in). The resulting plasmid (pAAV2-CMV-mTACI-mIgG1) was transfected into human embryonic kidney (HEK 293) cells and protein secretion into the supernatant was quantified by performing a regular ELISA for mouse TACI (R&D systems, Minneapolis, MN, USA) and by a modified ELISA in which the capture antibody was directed against TACI (R&D systems) and a biotin-labeled detection antibody was directed against mouse IgG (Bethyl Laboratories, Montgomery, TX, USA). The size of the secreted fusion protein was confirmed by western blotting under reduced conditions using a 10% SDS gel and a labeled (IRDye 800 CW) anti-mouse IgG (Li-Cor, Lincoln, NE, USA).

Vector production

rAAV serotype 2 vectors (rAAV2) were generated as previously described. The titer of DNA physical particles in rAAV stocks was determined by quantitative (q)-PCR using primers for the CMV-promotor region and the vectors were stored at -80°C. On the day of vector administration to non obese diabetic (NOD) mice, the vector was dialyzed for 3 hours (hrs) against saline. Construction and vector production of the control vector rAAV-LacZ encoding β-galactosidase was previously described.

Animals, vector administration and detection

Female NOD mice (Jackson Laboratory, Bar Harbor, ME, USA) were kept under specific pathogen-free conditions in the animal facilities of the National Institute of Dental and Craniofacial Research (NIDCR). Animal protocols were approved by the NIDCR Animal Care and Use Committee and the National Institutes of Health (NIH) Biosafety Committee. Vectors were delivered into the submandibular SG by retrograde instillation as previously described. In short, 10 week old female NOD mice were anesthetized with a mild anesthesia (a combination of ketamine and xylazine) and 50 µl containing $1 \times 10^{11}$ vector particles was administered to each submandibular gland by retrograde ductal instillation using a thin cannula (Intermedic PE10, Clay Adams, Parsippany, NJ, USA). Mice were sacrificed at 20 weeks of age. At time of sacrifice, submandibular SGs were removed and cut in 4 equal parts. One part was homogenized and total genomic DNA was isolated using DNeasy blood & tissue kit (Qiagen, Venlo, the Netherlands). Vector was detected using q-PCR on an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).
Saliva and serum collection and fusion protein detection

Saliva was collected at 20 weeks of age under anesthesia (procedure described above). Saliva secretion was induced by subcutaneous (sc) injection of pilocarpine (0.5 mg/kg BW; Sigma-Aldrich, St. Louis, MO, USA) and stimulated whole saliva was collected for 20 minutes (min) from the oral cavity by gravity with a hematocrit tube (Drummond Scientific Company, Broomall, PA, USA) placed into a pre-weighed 0.5 ml micro centrifuge tube. Saliva volume was determined by weight and expressed as μl/gram body weight*20 min. Blood was collected by heart puncture during sacrifice. Blood was left to clot on ice for 3 hours and centrifuged at 2,500 x g for 25 minutes at 4°C to obtain serum. Murine TACI was measured in both SG protein homogenates and serum using a commercial ELISA-kit (R&D systems).

Histological assessment and immunohistochemistry

One cross-section of the submandibular SG was embedded in paraffin and sections were cut at 5 μm. Three sections were stained with hematoxylin and eosin (H&E) according to standard protocol. Focus score (FS) was determined by averaging the number of aggregates (>50 lymphocytes) per 4 mm² of SG tissue per mouse and were scored blindly by 2 different examiners. Other slides were stained with anti-CD138 (clone 281-2, BD, Breda, the Netherlands), anti-BAFF (clone L17835/b, Alexis Biochemicals, San Diego, CA, USA), and anti-APRIL (Abcam, Cambridge, MA, USA) after heat-induced antigen-retrieval with citrate. Another cross-section of the submandibular SG was collected frozen in OCT compound. Sections (5 μm) were stained with anti-CD4 (clone L3T4, eBioscience, San Diego, CA, USA), anti-CD8 (clone 53-6.7, eBioscience), anti-CD19 (clone 1D3, BD), and anti-IgD (clone 11-26c, eBioscience), followed by goat anti-rat-HRP (Southern Biotechnology, Birmingham, AL) and developed with AEC substrate (Dako, Glostrup, Denmark; Detailed staining procedure as previously described30). Images of the foci in the salivary gland sections were taken, 18 high-power fields (taken with a 40X objective lens) for each cell subset per mouse, and were analyzed using the Qwin analysis system (Leica, Cambridge, UK) as previously described31, evaluating foci and directly surrounding salivary gland tissue in one analysis. Positive staining for the cellular markers was expressed as the number of positive cells/mm² and the staining for the cytokine markers as integrated optical density (IOD)/mm², an arbitrary unit representing the intensity of staining per mm²32.

Protein extraction and determination of immunoglobulin levels

One part of the submandibular SG of each mouse was crushed, placed in 2 mL tubes containing 1 mL HEPES lysis buffer (20 mM HEPES, 0.5 M NaCl, 0.25% Triton X-100 and 1 mM EDTA) and complete protease inhibitor (Roche, Mannheim, Germany), and was lysed shaking at 4°C overnight. The next day, samples were centrifuged at 1,500 x g at 4°C for 10 minutes and supernatant was collected. Total protein was determined with BCA™ protein assay kit (Pierce, Rockford, IL, USA). IgG, IgA and IgM were measured in serum and SG homogenates by commercially available ELISA kits (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer’s protocol. Values were corrected for total protein content for the SG protein homogenates.
Quantification of cytokines

Cytokines in serum and homogenates of SG were measured commercially using a multiplex sandwich-ELISA assay (Aushon Biosystem, Billerica, MA, USA). Values were corrected for total protein content in the SG protein homogenates.

Statistical analysis

Differences in cytokine levels between experimental groups were assessed using the non-parametric Wilcoxon’s rank-sum test or parametric Student’s t-test depending on data distribution. Differences in all the other experiments were assessed using Student’s t-tests. Correlations between vector copy number and FS were assessed using Spearman’s Rho test (non-parametric) and between BAFF and APRIL using Pearson correlation coefficient (parametric). Both were performed with SPSS for Windows (SPSS version 16.0.02, Chicago, IL, USA). All other analyses were performed with GraphPad Prism statistical software (GraphPad Software Inc. version 5.01, La Jolla, CA, USA). A p value ≤ 0.05 was considered to be statistically significant.

RESULTS

**In vitro** AAV2-TACI-Fc transduction leads to expression of full length TACI-Fc fusion protein

A TACI-Fc construct was synthetically designed and cloned into an AAV2 serotype viral vector. We confirmed that the full length fusion protein was expressed and secreted in vitro after transfection of HEK293 cells with the viral construct. Both TACI and the Fc part could be detected in the supernatant after transfection by sandwich ELISA using rat anti-murine TACI or HRP labeled anti-rat antibodies and biotin-labeled goat anti-murine IgG as secondary antibodies (data not shown). In a western blot, secreted TACI-Fc migrated as a monomer with an expected molecular mass of approximately 40 kDa (Figure 1A). The concentration of the secreted protein in supernatant from TACI-Fc expressing cells was measured at 562 ng/ml, which is, based on previous work, within the expected range for in vitro transgene expression.

AAV2-TACI-Fc stably transduces the salivary gland and results in significant soluble receptor expression

The NOD mouse is one of the models used to study a SS-like disease33. Infiltrates, resembling the morphology and composition of infiltrates seen in humans with SS, are detected in the SGs of these mice starting at 8 weeks of age and the number and size of the infiltrates increase thereafter. In addition, this mouse strain is known to spontaneously develop SG dysfunction over time. In our facility, SG inflammation in NOD mice becomes histologically evident at the age of 8 weeks, and progressively increases with age30. In order to evaluate the therapeutic potential of soluble TACI-Fc to treat rather than to prevent the disease, which would be the most relevant for a clinical application in human SS, the vector was delivered to the mice by retrograde instillation of the submandibular SGs after the onset of disease at 10 weeks of age.
Vector delivery was confirmed by q-PCR detection of vector DNA in SG DNA isolated from treated mice at the end of the experiment (data not shown). Local expression of the fusion protein transferred to the SG by AAV2 is expected from 3 days after gene transfer into the SG of NOD mice (based on transduction with AAV-luciferase followed by in vivo imaging of luciferase activity, unpublished observations) and previous work in healthy mice has shown that it can still be detected up to one year after instillation\textsuperscript{34}. We determined TACI levels in protein homogenates from the SGs of treated mice and LacZ-treated mice 10 weeks after injection and found significantly higher TACI levels in TACI-Fc-treated mice compared with control mice (mean ± SD; 369.2 ± 110.7 versus 201.7 ± 172.2 pg/ml; p = 0.03; Figure 1B). Moreover, additional analysis of vector detection in the liver showed no spread to this organ and TACI-Fc protein could not be detected in serum. No effect on the incidence of diabetes was seen when LacZ treated mice were compared with mice that received TACI-Fc (data not shown).

Expression of TACI-Fc dampens salivary gland inflammation in NOD mice and reduces the number of infiltrating mature B and plasma cells
To assess the effect of local expression of TACI-Fc on overall SG inflammation, the FS was determined. Initially, a pilot experiment was set up in which 4 NOD mice were treated with LacZ and 4 mice with TACI-Fc. The results showed a reduction in FS in the TACI-Fc treated mice (data not shown). To confirm this, and to analyze the effect of the treatment on the cells within the infiltrates on more detail, we set up a second experiment with 9 mice per treatment group. In this group we confirmed that local SG treatment with TACI-Fc reduced FS compared with controls (mean ± SD; 2.63 ± 0.57 versus 3.79 ± 0.78; p <0.005; Figure 2A). The decreased FS correlated with the detected number of vector copies (r = -0.833 and p = 0.01, data not shown).
Focal infiltrates were characterized by quantitative immunohistochemical analysis. CD4+ and CD8+ T cell population numbers were similar for TACI-Fc and LacZ groups (p = 0.94 and p = 0.54 for CD4+ and CD8+ respectively; Figure 2B). CD19+ B cell numbers tended to be lower in the TACI-Fc-treated mice, nearly reaching significance (p = 0.06; Figure 2C). Moreover, non-switched mature B cells (IgD+, p = 0.02) and plasma cells (CD138+, p = 0.03; Figure 2C and D) were significantly decreased in TACI-Fc-treated SGs. BAFF and APRIL expression was found in ductal epithelial cells and infiltrating foci (Figure 3A and B). BAFF expression in the SGs of TACI-Fc-treated mice was significantly increased compared with LacZ-treated mice (p <0.005), APRIL expression in TACI-Fc-treated mice was unaltered compared to LacZ control mice (p = 0.18; Figure 3A and B).

TACI-Fc decreases IgG and IgM, but does not change IgA levels in the salivary gland

BAFF and APRIL stimulate B cells to produce immunoglobulins and, in autoimmune diseases, to produce autoantibodies9-10. Therefore, we investigated whether TACI-Fc affects immunoglobulin levels. Treatment with TACI-Fc resulted in significantly lower SG concentrations of IgG (1.45 ± 0.54 μg/ml) and IgM (0.32 ± 0.19 μg/ml) compared...
with SG levels of IgG (3.05 ± 1.80 μg/ml) and IgM (0.54 ± 0.21 μg/ml) in LacZ treated mice (p = 0.03 and p = 0.05 respectively), while IgA concentrations did not change (7.62 ± 5.00 versus 6.57 ± 2.43 μg/ml respectively; p = 0.60; Figure 4A). In serum, no significant difference in immunoglobulin levels was found between the TACI-Fc treated and the control groups (Figure 4B). The reduced IgG levels after treatment of SGs with TACI-Fc resulted in an overall increased IgA/IgG ratio for treated mice compared with controls (Figure 4C).

Lower IL-2 levels in TACI-Fc-treated salivary glands

BAFF and APRIL have effects on both B and T cells, and might influence the cytokine balance. To investigate if local expression of TACI-Fc in the SG could change cytokine levels either systemically in the serum or locally in the SGs, serum samples and SG protein extracts were obtained from mice at time of sacrifice and cytokine levels were measured by multiplex analysis. TACI-Fc-treated SGs showed decreased levels of IL-2 (p = 0.05), a cytokine which modulates activated T cells and immunoglobulin synthesis. Moreover, levels of other cytokines (IL-1β, IL-4, IL-6, IL-10 and IL-17) were suppressed after TACI-Fc delivery, although this did not reach statistical significance. No differences were detected for IFNγ and IL-12p40 (Figure 5). Serum levels of cytokines were unaffected (data not shown).

Stimulated salivary flow is unchanged after treatment

To investigate if TACI-Fc mediated reduction in SG inflammation affected SG function, pilocarpine-stimulated salivary flow was measured at 20 weeks. Healthy mice and adult NOD mice of 10 weeks of age have a salivary flow of 4-5 ul/gram body weight*20 minutes. We found a decline in salivary flow in both the control group and the TACI-Fc treated mice at 20 weeks of age compared to 10 week old mice (2.18 ± 0.67 and 1.74 ± 0.71 versus 4.3 ± 1.4 μl/gram bodyweight*20 minutes respectively). The SFR of 20 week old treated mice did not differ from untreated controls (Figure 6).

DISCUSSION

SS patients have elevated serum levels of BAFF and APRIL, which may be involved in pathogenic B cell differentiation, survival and an increased risk of the development of lymphoma associated with the disease. There is no universally effective therapy, however some patients have benefited from the B cell depleting agent rituximab, validating the B cell compartment as a therapeutic target. Besides B cell depletion, another B cell-directed therapeutic approach is the neutralization of BAFF and APRIL by expression of soluble TACI, a common receptor. In this study, we are the first to evaluate the effects of soluble TACI in an animal model of SS. We show that expression of soluble TACI in the SG of NOD mice, which spontaneously develop a SS-like syndrome, reduces autoimmune SG inflammation via reduction of B and plasma cells, and immunoglobulins.

Expression of soluble TACI-Fc locally in the salivary glands of NOD mice resulted in a two-fold increase in detectable TACI in the SG. Additionally, we detected a significant
Figure 3. Increased BAFF detection in TACI-Fc-treated mice. SGs (N = 10 for LacZ and N = 9 for TACI-Fc) were stained and quantified for BAFF (A) and APRIL (B) expression. For each isotype and staining a representative picture, taken with a 40X objective lens. Arrows indicate a positive staining of ductal epithelial cells. Data shown are the mean +/- SD integrated optical density (IOD) per mm² tissue. The p-values were determined by Student's t-tests and significant differences are indicated (*).
increase in BAFF levels, while APRIL levels were unaffected. We did not measure free BAFF separate from soluble receptor-bound BAFF levels, but we attribute this increase to cytokine-soluble receptor binding and stabilization. This phenomenon, in which the therapeutic drug leads to a seemingly paradoxical increase of the targeted cytokine, has been described previously in a clinical trial with atacicept in RA patients. Herein, the atacicept-BlyS complex has been shown to accumulate in the high dose atacicept group. Additionally, in clinical trials with soluble TNF-receptors, the amount of circulating TNF in patients was seen to be increased up to 7-fold. Most of this TNF lacked TNF bioactivity.

Treatment with TACI-Fc lead to a decreased number of SG infiltrates in our initial pilot study, which was confirmed in our follow-up study. Analysis of the infiltrates showed that T cell numbers were not affected, but overall B cell numbers tended to be lower and treated mice had significantly reduced numbers of IgD⁺ non-switched mature B cells and plasma cells. This decrease in B cells in the SG can be explained by a shortened survival of B cells infiltrated in the gland or, since BAFF is also known to play a role in chemotaxis, reduced B cell recruitment to the SG due to the decreased availability of (free) BAFF. Since it is not known whether plasma cell differentiation takes place in the SG, the observed decrease of plasma cells within the SGs, can not be directly explained. It could be the direct result of a reduction in the recruitment of peripheral plasma cells into the SG, for instance by decreased bioavailability of APRIL after binding to TACI. Alternatively, since BAFF levels correlate with ectopic GC formation, a pathological feature commonly found in the SG of SS patients.

**Figure 4. Decreased salivary gland IgG and IgM after TACI-Fc delivery.** SG homogenates (N = 7 for LacZ and N = 8 for TACI-Fc) (A) and serum (N = 6 for LacZ and N = 9 for TACI-Fc) (B) from 20-week-old mice were analyzed for IgG, IgA and IgM. Data shown are mean +/- SD, the P-values were determined by Student’s t-tests and significant differences are indicated (*). Additional analysis showing the IgA:IgG ratio in the SGs (C).
Figure 5. Lower IL-2 levels in the salivary glands of TACI-Fc treated mice. Data shown are the mean values from replicate assays (pg/ml) (N = 9 for LacZ and N = 8 for TACI-Fc) after correction for total protein content. The p-value was determined by the Student’s t-test or the non-parametric Wilcoxon’s rank-sum test depending on data distribution for each cytokine and significant differences are indicated (*).
and NOD mice\textsuperscript{41}, the decrease could be the result of altered ectopic GC formation or interactions within these GCs in the SG resulting in decreased B cell to plasma cell switching. Future research will have to elucidate the exact mechanism. Taken together, these data suggest that expression of soluble TACI in the SG of NOD mice reduces the number of B cells, possibly by inhibiting proliferation and differentiation while T cell numbers were not affected.

The reduction in plasma cells was accompanied by decreased levels of IgG and IgM, but not IgA, in the SGs. These findings are supported by prior studies utilizing systemic administration of soluble murine and human TACI to murine models of SLE, which showed decreased B cells and plasma cells in the spleen as well as decreased serum levels of IgM and IgG\textsuperscript{20-21,42}. IgA is known to have an important role in mucosal immunity and the majority of plasma cells in the mucosal immune system of the SG express IgA. A lower percentage of IgA and a higher percentage of IgG-producing plasma cells resulting in a decreased IgA/IgG ratio has been found in the SGs of patients with SS\textsuperscript{43}. In our study, expression of soluble TACI in the SGs led to a statistically significant decrease in IgG levels and therefore an improved IgA/IgG ratio when compared with the control group, suggesting restoration of the mucosal immune balance. The APRIL-TACI axis is thought to be critical in the class switching to IgA, but not IgG and IgM\textsuperscript{44-45}. Soluble TACI binds to both APRIL and BAFF with varying affinities depending on dimerization/heteromerization of the ligands\textsuperscript{46-47}. Since soluble TACI affected IgM and IgG but not IgA, it can be speculated that soluble TACI affects BAFF more than APRIL, possibly due to a difference in binding affinity for the soluble receptor. This is supported by the significant increase in BAFF, but not APRIL, protein levels in treated SGs, suggesting that more BAFF is stably bound by soluble TACI compared with APRIL.

We detected an overall mild reduction in pro-inflammatory cytokines with IL-2 reaching statistical significance (p = 0.05). IL-2, a classical Th1 cytokine, is produced by activated T cells and leads to both activation of T cells and B cell differentiation. Besides B cell activation and stimulation, BAFF and APRIL were also found to induce IL-2 secretion by T cells\textsuperscript{17,48}. We did not analyze T cell activation specifically, but the

Figure 6. Salivary flow rates are unaffected by TACI-Fc treatment. Pilocarpine stimulated salivary flow was measured for 20 minutes for TACI-Fc and LacZ control treated mice at 20 weeks of age. Both groups showed equal reduction in SFR when compared to the 20 week old NOD mice that were not treated when compared with 10 week old mice. (n = 10 for NOD at 10 and 20 weeks, n = 9 for LacZ and n = 8 for TACI-Fc). Data shown is corrected for body weight (gram) and bars represent mean + SD.
decrease in IL-2 in the SGs of treated mice in our study implies inhibition of T cell activation state and cytokine secretion without a change in T cell numbers.

Although SG inflammation was reduced, stimulated salivary flow was not increased after treatment. This causes some major concerns for the use of TACI-Fc in a clinical setting, since dryness is one of the most debilitating symptoms of SS. The lack of effect on the salivary flow may be the result of the following. At first, it may be due to insufficient inflammatory suppression; although treated mice showed a reduced FS, there were still inflammatory foci present in the SG after 10 weeks of treatment. Second, the timing of our treatment may be a reason for lack of physiological improvement. A decline in salivary flow is usually not detected until 16-20 weeks of age; however, at the initiation of treatment, NOD mice already had evidence of inflammation and the processes leading to SG dysfunction may already have been initiated and the pathology involved may be beyond repair. Third, the lack of improvement in the SG function may also be due to unchanged levels of IFN\(\gamma\) and IL-12 in the SGs. In a previous publication our group has shown physiological and immunological effects of IFN\(\gamma\) on human salivary gland (HSG) cells\(^{49}\). Moreover, we have shown that overexpression of IL-12 in mice leads to salivary gland dysfunction\(^{50}\) suggesting important roles for IFN\(\gamma\) and IL-12 in the pathogenesis and function of the SGs. One last reason for the lack of efficacy of TACI-Fc could be related to the role of APRIL. Little is known about the role of APRIL in SGs of normal individuals and SS patients. And in contrast to the related autoimmune diseases RA and SLE (reviewed in\(^{51}\)) APRIL levels in SG of SS patients are detected in lower or similar instead of higher levels compared to healthy controls (J.L. Vosters et al, unpublished observations and\(^{52}\)). In addition, APRIL levels have been shown to inversely correlated with disease activity\(^{53-54}\) and BAFF levels\(^{54}\) in SLE. This raises the question whether the role of APRIL is solely pathogenic and, as a consequence, whether blocking APRIL is desirable in the treatment of SS. Our study does not prove that blocking APRIL prevented restoration of salivary flow, however future studies will have to show an effect on dryness before considering this therapy for humans with SS.

Patients commonly have autoantibodies to the nuclear antigens Ro and La and this may be related to the level of BAFF and APRIL\(^{11}\). We did not study these antibodies in the treated NOD mice, since the penetrance of this feature is highly variable in NOD mice. Previously, we (unpublished observations) and others\(^{55}\) found no or only low serum levels, making this mouse model unfit for the study of these antibodies.

In conclusion, local delivery of soluble TACI-Fc to the SGs of NOD mice leads to beneficial effects on autoimmune SG inflammation at both cellular and immunoglobulin levels, but treatment did not lead to an improvement in salivary flow. BAFF and/or APRIL blockade may prove useful in the treatment of autoimmune-inflammation of the SG, but future studies will have to show an effect on dryness before considering this therapy for humans with SS.
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THE EXPRESSION OF APRIL IN SJÖGREN’S SYNDROME: ABERRANT EXPRESSION OF APRIL IN THE SALIVARY GLAND

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ABSTRACT

Objective: A proliferation inducing ligand (APRIL) and B cell-activating factor (BAFF) are B cell-related mediators and may play a role in the pathogenesis in Sjögren’s syndrome. In this descriptive study we assessed the expression of APRIL and BAFF in the minor salivary gland and serum from Sjögren’s syndrome (SS) patients.

Methods: Paraffin-embedded minor salivary gland sections from SS, sicca controls and healthy volunteers were analyzed by immunohistochemistry. Digital image quantification was performed to evaluate the expression of BAFF, APRIL, and their receptors. Furthermore, serum was analyzed for soluble BAFF and APRIL levels by enzyme-linked immunosorbent assay. All the data were also separated based on decreased and normal stimulated flow and analyzed independent of their classification.

Results: APRIL expression was lower in minor salivary gland biopsies from SS patients compared with healthy volunteers and to a lesser extend sicca controls, whereas BAFF expression was similar in both groups. Soluble APRIL levels in serum were increased in autoantibody positive SS patients and in subjects with decreased salivary flow independent of the classification.

Conclusions: APRIL salivary gland tissue levels are decreased, suggesting that targeting this cytokine locally in the salivary glands would not benefit SS patients. Moreover, the discrepancy between local and systemic levels is striking and future research should assess this in more detail.
INTRODUCTION

Sjögren’s syndrome (SS) is a systemic autoimmune disorder characterized by lymphocytic infiltration in the exocrine glands and the recruitment of activated and memory B cells in the salivary gland infiltrates\(^1\). A proliferation inducing-ligand (APRIL) and B cell-activating factor of the tumor necrosis factor family (BAFF) have powerful roles in B cell biology. They are involved in augmentation of B cell antigen presentation, co-stimulation of B cell activation, enhancement of B cell survival, regulation of B cell tolerance and germinal center maintenance\(^2\). These ligands share two tumor necrosis factor (TNF) family member receptors, transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA). In addition, BAFF binds BAFF-receptor (BAFF-R) and APRIL binds heparin sulphate proteoglycans (HSPGs). Unlike BAFF, APRIL does not exist in a membrane-bound form, although a cell surface fusion protein with TWEAK is described as TWE-PRIL\(^3,4\).

Recent studies have localized APRIL to immune cell subsets that also produce BAFF; monocytes, macrophages, dendritic cells, and T cells. Moreover, stimulation of these cells with interferon (IFN)\(\gamma\) and IFN\(\alpha\), results in upregulation of APRIL and BAFF mRNAs\(^5,6\). Additionally, non-immune cells can express APRIL, including osteoclasts\(^7\) and tumor tissues\(^8\). BAFF is also expressed by cells outside the immune system, such as fibroblast-like synoviocytes in the synovium of patients with rheumatoid arthritis (RA)\(^9\) and salivary gland epithelial cells in both patients with SS and healthy individuals\(^10\). IFN\(\alpha\) and IFN\(\gamma\) induce BAFF mRNA and protein secretion in salivary gland epithelial cells and the human salivary gland cell line, with a higher increase in salivary gland epithelial cells derived from SS patients compared with controls. BAFF induction is independent of interleukin-10 (IL-10) and TNF alone\(^11\). This has never been described for APRIL.

In SS, increased BAFF has been reported in serum and salivary glands, and correlates with disease parameters\(^12-15\). Similarly, increased serum APRIL levels correlate with focus score and serum IgG\(^14\). Recently, APRIL production in the salivary gland was found to be low in SS patients\(^16\). In this study, sicca controls were not investigated and no associations with other systemic and local symptoms were made. The reduced or absent expression of APRIL in the salivary glands from SS patients is surprising since it is thought to enhance autoimmune disease by sustained B cell activation\(^17\). Other data suggesting a complicated role for APRIL in autoimmune diseases include the description of an inverse association between circulating APRIL levels and BAFF levels and APRIL levels and other disease parameters in systemic lupus erythematosus (SLE) patients\(^18,19\).

Although the expression level of APRIL in SS compared to sicca controls, which did not fulfill the European-American (EA) criteria for primary SS (pSS), and healthy volunteers and the correlation with systemic features or salivary flow is largely unknown, APRIL has been brought up as a therapeutic target for SS\(^20\). Therefore, we studied the expression of APRIL, BAFF and their receptors in minor salivary gland biopsies and APRIL and BAFF in serum from SS patients, sicca controls and healthy volunteers and correlated these levels with the salivary flow.
MATERIAL AND METHODS

Patients and controls

All 17 patients included in this study fulfilled the European-American (EA) criteria\textsuperscript{21} for primary SS (pSS) and were recruited from the Sjögren's syndrome clinic at the National Institutes of Dental and Cranial Research (NIDCR), National Institutes of Health (NIH), Bethesda, MD, USA. The control group consisted of 5 gender and age-matched healthy volunteers (HV) and 9 gender and age-matched patients evaluated for sicca symptoms not meeting the European-American (EA) criteria for pSS (N; Table 1). All subjects signed an informed consent and the study was approved by the Institutional Review Board (IRB) of the National Institutes of Dental and Craniofacial Research (NIDCR).

Laboratory assays

Data on serum autoantibodies, immunoglobulins (IgG, IgM, IgA), erythrocyte sedimentation rate (ESR), minor salivary gland focus score, and stimulated salivary flow were obtained by NIDCR’s Sjögren’s clinic staff and evaluated as part of the routine diagnostic evaluation for SS. A focus score is defined as a number lymphocytic foci, which are adjacent and normal-appearing mucus acini and contain more than 50 lymphocytes, per 4mm\textsuperscript{2} of glandular tissue\textsuperscript{21}. BAFF and APRIL levels in serum were determined using a commercial ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Before BAFF detection, serum samples were pre-treated with protein-A sepharose (Sigma-Aldrich, St. Louis, MO, USA) to prevent possible interference with rheumatoid factor immunoglobulin.

Immunohistochemistry and digital quantification

Paraffin sections of minor salivary gland biopsies were stained after heat-induced citrate antigen retrieval with the following antibodies: mouse anti-human APRIL (Aprily-2, Alexis Biochemicals, San Diego, CA, USA), rabbit anti-human BCMA (ProSci Incorporation, Poway, CA, USA), mouse anti-human TACI (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human BAFF-R (Alexis Biochemicals), and rabbit anti-Perlecan (H-300, Santa Cruz Biotechnology). For each staining, isotype controls were included; mouse IgG1 (Dako, Carpinteria, CA, USA), rat IgM (BD biosciences, San Jose, CA, USA), and rabbit IgG (R&D systems). The secondary antibodies goat anti-mouse HRP (Dako), goat anti-rat HRP (Southern Biotechnology, Birmingham, AL, USA), and goat anti-rabbit HRP (Dako) were used. Staining was developed with AEC substrate (Vector Laboratories, Burlingame, CA, USA). All sections were randomly analyzed by computer-assisted image analysis using 400x magnification. The images of the high-power fields were analyzed both within and outside infiltrates, using the Qwin analysis system (Leica, Cambridge, UK), as described previously\textsuperscript{22}. Positive staining for the cellular markers was expressed as the number of positive cells/mm\textsuperscript{2} and the staining for the cytokine markers as integrated optical density (IOD)/mm\textsuperscript{2}.

Statistical analysis

Differences in immunohistochemistry, serum APRIL and serum BAFF between experimental groups (>2 groups) were assessed using the Kruskal-Wallis test followed
Table 1. Clinical, laboratory and histological characteristics of study subjects.

| Characteristics            | HV (n=5) | N (n=9) | SS (n=17) |
|---------------------------|----------|---------|-----------|
| Age (yr), mean (sd)       | 53.0 (12.0) | 46.8 (12.2) | 53.4 (15.0) |
| Female, n (%)             | 5/5 (100.0) | 9/9 (100.0) | 16/17 (94.1) |
| ANA positive, n (%)       | 1/5 (20.0) | 3/9 (33.3) | 13/16 (82.3) |
| ENA positive, n (%)       | 0/5 (0.0) | 0/9 (0.0) | 11/16 (68.8) |
| SSA/Ro positive, n (%)    | 0/5 (0.0) | 0/9 (0.0) | 8/17 (47.1) |
| SSB/La positive, n (%)    | 0/5 (0.0) | 0/9 (0.0) | 10/17 (58.8) |
| SSA+SSB positive, n (%)   | 0/5 (0.0) | 0/9 (0.0) | 8/17 (47.1) |
| ESR, mean (sd)            | 17 (11) | 29 (27) | |
| IgG (mg/dL), mean (sd)    | 971 (188) | 1447 (669) | |
| IgA (mg/dL), mean (sd)    | 165 (99) | 331 (155) | |
| IgM (mg/dL), mean (sd)    | 108 (41) | 156 (90) | |
| Treatment                |          |         |           |
| Hydroxychloroquine, n (%)| 4/9 (44.4) | 2/17 (11.8) | |
| Dose                     | 1/17: 200 mg/day |          |          |
|                          | 3/9: 400 mg/day |          |          |
|                          | 1/9: 600 mg/day | 1/17: 600 mg/day | |
| Oral steroids, n (%)     | 2/9 (22.2) | 2/17 (11.8) | |
| Dose                     | -         | 1/17: 5 mg/day |          |
|                          | 1/9: 10 mg/day |          |          |
|                          | 1/9: 40 mg/day |          |          |
|                          | -         | 1/17: 60 mg/day |          |
| Methotrexate, n (%)      | 1/9 (11.1) | 0/17 (0.0) | |
| Dose                     | 10 mg/week |          |          |
| MALT lymphoma, n (%)     | 0/5 (0.0) | 0/9 (0.0) | 1/15 (6.7) |
| Focus score, mean (sd)   | 1.00 (0.45) | 0.44 (0.88) | 4.24 (3.47) |
| EGM, n (%)               | 0/5 (0.0) | 0/9 (0.0) | 5/17 (29.4) |
| Vasculitis               | 1/17 (5.9) |          |          |
| Peripheral neuropathy    | 2/17 (11.8) |          |          |
| Arthritis                | 2/17 (11.8) |          |          |

ANA: antinuclear antibodies; ENA: antibodies to extractable nuclear antigens; EGM: extra glandular manifestation; ESR: erythrocyte sedimentation rate; SSA: antibodies to Ro antigens; SSB: antibodies to La antigens; SS: patients fulfilling the European-American (EA) criteria for primary Sjögren’s syndrome (pSS); HV: healthy volunteers (n=5); N: patients evaluated for sicca symptoms not meeting the EA criteria for pSS (n=9).
by the non-parametric Mann Whitney test. The p-values were not adjusted for multiple comparisons. All analyses were performed with GraphPad Prism statistical software (GraphPad Software Inc. version 5.01, La Jolla, CA, USA) using a p-value ≤ 0.05 as statistically significant.

RESULTS
Decreased APRIL and TACI expression in the SGs from SS patients
Minor salivary gland sections from healthy volunteers (HV), sicca controls (N) and SS patients were analyzed using immunohistochemistry and quantitative digital image analysis to locate and quantify the expression of APRIL, BAFF and their receptors, TACI and BCMA, respectively. In each subject from each group, eighteen high-power fields with foci and eighteen high-power fields without foci were analyzed and averaged to randomize the expression of the markers for localization within the salivary gland. APRIL, BAFF, TACI and BCMA were expressed in the salivary duct cells of each group (Figure 1 shows representative pictures). In SS patients, BAFF and BCMA showed a different

Figure 1. APRIL, BAFF, TACI and BCMA expression in the minor salivary glands. Immunohistochemical analysis of APRIL, BAFF, TACI and BCMA expression (red staining) in minor salivary gland biopsies from healthy volunteers (HV), sicca controls (N) and SS patients. For each antibody staining (in red) the isotype control and representative examples are shown. Original magnification: 400x. For reference, arrows indicate ductal epithelium, triangles indicate acinar cells and asterisks indicate focal infiltrates.
staining pattern when compared with APRIL and TACI, with more pronounced BAFF and BCMA staining in ductal cells and inflammatory foci (Figure 1). Quantification of the immunohistochemical staining revealed decreased APRIL expression in the minor salivary glands of SS patients compared with healthy volunteers (18123 IOD/mm² and 41283 IOD/mm² respectively, p = 0.02), and the expression in sicca patients was similar to those with healthy volunteers (p = 0.52; Figure 2A). In contrast, BAFF expression was similar between both SS (320655 IOD/mm²) and healthy volunteers (411685 IOD/mm², p = 0.35), and SS and sicca controls (269056 IOD/mm², p = 0.36; Figure 2A). TACI expression, like APRIL, was decreased in SS compared with healthy volunteers (16.0 counts/mm² and 158.0 counts/mm² respectively, p = 0.02), but not in sicca controls (29.0 counts/mm², p = 0.55). Although numerically more individuals with sicca complaints had lower TACI levels compared with healthy volunteers, no statistical significance was reached (p = 0.08; Figure 2B). The other shared receptor for

Figure 1

Figure 2. Decreased APRIL and TACI expression is correlated in ductal cells from SS minor salivary glands. Quantification of APRIL, BAFF (A), TACI, and BCMA (B) expression using digital image analysis. Soluble cytokines (APRIL and BAFF) are expressed as integrated optical density (IOD)/mm² and receptors (TACI and BCMA) are given as positive cell count/mm². The horizontal bar is the median value and the p-values were determined by the Kruskal-Wallis test followed by the non-parametric Mann Whitney test and significant differences were indicated (*). HV = healthy volunteers, N = sicca controls, SS = Sjögren’s syndrome patients.
APRIL and BAFF, BCMA, was not differentially expressed in SS patients compared with healthy volunteers ($p = 0.48$). Interestingly, the expression of BCMA was significantly different for SS compared with sicca controls (2035 counts/mm$^2$ and 813 counts/mm$^2$ respectively, $p = 0.02$; Figure 2B). Heparan sulfate proteoglycans (HSPG, Perlecan) on the cell surface have recently been described as interactors with APRIL$^{23}$, but staining for these molecules did not show any difference for the 3 groups (data not shown).

Increased APRIL serum levels in anti-Ro and -La positive SS patients

APRIL and BAFF are elevated in serum in other autoimmune diseases, e.g. RA and SLE$^{24}$. Therefore, we measured APRIL and BAFF levels in the serum from SS patients. SS patients showed higher systemic levels of APRIL compared with healthy volunteers (3.95 ng/ml versus 1.83 ng/ml; $p = 0.02$) and sicca controls (2.16 ng/ml; $p = 0.03$; Figure 3A). In 41% (7 out of 17) of the SS patients, APRIL was detectable above baseline levels, and sub-analysis of the SS group showed that 71% (5 out of 7) of the patients with positive autoantibodies against Ro/SSA and La/SSB had high (> 20 ng/ml) APRIL levels (Figure 3B). APRIL expression was not different between SS patients with and without extra glandular manifestations (EGM, see table 1). Serum BAFF levels in SS patients

![Figure 3](image_url)

**Figure 3. Increased APRIL serum levels in anti-SSA/SSB positive SS patients.** Serum from healthy volunteers (HV), sicca controls (N), and Sjögren’s syndrome patients (SS) were analyzed for APRIL (A) and BAFF (C) levels using ELISA. In the SS group, the serum APRIL levels were subdivided for the presence of autoantibodies (B) against SSA and SSB. The horizontal bar is the median value and the p-values were determined by the Kruskal-Wallis test followed by the non-parametric Mann Whitney test and significant differences were indicated (*). Data on the y-axis for figure B is plotted in log-scale. SSA = Sjögren’s syndrome A/Ro, SSB = Sjögren’s syndrome B/La.
(759 pg/ml) were not significantly different when compared with healthy volunteers (743 pg/ml, p = 0.59), or with sicca controls (545 pg/ml, p = 0.12; Figure 3C).

Patients with decreased salivary flow have increased serum APRIL and TACI expression in the salivary glands

One of the hallmarks of SS is decreased salivary flow. Therefore, data from all the markers in the salivary gland and serum were analyzed for decreased and normal stimulated salivary flow, using 1.5ml/15min as a cutoff, independent of the classification. Serum APRIL and salivary gland TACI levels were significantly increased in subjects with decreased salivary flow (Figure 4 A and B respectively, both p = 0.02). For all the other markers, no significant changes were found.

DISCUSSION

APRIL has been shown to be up-regulated in RA and SLE\textsuperscript{24}. Recently, APRIL expression in the salivary gland of SS patients was found to be low compared with healthy controls. In that study, sicca controls were not tested and APRIL levels were not correlated with systemic features or salivary flow\textsuperscript{16}. It is known that epithelial salivary gland cells of SS patients play a role in the presentation of antigens\textsuperscript{25}, and are potent producers and secretors of BAFF\textsuperscript{11}. In contrast, the expression level and role of APRIL in epithelial cells is less well known. Therefore, we have setup a descriptive study for the APRIL expression in minor salivary gland biopsies and serum of SS patients, sicca controls and healthy volunteers. In addition, we assessed the expression of the receptors for BAFF and APRIL. We found by immunohistochemical analysis of minor salivary gland
tissue that APRIL was expressed in the ductal epithelium of all individuals. Interestingly, minor salivary gland biopsies of SS patients showed decreased APRIL expression compared with control groups. Systemically, APRIL levels were high in 41% of the patients and all these patients were double positive for Ro and La autoantibodies. Furthermore, APRIL serum levels correlated with reduced salivary flow independent of the classification criteria.

In contrast to BAFF expression, minor salivary gland biopsies showed APRIL expression mainly in the ductal epithelial cells, and not in the inflammatory foci. Moreover, the overall APRIL expression was decreased in SS patients compared with healthy volunteers. Previously, gene expression profiling of minor salivary glands by microarray, showed lower APRIL expression in SS patients, but this change was not significant. Our data are in line with a recent report, showing no up-regulation of APRIL in Sjögren’s sialadenitis lesions. In contrast to the expression in salivary glands, APRIL levels in serum were elevated in SS patients, especially in anti-Ro/La positive patients. The discrepancy between serum and salivary gland levels of APRIL raises the question where APRIL is produced. Additional research will have to answer this question, but based on our data and on a previous study, it is unlikely that this comes from the SG, since immunohistochemistry showed that APRIL is primarily present inside the epithelial cells and not in the inflammatory foci.

Interestingly, serum APRIL levels and TACI expression in the salivary glands were also increased in subjects with decreased salivary flow, independent from the diagnosis of SS. The relationship of systemic APRIL and salivary gland dysfunction is puzzling and more research on the function of APRIL in SS needs to be done to elucidate any possible connection. One may postulate that the association with TACI and salivary gland dysfunction is based on increased BAFF binding to TACI in an inflammatory environment in SS that results in internalization of the receptor or may mask the receptor from antibody binding in IHC. Further studies are needed to also evaluate this relationship.

We showed the presence of the receptors TACI and BCMA in the salivary glands of SS patients, sicca controls, and healthy volunteers. Staining of these receptors was primarily seen in epithelial duct cells. In addition, BCMA was also detected in the infiltrating cells. Although these receptors are known for their expression on lymphocytes, BAFF/APRIL receptors are also detected on non-immune cells. Expression of BAFF-R and TACI, for instance, has been observed in mammary epithelial cells and normal thyroid gland. Adipocytes also express BAFF and APRIL, together with their receptors, and are thought to act in an auto/paracrine way for regulation and differentiation of these cells. It is possible that a similar regulatory mechanism takes places in the salivary gland epithelial cells. Further research is necessary to confirm this finding. TACI expression in minor salivary gland biopsies from SS patients was decreased compared with healthy volunteers. In contrast, BCMA expression was up-regulated in SS minor salivary gland compared with sicca controls, but showed no difference when compared with healthy volunteers. Previously, microarray studies showed increased BCMA gene expression in SS minor salivary glands compared with
sicca controls. The different expression pattern suggests a different function for these receptors in the salivary gland.

Previous studies have shown overexpression of BAFF in lymphocytes in minor salivary glands from SS patients and equal expression in epithelial cells compared with healthy volunteers. Our data showed expression of BAFF in lymphocytic infiltrates in SS as well as duct epithelial cells of both healthy volunteers and SS patients. However, we did not detect an overall difference in the expression of BAFF in SS minor salivary glands compared with our control group. It is possible that the total BAFF expression did not change, when infiltrates and epithelium were assessed. In addition, the similar levels may be due to intrinsic limitations of immunohistochemistry, which is a very informative method to detect overall expression levels in tissue, but does not distinguish the specific cell types expressing the protein of interest. In addition, it may be that BAFF is only transiently expressed in patients. This is supported by a previous study showing that mRNA levels of BAFF are similar in SS and control salivary epithelial cells. Only ex vivo stimulation with TNFα and IFNγ resulted in increased BAFF mRNA expression in SS patients compared with sicca controls.

We showed no difference in serum BAFF levels between SS patients and controls. Literature shows conflicting results on the BAFF levels in sera from SS patients; some find higher levels in patients, while others do not detect differences. A reason for these opposing results may be that BAFF fluctuates with changes in disease activity. Technical limitations of the chosen assay, interference with rheumatoid factor and the stability of the BAFF protein may also explain the differences. Recently, a new ELISA has been developed for detection of both glycosylated and nonglycosylated BAFF, which should not be affected by rheumatoid factor. This ELISA may lead to more consistent results.

In conclusion, APRIL levels were low in the minor salivary glands of SS patients whereas high serum levels were found compared to sicca controls and healthy volunteers. This suggests that compared to BAFF, APRIL may have a less pronounced pro-inflammatory role in minor salivary gland pathogenesis in SS patients. Moreover, it is tempting to suggest that APRIL has anti-inflammatory effects in the minor salivary glands, and that SS patients may therefore benefit more from BAFF blockade alone, instead of targeting both APRIL and BAFF, but more research needs to explore this hypothesis.

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SUMMARY AND DISCUSSION

Sjögren’s syndrome

Sjögren’s syndrome (SS) is a common autoimmune disease. It affects many organs in the body, including and foremost the secreting glands such as the lachrymal and salivary glands. The diagnosis is based on subjective symptoms and objective measurements of the function of the lachrymal and salivary glands and the presence of signs of inflammation. The disease is nine times more common in women than in men and manifests itself mainly in the menopausal age. Patients suffer from (often severe) dry eyes and dry mouth leading to increased risk of infections, dyscomfort and pain in these areas. Furthermore, patients may also have signs of vascular inflammation (vasculitis), joint pain and can experience extreme fatigue. The cause of SS, as in many autoimmune diseases, remains unknown. There is currently no universally effective therapy and patients have to rely on symptomatic treatment. One of the main reasons for the lack of a good remedy is the limited existing knowledge on the pathogenesis and disease progression.

Inflammation in SS; a disordered system

One of the hallmarks of SS is inflammation of the salivary gland characterized by the influx of immune cells and their clustered organization. The recruitment into the salivary gland and the development, survival and function of these immune cells is in part controlled and influenced by cytokines, protein messengers that can inhibit, stimulate and induce other cytokines and that can pass signals to other cells. In the salivary gland, blood and saliva of patients with SS, differences in the presence of these cytokines can be detected when compared with healthy volunteers. Moreover, specific differences can be found with people who have a dry mouth and eyes, but not SS (sicca patients). A number of therapies based on normalizing the cytokine balance –such as anti-TNF therapy, of which some work well in other related autoimmune diseases such as rheumatoid arthritis– were not effective in the treatment of SS. Chapter 2 in this thesis describes the dysregulated cytokine network and the cytokine-based therapeutic studies in humans that mostly failed.

Gene therapy in SS

The reason why the described cytokine targeted therapies do not work is only partially understood. It is possible that the drug did not penetrate the most affected organs and/or prolonged therapy may be needed to elicit a substantial beneficial effect. Gene therapy, in particular local gene therapy in the salivary gland, could overcome these problems. Through the introduction of certain genes in the salivary gland, proteins can be produced and secreted by the salivary gland cells which can then influence the local inflammatory environment. So-called adeno-associated viral viruses (AAV’s) can infect epithelial cells of the salivary gland; recombinant AAVs can not multiply and are not pathogenic. These viruses can be made to carry genes that encode for proteins that can affect the inflammatory milieu. When introduced into the salivary gland, local, long-term and stable production of these therapeutic proteins can be achieved.
Chapter 3 discusses in detail the possibilities and limitations of gene therapy in SS and proposes a number of candidate targets that can be addressed. These targets include cytokines, adhesion molecules, and the co-stimulatory pathway.

Identifying targets for the treatment of SS; studies in mice

Some of the targets proposed in chapter 3 were studied in the non-obese diabetic (NOD) mouse. The NOD mouse spontaneously develops a large number of immune-mediated disorders in life, of which diabetes is most well known. Besides diabetes it also develops a syndrome that resembles human SS; with age these mice develop inflammation of the salivary and lachrymal glands. In addition, salivary flow may decrease with age, predominantly in female mice. Chapter 4 describes a number of observations in this spontaneous course of the disease in the NOD. We investigated in detail the inflammation of the salivary gland and found that macrophages are infiltrated in the gland before other immune cells enter and possibly, in cooperation with the epithelial cells, produce cytokines that attract other inflammatory cells. In addition, we also found that the adhesion molecule intercellular adhesion molecule type 1 (ICAM-1) is strongly present before infiltration of cells. ICAM-1 aids immune cells to exit the bloodstream and is involved in co-stimulatory pathways, crucial for reciprocal activation of B and T cells. CD40, also involved in co-stimulatory pathways was found to be upregulated in focal infiltrates (foci) but was not detected prior to the formation of foci. The growth factor B cell activating factor, BAFF, which is important for the development and survival of subsets of B lymphocytes, is strikingly present in the epithelial cells of the ductal structures of the salivary gland. In late stage disease, BAFF production significantly increases in these epithelial cells suggesting an important role for this growth factor at the more advanced phase of inflammation. These data show that autoimmune inflammation in the NOD mice is a multi-step process directed by many different players at different stages of the disease. Understanding these stages and the individual key players important in each of these stages is crucial for the discovery of an effective therapy in mice and may guide us in the development of a future therapy in humans.

Based on the immunological aberrations found in the salivary glands of NOD mice, gene therapeutical approaches aimed at these abnormalities were tested and described in chapter 5, 6 and 7. AAVs were used to deliver specific inhibitors/modulators to the salivary glands and their effect on inflammation and salivary gland function was determined. In chapter 5, an AAV was constructed encoding soluble ICAM-1 (sICAM-1). Expression of the soluble molecule competes with endogenous cell bound ICAM-1 for binding with its ligands and should therefore inhibit cell-adhesion and co-stimulation. When sICAM-1 was administered before focal inflammation was present in the salivary gland, less inflammation was observed 12 weeks later. However, if the AAV vector was given when focal inflammation was present, sICAM-1 did not positively affect the disease and the number of T cells in the salivary gland was increased. This indicates that early intervention with the ICAM-1-ligand binding can positively affect disease outcome, but late treatment may worsen the disease and increase inflammatory T cells in the glands. In chapter 6, CD40, also involved in the co-stimulatory pathway,
was expressed in a soluble form at different disease stages. Although the treatment resulted in changes in mRNA profiles related to inflammation, no effect was seen on focal inflammation or on salivary gland function. This study suggests that CD40 is not a good target in the treatment of SS. In chapter 7 gene therapy with an AAV-vector encoding for the transmembrane and CAML interactor receptor (TACI) of BAFF and a proliferation inducing ligand (APRIL), two cytokines involved in B cell stimulation and survival, is described. The soluble form of this receptor should scavenge these cytokines and prevent it from binding to the endogenous receptor on cells. This therapy reduced inflammation in the salivary gland based on B cell and immunoglobulin (type IgG) reduction. This data suggests that the use of soluble TACI may be effective in the treatment for SS. Taken together, these three studies indicate that timing and choosing the correct target are of considerable importance in the treatment of SS-like disease in NOD mice. This will likely also be the case in humans.

Novel therapeutic targets in patients with SS

In chapter 8 we made the transition from mice to humans. The salivary glands and peripheral blood of patients with SS, those with complaints of dry mouth and eyes but without SS (sicca patients) and healthy individuals were studied. We showed how they differ in the presence and distribution of two key inflammatory related cytokines, BAFF, previously also described in the NOD mouse, and APRIL. We found that APRIL is expressed at lower levels in the salivary glands of SS patients compared with healthy volunteers and sicca patients. At the same time some patients had strongly increased APRIL levels in the peripheral blood. BAFF was detected in the ductal cells and in infiltrating cells which confirms previous observations by other researchers. The receptors to which these two cytokines bind were also present in varying degrees when compared between patients and healthy people. These abnormalities indicate that a therapy inhibiting BAFF in the salivary gland may be beneficial, but local inhibition of APRIL may not be effective in the treatment of SS.

Future directions and considerations

Our proof-of-principle studies show that gene therapy can be effective for the treatment of the inflammatory component of SS in NOD mice. However, none of the gene therapy studies in NOD mice increased salivary flow. This is a major concern, since dryness is an important and very debilitating component of SS. Therefore, the quest for an ideal target in the treatment of SS has not ended with this thesis. It is possible that a combination of therapies could lead to better results including improved salivary gland function. In addition, other approaches besides the use of a soluble receptor needs to be explored. The silencing of genes in the cell resulting in decreased production of proteins such as receptors, cytokines and chemokines should also be further investigated. In addition the overexpression of cytokines that are naturally low in SS and have anti-inflammatory properties should also be explored in the future. The application of AAV-vectors is not limited to the expression of soluble receptors (as in this study) and could be used for these purposes. In parallel, more research is needed in patients with SS aimed at the identification of novel targets, and to provide more insight into the natural progression
of the disease. Attempts should be made to identify different natural disease stages, since certain therapies may be beneficial at an early phase but could be detrimental at a more progressed disease stage.

Conclusion
The research presented herein summarizes the current knowledge on cytokines and their presence in SS, it identifies immunological targets in mice with an SS-like disease and shows that local gene therapy can be successful for the treatment of the inflammatory salivary gland component of a SS-like disorder in mice. The chance for success of this approach depends on proper timing and should be aimed at the right target.
Sjögren’s syndrome

Patiënten die lijden aan het syndroom van Sjögren (SjS) hebben vaak last van een zeer droge mond en droge ogen. Hiervan ondervinden ze ongemak en pijn, maar ook bestaat er een verhoogde kans op infecties in deze gebieden. Deze veel voorkomende autoimmuunziekte kan het hele lichaam aandoen, maar uit zich in het bijzonder in ontstekingen in de secernerende klieren zoals de traan- en speekselklieren. Verder komen er bij deze patiënten onder andere ook vaatontstekingen (vasculitiden), gewrichtspijnen en extreme vermoeidheid voor. De diagnose wordt gesteld op basis van subjectieve klachten en objectieve metingen van de werking van de traan en speekselklier en op basis tekenen van ontsteking. De ziekte komt 9 keer meer voor bij vrouwen dan bij mannen en openbaart zich voornamelijk in de leeftijd rond de menopauze. De directe oorzaak van SjS is, zoals bij zoveel auto-immuun ziekten, tot op heden onbekend. Er is op dit moment geen afdoende behandeling voor SjS en patiënten moeten zich overwegend behelpen met symptoombestrijdende middelen. Een van de belangrijkste redenen voor het gebrek aan goede geneesmiddelen is de beperkte kennis die we hebben over de ontstaansgeschiedenis en het natuurlijke beloop van deze ziekte.

Ontsteking in SjS; een ontregeld systeem

De ontsteking in de speekselklier bij SjS wordt gekenmerkt door de het migreren van ontstekingscellen vanuit het perifere bloed naar de speekselklieren. Dit proces, als ook de overleving en de functie van deze cellen in het ontstoken weefsel, worden deels gestuurd en bijgevolg door cytokines, eiwitten die als boodschapper remmende of stimulerende signalen door kunnen geven aan andere cellen en de productie van andere cytokines kunnen beïnvloeden. In de speekselklier, het bloed en het speeksel van patiënten met SjS, worden verschillen in de aanwezigheid van deze cytokines waargenomen in vergelijking met gezonde vrijwilligers. Daarnaast kunnen er ook verschillen gevonden worden met mensen die wel klachten hebben van droge mond en ogen, maar geen SjS hebben. Dit geeft aan dat SjS een afzonderlijke ziekte is, én meer is dan het hebben van een droge mond. Een aantal therapieën gebaseerd op het normaliseren van de cytokinebalans -en waarvan sommige, zoals anti tumor necrosis factor (TNF) therapie, goed werken in andere auto-immuun ziekten zoals reumatoïde artritis- bleken geen goed effect te hebben op SjS. Hoofdstuk 2 beschrijft het ontregelde cytokinenetwerk en de hierop gebaseerde grotendeels falende therapeutische studies in mensen.

Gen-therapeutische opties voor SjS

De reden waarom de beschreven cytokine-gerichte therapieën niet werken is deels onbegrepen. Het is mogelijk dat het medicijn niet goed vanuit de bloedbaan in de meest aangedane organen doordringt of dat bijvoorbeeld langdurige therapie nodig is om een effect te kunnen verkrijgen. Gentherapie, en in het bijzonder lokale gentherapie van de speekselklier, kan gebruikt worden om deze problemen te
omzeilen. Door het inbrengen van bepaalde genen in cellen van de speekselklier kan deze klier zelf eiwitten gaan produceren waarvoor deze genen coderen. Deze eiwitten kunnen vervolgens een effect uitoefenen op het lokale ontstekingsmilieu. Als er wordt gekozen voor een adeno-associated virus (AAV) als vector die het DNA in de cel aflevert, kan dit leiden tot langdurige, locale en stabiele productie van therapeutische eiwitten. AAV's zijn virussen die zodanig zijn aangepast dat ze zich niet kunnen vermenigvuldigen en geen ziekmakend effect hebben. In hoofdstuk 3 wordt er uitgebreid ingegaan op de mogelijkheden en onmogelijkheden van gen therapie in SjS en wordt een aantal kandidaat-genen voorgesteld.

Doelwitten in de behandeling van SjS; studies in muizen
Om deze genen verpakt in AAV's te kunnen testen op hun werkzaamheid, is er een model nodig waarin deze genen kunnen worden uitgetest. In het SjS onderzoek wordt al jaren gebruik gemaakt van muismodellen die (al dan niet spontaan) een SjS-achtige ziekte ontwikkelen. In dit proefschrift wordt gebruik gemaakt van de non-obese diabetic (NOD) muis. Deze muis ontwikkelt spontaan een groot aantal immuun-gemediaerde afwijkingen, waarvan suikerziekte de bekendste is. Naast diabetes ontwikkelt de NOD muis ook een syndroom wat aan SjS in patiënten doet denken; met het ouder worden vertonen deze muizen ontstekingsverschijnselen in de speeksel- en traanklieren. Onder de microscoop lijken de cellen en de manier waarop de cellen georganiseerd zijn sterk op de ontstekingsinfiltraten bij SjS. Voor de vrouwelijke muizen geldt bovendien dat de speekselvloed vaak afneemt met de leeftijd. Hoofdstuk 4 beschrijft een aantal observaties bij het bestuderen van het spontane ziektebeloop bij deze muizen om zo meer inzicht te krijgen processen en eiwitten die belangrijk zijn voor het ontstaan van de ziekte. Hierbij viel het op dat bepaalde ontstekingscellen, macrofagen, al in een vroeg stadium in de klier infiltreren; mogelijk produceren deze in interactie met epitheliale cellen (cellen die het kanalenstelsel van de speekselklier vormen) cytokines die andere ontstekingscellen aantrekken. Ook vonden we dat een bepaald adhesiemolecuul, intercellular adhesion molecule type 1 (ICAM-1), dat een rol speelt in het migreren van ontstekingscellen vanuit het perifere bloed naar het ontstoken weefsel, al voor deze immuuncellen binnendringen in verhoogde mate aanwezig is in de speekselklier. Een cytokine dat een rol speelt de ontwikkeling en overleving van B lymfocyten, B cell activating factor (BAFF), was ook in een vroeg stadium van de ziekte en in verhoogde mate aanwezig in de epitheliale cellen. Gedurende de latere fase van de ziekte is er een verdere toename van BAFF. Om de hypothese te testen dat een therapie gericht op het herstellen van de afwijkingen gevonden in de NOD muis het SjS-achtige ziektebeeld zou kunnen verbeteren, hebben we een aantal genen gericht op de afwijkingen die we vonden met behulp van AAV's in de speekselklier tot expressie gebracht.

Hoofdstuk 5 beschrijft een onderzoek waarbij gebruik werd gemaakt van een AAV construct dat codeert voor het adhesiemolecuul ICAM-1. Dit is een celgebonden molecuul dat ervoor zorgt dat ontstekingscellen uit de bloedbaan kunnen treden en het speelt een rol in de activatie van witte bloedcellen (zogenaamde co-stimulering). In de NOD muis was ICAM-1 al vroeg in het ontstekingsproces aanwezig. Doordat
expressie van de oplosbare vorm van ICAM-1 (sICAM-1) voor wat betreft de binding aan liganden concureert met het van nature aanwezige ICAM-1 zal het cel-adhesie en co-stimulerende signalering kunnen remmen. Het bleek dat wanneer sICAM-1 werd toegediend vóórdat focale ontsteking aanwezig was, er 12 weken later minder ontsteking in de speekselklier werd gezien. Echter, wanneer de AAV-vector werd gegeven op het moment dat ontsteking al duidelijk aanwezig was, was sICAM-1 niet van positieve invloed op de ziekte en nam het aantal T-cellen in de speekselklier zelfs toe. Dit geeft aan dat therapie gericht op ICAM-1 in een vroeg stadium van de ziekte mogelijk een positief effect kan hebben. Later in de ziekte is het minder effectief en kan het zelfs in verergering van de ontsteking resulteren. In het in hoofdstuk 6 beschreven onderzoek werden genen die coderen voor de oplosbare vorm van CD40, dat ook betrokken is in co-stimulerende mechanismen, met behulp van een AAV-vector lokaal toegediend in verschillende fasen van de ziekte. Hoewel de behandeling resulteerde in veranderingen in expressieprofielen van genen die een rol spelen in ontstekingsprocessen, werd er geen effect gezien op de uiteindelijke ontsteking of op de speekselklier functie. Op grond hiervan concluderen wij dat CD40 waarschijnlijk geen goed aangrijpingspunt is voor de behandeling van patiënten met SjS.

In hoofdstuk 7 richt zich op locale gentherapie met een AAV-vector die codeert voor een receptor; de transmembraan-en CAML interactor receptor (TACI). Deze receptor bindt normaal aan BAFF en APRIL, twee cytokinen die betrokken zijn stimulatie en overleving van B cellen. De oplosbare vorm van deze receptor bindt deze cytokines en voorkomt zo dat BAFF en a proliferation inducing ligand (APRIL) aan de natuurlijk voorkomende receptor op de cellen bindt. Hierdoor kunnen de cytokines hun inflammatoire effect niet uitoefenen. Deze therapie leidde tot verminderde ontsteking in de speekselklier met name door een afname van B-cellen en immunoglobulines (type IgG), geproduceerd door B cellen en plasma cellen. De onderzoeken beschreven in bovengenoemde hoofdstukken laten zien dat het cruciaal is niet alleen het juiste therapeutische ‘target’ te kiezen, maar ook de timing in het ziekteverloop is van belang voor het uiteindelijke effect.

Op zoek naar nieuwe therapeutische doelwitten in patiënten met SjS

Hoofdstuk 8 ten slotte beschrijft de overstap van muis naar mens. We bestudeerden de speekselklier en het bloed van patiënten met SjS, mensen met klachten van droge mond en ogen maar die geen SjS hebben (sicca patiënten) en gezonde vrijwilligers. We zochten naar verschillen in de aanwezigheid van twee belangrijke ontstekingsgerelateerde cytokines BAFF en APRIL, eerder beschreven en als therapeutisch doelwit gebruikt in onze experimentele therapie in de NOD muis. We vonden dat APRIL minder tot expressie komt in de speekselklier van SjS patiënten in vergelijking met gezonde vrijwilligers en sicca patiënten terwijl sommige patiënten sterk verhoogde APRIL waarden hadden in het bloed. BAFF werd zowel in de ductale cellen als in de infiltrerende cellen van patiënten gevonden. Deze observatie bevestigde wat eerder ook door andere onderzoekers was waargenomen. Ook receptoren, zoals TACI, waaraan deze twee cytokines kunnen binden kwamen in verschillende mate voor in patiënten in vergelijking met gezonde mensen. Deze bevindingen geven aan
dat lokale therapie gericht tegen BAFF mogelijk zin heeft in patiënten met SjS, maar dat APRIL remming vermoedelijk niet leidt tot een therapeutisch effect.

Conclusies en verder onderzoek
Het hier gepresenteerde onderzoek vat de stand van zaken samen op het gebied van de rol van cytokines in de pathogenese van SjS. Verder hebben wij locale gentherapie gebruikt in het kader van experimenteel ‘proof of principle’ onderzoek in een diermodel voor SjS om meer inzicht te krijgen in de waarde van mogelijke therapeutische ‘targets’. Hieruit blijkt dat timing in het ziektebeloop belangrijk is, waarbij wij een beter therapeutisch effect vonden als de behandeling in een zeer vroeg stadium van de ziekte werd gestart. Verder ondersteunt dit onderzoek therapeutische interventies die gericht zijn op het interfereren met de humorale respons voor de behandeling van SjS.
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Jay, my co-promotor, I showed up in your lab in 2005, with hardly any knowledge on gene therapy or Sjögren’s and I left feeling an expert (whether that is justified or not…). Thank you for letting me find my way in this research project and for supporting me during this, sometimes bumpy, process. I appreciate the fact that you were/are always approachable and that you almost always instantly found time to help, advise and correct. Gabor, thank you for sharing your knowledge on cytokines, for your valuable comments on the various study designs, your new ideas and for teaching me how to be a better writer.

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Kathrina, dear friend in- and outside the lab. It was so nice to have someone to talk (whine!) to, whether it was about the future, research or no research, career, life and love. I miss you and think you should move to Europe so we can see each other more often! Hongen, I admire your determination and your incredible strength in combining a demanding research career with the care for your daughter. I am sure she will grow up being a strong woman. Thanks for assisting me with those aggressive NOD mice. Giovanni, Hiroshi, Sandra, Beverly, Melodie, thank you all for the fun lab parties, the all-you-can-eat sushi lunches and all the help in the lab when I did not know what to do or how to do it. Marsha, you were always super fast whenever I needed papers, signatures and confirmations. You sure have a talent for organization! Ana, thanks for being there when I needed help and for the BBQ’s you always managed to organize somehow despite your busy life. Milton Papa, Jamie Schetrompf and the other animal technicians; you are all invaluable. I am very grateful for your work with the animals.

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er een mooi artikel uitgesleept. Dank je voor je hulp en vriendschap! Frieda en Caroline, ik bewonder jullie daadkracht, enthousiasme en ontzettende volwassenheid. Jullie en alle andere Arthrogenners, Margriet, Niels, Ciska, Karin, Jan, Lisette, Gerdien, Ingrid, Scott, Corinne, Willem en Marilyn; dank dat ik, toch een beetje een buitenstaander, een heel jaar deel van jullie team uit mocht maken. Ik mis de borrels!

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nu niet meer mijn ‘kleine’ broertje, letterlijk en figuurlijk, misschien moet ik er vanaf nu maar ‘bro’ van maken. Ik ben ontzettend trots op je en bof maar met zo’n ‘bro’ als jij!

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CURRICULUM VITAE

De auteur van dit proefschrift kwam op 9 januari 1976 ter wereld in Groningen. Het opleidingstraject van Nienke Roescher begon op 4 jarige leeftijd op de Brandaris in Groningen en leidde via de Scheepskameel en de Meergronden in Almere tot een VWO diploma in 1994. Na een zeer leerzaam jaar reizend door Australië werd begonnen met de opleiding medische biologie aan de Universiteit van Amsterdam. Het daaropvolgende jaar kwam daar de studie geneeskunde bij. Via twee immunologisch georiënteerde stages aan het Nederlands Kanker Instituut in Amsterdam en aan de Universiteit van Florence en het Karolinska Institutet in Stockholm behaalde zij haar doctoraal geneeskunde en medische biologie in 2002. Na ruim twee jaar co-schappen, inclusief een oudste co-schap van 6 maanden in Kenia, werd in 2004 het artsexamen behaald.

Om klinische ervaring op te doen, werkte Nienke een jaar in het Antoni van Leeuwenhoekziekenhuis als arts-assistent. De wetenschap trok en eind 2005 begon ze aan een promotieonderzoek onder leiding van professor Paul Peter Tak. De eerste 4 en een half jaar van dit onderzoek werd uitgevoerd aan het National Institutes of Health in de Verenigde Staten onder directe begeleiding van dr. Jay Chiorini en dr. Gabor Illei. Het laatste jaar werd het onderzoek afgerond in het Academisch Medisch Centrum aan de Universiteit van Amsterdam. Op 10 februari 2012 om 12 uur wordt het proefschrift dat voortkwam uit dit onderzoek verdedigd te Amsterdam. Nienke is momenteel in opleiding tot arts-medisch microbioloog in het Academisch Medisch Centrum in Amsterdam.
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