Interferon Type I Driven Immune Activation in Generalized Autoimmune Diseases

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The studies described in this thesis were performed at the Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands.

The studies were financially supported by the Netherlands Organization for Scientific Research (NWO) Mosaic grant and the Dutch Arthritis Foundation.

The printing of the thesis was supported by J.E. Jurriaanse Stichting, Nationale Vereniging Sjögrenpatiënten and the Dutch Arthritis Foundation

ISBN: 978-90-5335-713-2

Illustrations: Zana Brkić, Sandra de Bruin-Versteeg, Odilia Corneth
Cover: Shilpi Ahmed-van der Pool
Lay-out: Simone Vinke, Ridderprint B.V., Ridderkerk, the Netherlands
Printing: Ridderprint B.V., Ridderkerk, the Netherlands

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Interferon type I gemedieerde immuun activatie in gegeneraliseerde auto-immuunziekten

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties
De openbare verdediging zal plaatsvinden op
donderdag 19 september
om 11.30 uur

door

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geboren te Livno, Bosnië en Herzegovina

ERASMUS UNIVERSITEIT ROTTERDAM

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Voor mijn ouders

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CHAPTER 1

GENERAL INTRODUCTION

This thesis describes research performed on several generalized autoimmune diseases with the main focus on primary Sjögren's syndrome. Interferon type I has been implicated in the pathogenesis of these diseases and will be introduced in this chapter together with other important immune factors in these diseases such as BAFF and Th17 cells. In this chapter first a general introduction about the immune system will be given, followed by introduction about Interferon type I, BAFF, Th17, Sjögren's syndrome and finally the other two studied autoimmune diseases.

1.1 THE IMMUNE SYSTEM

1.1.1 Innate immune system

Humans live in a microbial world composed of various danger signals. The community of microbes includes both pathogenic and nonpathogenic commensal organisms, which the host must tolerate in order to support normal tissue and organ function. Next to microbes, our environment contains a spectrum of possibly harmful toxic or allergenic substances. The appearance of the first multicellular organisms, has probably led to the need for a robust innate immune system. Innate immunity is present in all multicellular organisms. The innate immune system reacts immediately, however in a stereotype non-specific kind of way. The innate immunity includes the barriers of the body consisting of the epithelial surfaces of the skin, gut and respiratory tract. These barriers can produce antimicrobial peptides, small chains of amino acids that are very potent at inhibiting many bacteria and fungi. Next to these barriers, the innate immune system is composed of different cell types which are activated upon identification of the microbes by pattern recognition receptors (PRRs) such as the Toll like receptor (TLR). Components of the complement system can also tag microbes for destruction by the cells of the innate system. Cell types involved in the innate immune system are granulocytes, natural killer (NK) cells, monocytes, macrophages and dendritic cells (DC). Peripheral blood monocytes are thought to be the precursors for macrophages and DCs in the peripheral tissues. However studies from our group indicate a contribution of local tissue precursors to the pancreatic DCs and macrophage populations as well [1, 2].

1.1.2 Adaptive immune system

The adaptive immune system, that is only present in mammals, changes with repeated exposure to the antigen and results in long-lasting antigen specific immunological memory. Key-players of the adaptive immune system are T cells and B cells. During adaptive immunity a virtually unlimited spectrum of antigen specific receptors is randomly formed on the surface of T cells (TCR) and B cells (BCR). Two major types of T cells are the CD4+ T helper (Th) cells and the cytotoxic CD8+ cells. Th cells recognize antigens bound to major histocompatibility complex class II (MHC class II) molecules and can be subdivided in Th1, Th2 and the recently

discovered Th17 subtypes. CD8+ T cells recognize antigens bound to MHC class I molecules and are important in killing infected or damaged cells. Since TCRs and BCRs are randomly formed, a significant part of these receptors recognizes harmless antigens from its own body, inducing an autoimmune response. Another T cell type, is the regulatory T cell (Treg). This CD4+FoxP3+ cell maintains tolerance to self-antigens, preventing autoimmune reactions. A primary function of B cells is to produce antibodies (immunoglobulins). After activation of naive B cells by specific antigens, the B cells develop into long living memory B cells and antibody secreting plasma cells. B cells can also use complement to tag pathogens for destruction and they can serve as antigen presenting cells (APC).

1.2 INTERFERON TYPE I

The family of interferons (IFN) comprises type I interferons, the type II interferon IFNγ and the recently described type III interferons, called IFNλ [3, 4]. The type I interferons were originally defined in 1957 by their capacity to interfere with viral replication [5]. This viral interference was the reason that the name 'interferon' was chosen [6]. IFN type I includes in humans more than 13 different members of IFNα as well as IFNβ, IFNε, IFNκ and IFNω [7]. Since this large group of structurally similar cytokines signal through the same receptor, the IFN type I receptor (IFNAR), these cytokines are characterized by the term IFN type I. IFNAR is a heterodimeric receptor consisting of 2 membrane spanning polypeptide chains, IFNAR1 and IFNAR2 [8].

In response to viral infection, all nucleated cells have the ability to produce relatively small amounts of IFN type I, but the "professional" IFN type I producing cell is the plasmacytoid DC (pDC). Althoug pDCs constitute only 0.2%-0.8% of peripheral blood cells, these cells secrete up to 1.000-fold more IFN type I compared to other cell types [9]. pDCs are round cells with a prominent rough endoplasmatic reticulum (ER) resembling that of immunoglobulin secreting plasma cells and are therefore called plasmacytoid [10]. pDCs are characterized by the presence of 2 dendritic markers termed blood dendritic cell antigens (BDCA 2 and 4) and the receptor FcγRII, which contributes to the internalization of immune complexes [11].

IFN type I production is induced by viruses, bacteria, or microbial nucleic acids when sensed by the PRRs, such as TLRs, RIG-I like receptors (RLRs) and NOD-like receptors (NLRs) [12]. Both TLR7 and TLR9 are expressed at the endosomal membranes of pDCs and can therefore become activated by pathogens invading the pDC through receptor-mediated endocytosis [6]. DNA/RNA containing immunecomplexes (ICs) can also get endocytosed via FcγRIIa, leading to activation of the endosomal TLR7 and 9. The first step in the production of IFN type I involves the association of MyD88 with TRAF6 and IRAK 1 and 4 (Figure 1, middle panel). Upon this complex formation, IFN regulatory factor (IRF)-3, 5 and 7 are activated, form homodimers or heterodimers and translocate to the nucleus where they

bind to regulatory elements in the promoter region of IFN type I genes and subsequently trigger transcription of these genes [13]. The third endosomal TLR is TLR3. TLR3 is expressed by DC, but not by pDC or monocytes [14]. TLR3 is the only TLR that does not signal via the MyD88. The signal from TLR3 is transduced by TIR containing adaptor molecule (TRIF) which activates the transcription factors IRF3 and IRF7 triggering IFN type I production [15].

In contrast to the pDC, the majority of cell types expresses the cytoplasmic RNA helicases belonging to the RLR family – RIG-1, MDA5 and LGP2. When a virus enters directly into the cytosol of the cell, RIG-1 and MDA-5 recognize the RNA which further interacts with the MAVS adaptor protein (Figure 1 left panel). MAVS assembles with a signaling complex including TRAF3, TBK1 and IKKE. Finally NF-KB is activated together with IRF3/IRF7 resulting in IFN type I production [15].

TLR4 can also initiate IFN type I production after binding of LPS. This happens in a TRIF dependent way involving IRF3 [16]. Interestingly, the constitutive expression of IRF7 and IRF5 in the pDCs makes a rapid onset of IFN type I production possible [15]. The expression of IRF7 and 5 is further enhanced by stimulation with IFN type I, the so called priming effect. In other cells without constitutive IRF5 or IRF7 expression, a signal through IRF3 phosphorylation is required to activate IFN type I expression and thereby upregulates the level of IRF7 and primes the cell to IFN type I production [9].

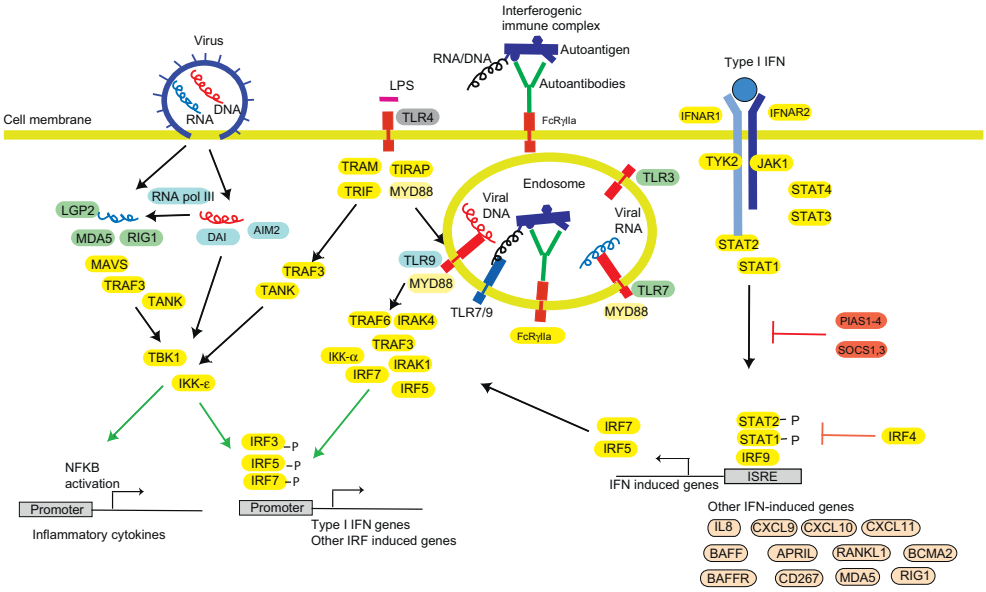

Figure 1. Adapted from Rönnblom L. et al. (2011)[15]. **Signaling pathways of the IFN type I system**. On the left the induction of IFN type I in response to viral RNA/DNA through PRRs (MDA5, RIG-1, DAI, AIM2) is depicted. In the middle IFN type I production by interferogenic ICs is depicted and by LPS binding on TLR4. On the right the IFN signaling via the IFNAR is depicted.

Binding of IFN type I to the IFNAR results in activation of Tyk2 and Jak1. These activated kinases recruit and phosphorylate the transcription factors STAT1 and STAT2, which translocate to the nucleus where STAT1:STAT1 homodimers bind to gene promoters and activate transcription [17] (Figure 1 right panel). In contrast, STAT1:STAT2 heterodimers associate with IRF9 forming a complex that binds to IFN-stimulated response elements (ISRE) and activates the transcription of hundreds of IFN type I induced genes (IFIGs). The exact function of many of these IFN type I inducible genes is far from clear. However, it is known that IFN type I induces and activates enzymes such as MxA (Myxovirus-resistance protein 1), which is a key mediator of the IFN-induced antiviral response [18-20].

Because of the anti-viral capacities, IFN type I has been widely used for the treatment of hepatitis B and C virus infection [21]. Next to these anti-viral functions, IFN type I inhibits tumor growth by suppressing proliferation and inducing cell apoptosis, IFN type I inhibits angiogenesis and activates cytotoxicity against tumor cells [21]. For these reasons, IFN type I has been used in the treatment of malignancies such as melanoma, leukemia and Kaposi's sarcoma [21, 22]. However, early on it was noted that upon IFN type I treatment, higher autoantibody prevalence occurred as well as the development of autoimmune diseases [23, 24].

In recent years, an increasing number of immunomodulatory effects of IFN type I has been reported including: activation of immature dendritic cells through upregulation of MHC class I, chemokines and costimulatory molecules; B cell activation and immunoglobulin (Ig) class switching through induction of BAFF and APRIL or enhancement of B cell receptor-mediated responses; stimulation of Fas ligand expression on NK cells and target cell apoptosis; enhancement of T cell proliferation and survival, skewing of the immune response to the Th1 type and triggering CD8+ memory T cell activation. Finally, IFN type I induces a wide array of chemokines including CXCL9, CXCL10, CXCL11, that are potent chemoattractants for CXCR3 expressing pDC and lymphocytes [10]. IFN type I can also induce increased expression of autoantigens, such as Ro52 [25, 26] and promote the release of autoantigens by induction of apoptosis [27].

1.3 BAFF

B cell activities are orchestrated by members of the tumor-necrosis factor (TNF) family [28]. This large group consists of three members: the TNF-like weak inducer of apoptosis (TWEAK), a proliferation-inducing ligand (APRIL) and the B cell activating factor of the TNF family (BAFF) [29]. BAFF and APRIL share the transmembrane activator and calcium modulator ligand interactor (TACI) and the B cell maturation antigen receptor (BCMA). Only BAFF binds to the BAFF receptor (BAFF-R) [30] (Figure 2).

BAFF is a type II transmembrane protein, which is mainly expressed on the cell surface of monocytes, macrophages, neutrophils and activated T cells [31-34]. Alternatively, BAFF

is proteolytically processed by proteases and found in the soluble form. Other cell types also express BAFF: stromal cells from the bone marrow [35], follicular dendritic cells [36], synoviocytes [37], astrocytes [38] and epithelial cells of the salivary glands in Sjögren's syndrome [39]. Next to membrane or soluble forms, BAFF offers a cluster of variants: glycosylated or non-glycosylated forms, monomer or trimers, homotrimers or heterotrimers, heterotrimers with APRIL or heterotrimers with BAFF variants, or even virus-like aggregates of 60 monomers [29, 40, 41].

BAFF has a major role in B cell differentiation/maturation and survival of peripheral B cells and emerges as a very important factor that allows B cells to mature and remain as peripheral B cells (Figure 2). Pathological excess of BAFF rescues autoreactive B cells from peripheral deletion and allows them to home into microenvironments where chances of inappropriate activation are greater [42, 43]. Excessive BAFF production leads to the survival of low affinity and self reactive B cells resulting in a decrease in B cell tolerance [40].

In 2003 an alternative splice isoform of BAFF was identified, ΔBAFF [44]. ΔBAFF suppresses BAFF function by competitive co-association. Studies in the ΔBAFF transgenic mouse showed that ΔBAFF and BAFF have opposing effects on B cell survival [45]. In humans, although the ΔBAFF transcript was found is some tissues, the corresponding protein has not been detected yet [38].

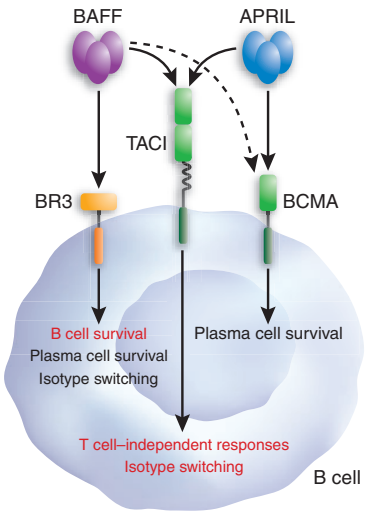

Figure 2. Adapted from Martin and Dixit (2005)[46]. **Receptors for BAFF and APRIL.** BAFF and APRIL share the transmembrane activator and calcium modulator ligand interactor (TACI) and the B cell maturation antigen receptor (BCMA). Only BAFF binds to the BAFF receptor (BR3 or BAFF-R). BAFF has a major role in B cell differentiation/maturation and survival of B cells.

1.4 TH17 CELLS

Th1 cells are promoted by IL-12 which induces expression of transcription factor T-bet and secretion of the hallmark cytokine IFN-γ [47-49]. Th2 cells are induced by IL-4 that upregulates the transcription factor GATA3, producing the cytokines IL-4, IL-5 and IL-13. [50, 51] (Figure 3). The discovery of Th17 cells formed an addition to the previous Th1/Th2 paradigm.

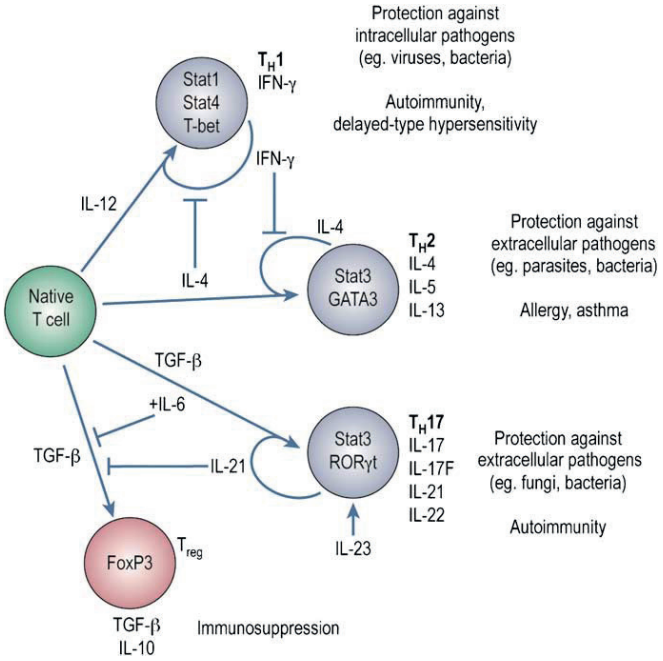

Figure 3. Adapted from Deenick and Tangye (2007)[52]. **Molecular requirement for Th17 cell differentiation**. Naive CD4+ T cells differentiate to Th17 cells under the influence of IL-6 and TGFβ. The expansion and stability of the Th17 population is regulated by IL-21 and IL-23, respectively. Th17 cells secrete IL-17A, IL-17F, IL17A/F heterodimers, as well as IL-21, IL-22, granulocyte macrophage-colony-stimulating factor (GM-CSF), and many other factors.

Naive CD4+ T cells differentiate to Th17 cells under the influence of IL-6 and TGFβ [53] (Figure 3). The expansion and stability of the Th17 population is regulated by IL-21 and IL-23, respectively [54, 55]. Th17 cells secrete IL-17A, IL-17F, IL17A/F heterodimers, as well as IL-21, IL-22, granulocyte macrophage-colony-stimulating factor (GM-CSF), and many other factors [56]. In this thesis we studied both chemokine receptor defined Th17 cells (defined as CD4+CD45RO+CCR6+CCR4+CXCR3-CCR10- cells) and IL-17A and IL-17F producing CCR6+ T memory cells. The latter enriches for Th17 cells, but contains also other cell types (Figure 4).

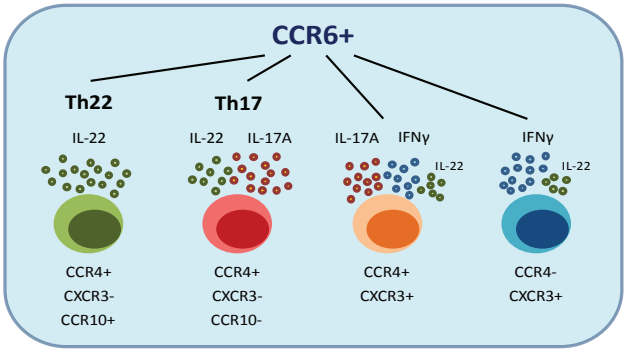

Figure 4. Composition of CCR6+ T memory cell subset with chemokine receptor expression. Chemokine defined Th17 cells are represented by the red cell (CD4+CD45RO+CCR6+CCR4+CXCR3-CCR10- cell). IL-17 producing CCR6+ cells are represented by both the red and the orange cell.

Next to Th17 cells, IL-17A is produced by several other immune cell types, including CD8+ T cells, CD4-CD8-CD3+ (double negative, DN) T cells, NK cells, γδ-T cells and mast cells. The proinflammatory effects of IL-17 are mediated through the IL-17 receptor (IL-17R), composed of IL-17RA and IL-17RD subunits [57]. IL-17R is widely expressed by immune cells like T cells, B cells and neutrophils, but also by other tissues such as epithelium, endothelium, fibroblasts, mesenchymal stromal cells and keratinocytes [57]. Il-17A promotes granulopoesis by triggering the secretion of granulocyte-colony-stimulating factor (G-CSF) in bone marrow stroma [58]. Moreover, IL-17 induces cytokines (GM-CSF, TNFα, IL-1β, IL-6), chemokines and chemokine receptors that act as powerful chemoattractants for granulocytes. Tissue damage by Th17 cells might be caused by direct recognition of the antigen-specific target, or it can result from the recruitment of neutrophils and macrophages into the microenvironment [59]. IL-17 also mediates the formation of inducible secondary lymphoid tissues following local infection [60] and plays an important role in immunity against a variety of micro-organisms [61].

Finally, Th17 cells have been implicated in the pathogenesis of autoimmune diseases in studies of experimental autoimmune encephalomyelitis (EAE) which is an animal model of multiple sclerosis (MS) [56, 62]. Since then, Th17 cells have been the subject of increasing attention in the context of systemic autoimmune diseases such as SLE, but also pSS, rheumatoid arthritis (RA) and psoriasis [63].

1.5 SJÖGREN'S SYNDROME

1.5.1 Nomenclature

Jan Mikulicz-Radecki, a Polish-Austrian surgeon, was the first to report in 1888 on a male patient with bilateral enlargement of the parotid and lacrimal glands and diminished production of tears and saliva. The term Mikulicz's syndrome could encompass all kinds of

diseases with swelling of the lacrimal and salivary glands such as tuberculosis, sarcoidosis and lymphoma. Consequently, this name gradually became a repository for a variety of diseases and fell into disuse. Also in 1888, Dr. W.B. Hadden described a woman with symptoms of dry eyes, mouth and skin which was successfully treated with pilocarpine. But it was not until 1933 that the Swedish ophthalmologist Henrik Sjögren described 19 patients with symptoms of dry mouth, dry eyes and inflamed joints. He introduced the term 'keratoconjuctivitis sicca' (dry eyes) to distinguish it from dry eyes caused by lack of vitamin A (xerophthalmia). Given the high number of patients Henrik Sjögren described, the syndrome has been characterized by the term 'Sjögren's syndrome' (SS).

1.5.2 Clinical manifestations — overview

SS is a generalized autoimmune disease, characterized by lymphocytic infiltrates of the salivary and lacrimal glands (also named sialoadenitis and dacryoadenitis, respectively). Patients suffer from dryness of the eyes (xerophthalmia) and dryness of the mouth (xerostomia) [64]. Characteristic eye symptoms are burning and/or itchy eyes as though there is sand or a foreign body in the eyes. The most characteristic symptom of the mouth is that patients need to drink when eating dry food in order to be able to swallow it (so called cracker-sign). Due to the lack of lubrication, the incidence of opportunistic infections in both the eyes and the mouth is increased and the poor quality of the saliva may be the cause of severe dental caries and periodontal disease [65].

Besides the ocular and oral complaints, symptoms can be observed in many other organs as well as systemically. Many patients are affected by general symptoms like fatigue, joint pain, muscle pain and Raynaud's phenomenon (abnormal vasoconstriction in digits). SS can be divided in primary and secondary SS, the latter being associated with another systemic autoimmune disease, such as rheumatoid arthritis, systemic lupus erythematosus (SLE) or systemic sclerosis (Ssc). In this thesis the focus will be mainly on primary Sjögren's syndrome (pSS).

According to Haugen *et al.* the prevalence of Sjögren's syndrome (in a large Norwegian study) is 0.2 % for people between 40-44 years and 1.4 % for people between 71-74 years [66]. Women are affected by Sjögren's syndrome 9x more frequently than men with the most common age of onset around menopause, however the disease may occur in patients of all ages [67, 68].

1.5.3 Extra glandular manifestations

For 40% of Sjögren's syndrome patients, fatigue has been described as the most severe symptom [69]. It often varies from day to day, may come on very suddenly and usually increases during the course of the day and improves after a rest. It is estimated that over 50% of patients suffer from extreme fatigue, in many cases leading to inability to work, and a strong decrease in quality of life [70-74].

Other frequent extraglandular manifestations are found in the joints and muscles. About 53% of pSS patients complain of arthralgia. Myalgias are reported in 22% of the pSS patients [75] and 7% of patients with fibromyalgia have pSS [76].

Skin problems are also a common feature of pSS. According to Bernacchi *et al*. [77] xerosis (skin dryness) and angular cheilitis (chronic inflammatory condition of the corners of the mouth) are the most frequent skin diseases in pSS. Bernacchi *et al.* also found a significant connection between xerosis erythema and the presence of anti-SSA and anti-SSB antibodies in pSS patients. Other manifestations include cutaneous Raynaud's phenomenon, vitiligo, anetoderma, alopecia, cutaneous lymphomas and vasculitis [78]. Vasculitis is an inflammation of small blood vessels and concerns mainly the blood vessels of the skin in the case of pSS [79-81]. Besides dryness of the skin, increased mucosal dryness is found in many pSS patients, in particular the vagina, resulting in an increased risk of local infections [67].

Cough due to tracheobronchial sicca and interstitial pneumonitis are the most common presentation of pulmonary involvement in pSS [82]. Other potential pulmonary complications include MALT (mucosa-associated lymphoid tissue) lymphoma or other types of lymphoma of the lung [83].

Renal manifestations include mostly interstitial nephritis, with or without renal tubular acidosis [84]. Unlike in other autoimmune diseases, the glomeruli in pSS are spared [85] and renal injury is due to lymphocyte infiltration of the interstitial space rather than immune complex deposition [86]. Symptoms indicating an interstitial cystitis are common in pSS and can be severe [87, 88].

Gastrointestinal manifestations include dysphagia (difficulty in swallowing) that is partly a result of xerostomia but also of oesophageal dysmotility. pSS patients can also suffer from functional dyspepsia (indigestion) causing symptoms of epigastric pain, early satiety, postprandial fullness and epigastric burning [89]. Coeliac disease is found in 10-14.7% of pSS patients [90, 91].

Neurological manifestations are reported in a relatively large subset of pSS patients. About 20% of patients suffer from neurological problems including central-nervous-system involvement, cranial neuropathies, myelopathy and peripheral (primarily sensory) neuropathies [92-94]. Sensory peripheral neuropathy is the most common form of neuropathy in pSS. Psychiatric disorders like depression and anxiety are reported in many pSS patients [95, 96]. The high frequency suggests that they are part of the underlying process rather than simply a response to the stress of a chronic disease [97].

Hypothyroidism is more common in pSS compared with the general population [98, 99]. In addition, pSS is present in about 10% of patients with autoimmune thyroid disease [100]. These patients are usually seropositive for anti-thyroid peroxidase (ant-TPO) and anti-thyroglobulin (ATG) antibodies [101]. In addition, in family members of pSS patients increased prevalence of thyroid diseases has been documented, suggesting genetic susceptibility [101].

Hematological abnormalities are not uncommon in pSS, although they rarely have clinical significance. Lymphopenia has been reported in 35.3% of pSS patients, leukopenia in 26.2% and thromobocytopenia in 7.1% [102].

Finally, a lack of control of inflammatory processes may lead to the formation of lymphomas. Malignant lymphoma is the only cause of premature mortality for which pSS patients are at increased risk [103]. Compared with the general population, patients with pSS have a 16-44 times higher relative risk for the development of a Non-Hodgkin lymphoma [104, 105]. Lifetime risk for the development of lymphoma is approximately 5%-10% in patients with pSS [106-108]. A retrospective study showed that the following factors could predict the development of Non-Hodgkin lymphoma: neutropenia, cryoglobulinemia, splenomegaly, lymphadenopathy and low C4 levels [109]. Furthermore, the detection of germinal centre-like structures in pSS diagnostic salivary biopsies is proposed as a highly predictive marker for the development of Non-Hodgkin lymphoma [110].

1.5.4 Diagnosis

An early, precise diagnosis of pSS can help to prevent or ensure timely treatment of many of the complications associated with the disease. Early recovery of salivary function can relieve oral dryness symptoms for example and may prevent or slow the progress of dental caries, oral candidiasis and periodontal disease. Since many symptoms in accordance with pSS develop gradually over a period of time, are deceptively non-specific and are frequently seen in middle-aged women, these symptoms may initially be unjustly attributed to menopause [67]. Until 2002 several sets of diagnostic criteria for pSS were used. The discrepancy in these different diagnostic criteria led to substantial confusion in research publications and clinical-trial reports.

Therefore an international consensus group suggested the latest set of criteria, the 2002 European-American classification criteria to confirm and unify the diagnosis of pSS [111]. This set consists of six criteria, both subjective and objective in nature (Figure 5). The subjective criteria contain symptoms of dry eyes and dry mouth. The objective criteria include clinical tests of lacrimal (eye tests) and salivary function (imaging or function investigation of the salivary glands) and two laboratory measurements. These laboratory measurements contain the presence of antibodies against Ro/SSA and La/SSB and the presence of focal lymphocytic infiltrates in biopsy material of the minor salivary glands. Establishment of the diagnosis of pSS requires four of these six criteria, including a positive minor-salivary-gland biopsy sample (focus score ≥ 1, defined as a number of lymphocytic foci which contain more than 50 lymphocytes per 4 mm^2 of glandular tissue) or antibody to SSA/SSB. All other possible causes of oral and ocular dryness such as previous radiotherapy to the head and neck, lymphoma, sarcoidosis, graft-versus-host disease and hepatitis C infection must be excluded.

1. Ocular symptoms—A positive response to at least one of the following questions:
a) Have you had daily, persistent, troublesome dry eyes for more than 3 months?

b) Do you have a recurrent sensation of sand or gravel in the eyes?

c) Do you use tear substitutes more than three times a day?

2. Oral symptoms—A positive response to at least one of the following questions:
a) Have you had a daily feeling of dry mouth for more than 3 months?

b) Have you had recurrently or persistently swollen salivary glands as an adult?

c) Do you frequently drink liquids to aid in swallowing dry food?

3. Ocular signs—Objective evidence of ocular involvement defined as a positive result for at least one of the following tests:
a) Schirmer's test for tear function, performed without anaesthesia (positive result ≤5 mm in 5 minutes)

b) Rose Bengal score or other ocular dye score (positive result score ≥4 on the van Bijsterveld scoring system)

4. Histopathology—In minor salivary glands (obtained through normal appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score of 1 (defined as the number of lymphocytic foci (which are adjacent to normal appearing mucous acini and contain >50 lymphocytes) per 4 mm^2 of glandular tissue)

5. Salivary gland involvement—Objective evidence of salivary gland involvement defined by a positive result for at least one of the following tests:
a) Unstimulated whole salivary flow (<1.5 mL in 15 minutes)

b) Parotid sialography showing presence of diffuse sialectasias (punctate, cavitary, or destructive pattern) without evidence of obstruction in the major ducts

c) Salivary scintigraphy showing delayed uptake, reduced concentration, or delayed excretion of tracer

6. Autoantibodies to Ro/SS-A or La/SS-B antigens, or both

Presence of primary Sjögren syndrome

Patients are classified as having primary Sjögren syndrome when they fulfil ≥4 of the 6 criteria; either criterion 4 (salivary gland pathology) or 6 (autoantibodies) is mandatory

Exclusion criteria—Past head and neck radiation treatment, hepatitis C infection, AIDS, pre-existing lymphoma, sarcoidosis, graft versus host disease, use of anticholinergic drugs

Figure 5. Adapted from Ramos-Casals M. et al (2012)[112]. **2002 American-European classification criteria for Sjögren's syndrome.**

Although these criteria result in more homogeneous and comparable patient populations in research, almost 60% of patients diagnosed by experienced clinicians as having pSS do not completely fulfil these criteria, even though the symptoms cannot be attributed to another disease [112]. The patients that do not fulfil the criteria but have pSS symptoms, frequently do not receive the proper treatment. Although it is a point of discussion, this group might be considered as pSS for clinical practice since this group is likely to benefit from the common treatment for pSS.

1.5.5 Treatment

Stimulation of salivary and lacrimal glands

Despite recent knowledge the treatment of pSS is rather symptomatic than causal. Treatment is aimed at recognizing and treating complications of the disease early. When patients still have some salivary and lacrimal function present, initial therapy is directed toward stimulating flow by sucking on flavoured lemon lozenges for example and chewing gum [113]. In patients where this type of stimulation is not sufficient, pharmacological intervention like muscarinic agonists such as pilocarpine may be needed.

Acetylcholine binds to muscarinic 3 receptors (M3Rs), which are present in the salivary and lacrimal glands, to stimulate saliva and tear flow. It has been proposed that acetylcholine does not bind successfully to M3Rs in pSS patients due to blocking antibodies against the M3R [114-117], although these findings are a matter of debate. Pilocarpine is a muscarin agonist that stimulates M3Rs to produce more saliva and tear flow. Two placebo-controlled trials showed pilocarpine induced improvement of both dry eye and dry mouth symptoms [118, 119], while a third trial found a similar improvement in solely dry mouth symptoms [120]. When pharmacological intervention does not suffice to increase tear flow, temporary or permanent occlusion can be used to block tear drainage and thus retain existing tears.

Replacement fluids

The use of replacement fluids may be helpful when pSS patients do not have sufficient remaining salivary and lacrimal function. Flushing water or using artificial saliva can alleviate oral dryness and artificial tears can be used to treat persistent symptoms of dry eyes [113].

Systemic therapy

Systemic manifestations such as arthralgia are generally treated with salicylates, non-steroidal anti-inflammatory drugs (NSAIDs) and hydroxychloroquine (HCQ). HCQ is an anti-inflammatory and disease-modifying drug and its inhibitory activity in autoimmune diseases has been attributed to the inhibition of endosomal acidification, since acidic pH is a prerequisite of endosomal TLR activation [121-123]. Strong interactions of nucleic acid TLR ligands with TLR occur only under pH 4.5-6.5. Recently Kuznik *et al*. showed that inhibition of pH acidification is not the underlying cause of inhibitory effect of HCQ in autoimmunity [124]. Instead, a direct binding of HCQ to nucleic acid TLR ligands was found, masking the TLR binding epitope and possibly explaining the efficiency of HCQ in autoimmune diseases. Only 1 double-blind placebo-controlled study on HCQ in pSS has been performed [125]. In this 2-year crossover trial 19 patients were treated with 400 mg HCQ per day. No significant differences in HCQ vs. placebo were found for clinical symptoms. In the HCQ group an improvement of hyperglobulinaemia, erythrocyte sedimentation rate (ESR) and IgM was found. An open label study of HCQ in 14 patients showed no effects on sicca symptoms and fatigue but significant reduction in ESR, C-reactive protein and IgG levels [126]. A retrospective study of 50 pSS patients showed improvement of painful eye and mouth symptoms, improvement of arthralgias and myalgias and a significant improvement in ESR and IgG levels [127]. Finally, a more recent retrospective analysis on 14 pSS patients showed beneficial effects of HCQ on xerostomia [128].

Corticosteroids are effective but limited by their common side effects and are therefore preserved for visceral involvement like vasculitis, pneumonitis, neuropathy and nephritis. There is limited evidence on the use of immunosuppressive agents, since controlled and prospective studies are small and are specifically designed to evaluate sicca features [113].

Biologicals

It is justified to use expensive biologic therapies in pSS patients with severe systemic involvement who fail to respond to conventional immunosuppression, despite the rare but potentially serious side effects of biologic therapy [129]. In the last decade anti-TNFs have taken the central stage for the treatment of rheumatic and autoimmune diseases. In contrast to the enormous success of anti-TNF agents in rheumatoid arthritis (RA), these agents have been found inefficacious in pSS [130-133]. Inefficacious results might be due to increased IFN type I response [134, 135], increased BAFF production [135] and increased Th17 response [136] upon anti-TNF therapy. The levels of circulating TNFα were even reported significantly increased in pSS patients after anti-TNF treatment [137]. How an antagonist of TNF might augment its levels has remained a puzzle. One explanation of diminished IL-10 in pSS, which is an inhibitor of TNFα production, has been suggested.

Rituximab, a monoclonal antibody against CD20 causing B cell depletion, has been considered to be effective in pSS. This antibody used for the treatment of B cell lymphoma, resulted in significant improvement in salivary gland function, decreased rheumatoid factor, improvement of extra-glandular manifestations and general well-being of treated pSS patients [138]. However after 36-48 weeks post treatment there is a reappearance of circulating B cells [139, 140] and relapse of clinical symptoms [138, 141-144]. The first reappearing B cells in the peripheral blood displayed a phenotype pointing toward transitional B cells, indicating that these cells are newly generated bone marrow derived B cells [145]. Contradicting data exist concerning the presence of CD20+ B cells in the salivary gland biopsies after rituximab [140, 146]. One group finds despite full depletion of circulating CD20+ B cells by rituximab still B cells present in parotid biopsies 12 weeks after initial treatment [146], while others demonstrated a complete absence of B cells in labial salivary gland biopsies up to one year after rituximab treatment [140]. Regarding retreatment with rituximab, most patients that were retreated reported a beneficial effect comparable to that of the initial treatment [138, 141, 142, 144]. Interestingly, following B cell depletion therapy with rituximab, BAFF protein levels were found elevated in pSS patients [147, 148]. Higher baseline BAFF levels were shown to lead to a shorter period of B cell depletion after rituximab treatment [140]. Moreover persistently elevated BAFF levels are also associated with resistance to rituximab treatment in pSS patients with lymphoma [149].

For this reason a new treatment for pSS has been proposed: Belimumab, a fully human IgG1 antibody directed against BAFF. pSS patients show an increased expression of BAFF in both serum [150, 151] and salivary glands [152], suggesting a critical role for BAFF in pSS. BAFF expression was furthermore found increased in peripheral blood mononuclear cells (PBMCs), epithelial cells, T cells and B cells within the salivary glands of pSS patients [151, 153, 154]. Belimumab was successful in clinical trials in SLE and is already in clinical use [155]. Although Belimumab trials in pSS have been registered (www.clinicalstudies.gov),

results of these studies are not known up till now. Another BAFF-blocking agent is Atacicept, which is a recombinant fusion protein designed to block the activity of both BAFF and APRIL. Initial study of Atacicept with mycophenolate in SLE patients had to be prematurely stopped due to infections in 3/6 SLE patients [156].

Epratuzumab, recombinant humanized monoclonal antibody, targets another B cell surface protein namely CD22. In contrast to anti-CD20 agents, Epratuzumab appears to function more by modulation of B cells rather than by their depletion capacity. Epratuzumab has been investigated in an open-label study of 16 pSS patients and showed clinical response in 52% to 67% of patients [157]. Further studies are needed to determine whether Epratuzumab is a promising therapy for pSS.

Abatacept is a fusion protein consisting of CTLA-4 linked to the Fc portion of IgG1. It is similar to CD28 in the way that it contains a high-affinity binding site for CD80 and CD86. For T cell activation next to binding of the T cell receptor to the MHC complex on APCs, a costimulatory signal is necessary as well. This costimulatory signal is provided by the binding of the T cell's CD28 protein to the CD80/86 protein on APCs. Abatacept binds to the CD80/86 protein on APCs and prevents the delivery of a costimulatory signal to T cells, resulting in prevention of T cell activation. Abatacept is licensed for the treatment of rheumatoid arthritis [158] and recently a pilot study with Abatecept was performed in 11 pSS patients. This study showed that Abatacept treatment significantly increased saliva production, reduced glandular inflammation, induced expansion of naive B cells, total lymphocytes and CD4+ cells in the peripheral blood [159].

1.5.6 Pathogenesis

The pathogenesis of pSS is multifactorial. A complex interplay exists between genetic factors, hormonal mechanisms and environmental events that involve innate and adaptive immunity including autoantibodies [160]. The first stages in the pathogenesis are still unclear.

Genetic factors

Some familial clustering of pSS has been identified, suggesting a genetic component of the disease. In fact, a family history of autoimmune diseases causes a seven fold increased risk of developing pSS [161]. The genetics of pSS involve HLA and non-HLA genes. pSS is associated with increased frequencies of HLA-B8, HLA-Dw3 and HLA-DR3 [162, 163]. Polymorphisms of non-HLA genes occur in the IFN type I pathway such as STAT4 and IRF5 [164-166]. A strong additive effect between the risk alleles of IRF5 and STAT4 was found for pSS [165].

Hormonal mechanisms

A role for sex steroids has been proposed given the female predominance among pSS patients and the fact that the most common age of onset is around menopause. Estrogen

receptor (ER) and mRNA of ER have been detected in salivary gland tissue and cultured salivary gland epithelial cells [167-169]. Mouse studies have shown that estrogen suppresses the development of pSS, while ovariectomy leads to a Sjögren-like disease [170]. Estrogen can ameliorate recruitment of T cells in the gland [171] and prevent cell apoptosis in the lacrimal glands in a murine model for pSS [172]. Interestingly, another group proposed that a defect in the synthesis of androgens in the salivary glands of pSS patients plays an important role and that the increased risk is due to a change in the androgen-estrogen ratio rather than absolute levels of estrogens [173] . Ovaries produce low levels of testosterone, which decrease at the time of menopause. The other significant source of androgens is the adrenal cortex, which produces dehydroepiandrosterone (DHEA) and its metabolite DHEA sulfate (DHEA-S) [160]. DHEA concentrations reach their peak in early adulthood and decline with age and are 40-50% lower in pSS compared with age and sex matched controls [174]. DHEA is a prohormone which can be converted to either androgens or estradiol locally in target organs including the salivary glands. Women may be particularly vulnerable to local androgen deficiency in the salivary gland in pSS as their local dihydrotestosterone production is completely dependent on local conversion of DHEA, whereas in men, systemic androgens may satisfy the local requirement [160]. Dillon et al. presented during the 11th international symposium in Athens data showing 4 times higher prevalence of pSS among females carrying the triple X chromosome (47XXX) compared with normal females (46XX). This would imply that genes on the X chromosome are responsible for the female bias of pSS rather than the sex hormones.

Environmental factors

The initial trigger that causes salivary gland injury is presently unknown, though one of the postulated triggers is a viral infection [175]. In this case, the infection provides antigens to TLRs that are expressed by DCs and epithelial cells of the salivary glands, initiating MHC class II presentation and secretion of chemokines and cytokines like IFN type I. Although no strict correlation has been found between pSS and one virus in particular, a few reports describe an association between pSS and EBV [176], HTLV1 [177], coxsackievirus [178] and HIV and hepatitis C viruses (HCV) [179]. Infections with HCV or HIV are associated with development of sialoadenitis resembling pSS, however they are not characterized by production of anti-SSA and anti-SSB autoantibodies nor by sex preference [180, 181].

Epithelial involvement

Several lines of evidence indicate that the target organ and in particular epithelial cells are not solely the innocent bystander targets of autoimmune responses but probably have their role as amplifiers of the inflammatory response [182]. In the salivary glands of the nonobese diabetic (NOD) mouse, which is a murine model for pSS, DCs were detected

before lymphocytic infiltration [183]. This suggests abnormalities in the exocrine gland itself that may contribute to the initiation of the autoimmune reaction.

The expression 'autoimmune epithelitis' has been proposed as an alternative for Sjögren's syndrome by Moutsopoulos back in 1994 [184]. Several studies show that salivary gland epithelial cells (SGEC) are activated in pSS and capable to function as non-professional APCs [185]. SGEC are reported to express MHC class I and class II as well as costimulatory molecules CD80 and CD86, thus providing both signals necessary for T cell activation [186-189] (Figure 6). SGEC also express intercellular adhesion molecules ICAM1 and VCAM [190, 191] and functional TLRs [192].

In addition, SGEC produce proinflammatory cytokines such as TNFα, IL-1 and IL-6 [193] and a number of chemokines involved in the recruitment of lymphocytes as well as the induction of germinal centers (GC). In 20% of pSS patients GC-like structures are found in the salivary glands [194, 195]. CXCL13, important for GC formation by attracting B and T cells through the receptor CXCR5, is reported to be elevated in pSS [196]. CCL19 and CCL21, involved in directing lymphocytes to lymphoid structures are also increased in pSS salivary glands. Furthermore, CXCL9 and CXCL10 are produced by SGEC from pSS patients, while most of CD3+ lymphocytes express CXCR3 (the common receptor for CXCL9 and CXCL10) [197]. CXCL12, constitutively expressed by SGEC, is the most efficient chemoattractant for pDCs and also synergizes with CXCL9 and CXCL10 [198]. Next to these chemokines, BAFF is expressed by SGEC in pSS after stimulation with IFN type I and type II, suggesting hypersensitivity of the salivary epithelial cells possibly after stimulation by innate immunity [153] (Figure 6).

Finally, SGEC may serve as a source of Ro/SSA and La/SSB autoantigens by virtue of increased apoptosis. The Ro/La proteins are intracellular and therefore normally not exposed to immune cells. To expose these proteins either elevated apoptotic death is required or the secretion of small endosomal vesicles, called exosomes [185]. Indeed, it has been shown that Ro/SSA and La/SSB undergo a redistribution during apoptosis and relocate to the surface of apoptotic cells [199, 200]. The pro-apoptotic molecules Fas and Fas ligand (Fas-L) are expressed by epithelial cells as well as by infiltrating mononuclear cells of the salivary and lacrimal glands of pSS patients [201-205]. Perforin and granzyme B expression was also observed in mononuclear cell infiltrates in pSS in contrast to HC [202] (Figure 6). Interestingly, X-chromosome-linked factors might influence apoptosis in salivary glands of patients with pSS [206]. The X-chromosome-linked inhibitor of apoptosis (XIAP) was found present in both pSS acinar and ductal epithelial cells and downregulated by TNFα. Next to apoptosis, the secretion of exosomes has been involved in the transfer of autoantigens to APCs [207]. Indeed, SGEC have been shown to release exosomes containing Ro/SSA and La/SSB [208].

Autoantibodies

In pSS patients a spectrum of autoantibodies to RNA binding proteins such as SSA, SSB, RNP, or SM can be found [209] (Figure 6). The anti-Ro/SSA and anti-La/SSB antibodies are considered typical and have been included in the American-European 2002 criteria. Other autoantibodies encountered in pSS patients are anti-nuclear antibodies (ANA), rheumatoid factor (RF), cryoglobulins and the less typical anti-mitochondrial antibodies (AMA), anti-centromere antibodies (ACA), anti-smooth muscle antibodies (ASMA), antibodies against cyclic citrullinated peptides (anti-CCP) and anti-parietal cell antibodies [210].

Anti-Ro/SSA and anti-La/SSB antibodies are directed against ribonucleoprotein complexes, comprising small RNAs non-covalently associated with the proteins Ro52, Ro60 and the La protein [211]. Ro-52, also denoted TRIM21 and SSA1, is a member of the TRIM family of single-protein E3 ligases and is known to be IFN inducible [212]. Ro52 was described to negatively regulate IFNβ production following TLR3/4 stimulation by promoting the ubiquitination and proteasomal degradation of IFN regulatory factor 3 (IRF3) [213]. Subsequent study demonstrated that Ro52 is also able to degrade IRF7 following TLR7 and TLR9 stimulation [212]. It was postulated that Ro52 is central to a negative feedback loop which protects the host from prolonged exposure to IFN type I. Ro60 protein, having a shape that resembles a doughnut, binds to misfolded, noncoding RNAs in vertebrate cells and acts as a quality checkpoint for misfolded RNA [214]. The misfolded RNAs are recognized and then tagged by Ro60 for degradation. Mice lacking Ro60 are found to develop autoantibodies and membrano-proliferative glomerulonephritis. The La protein is a polypeptide that exists abundantly in both the nucleus and cytoplasm and plays fundamental roles in diverse processes of RNA metabolism [215].

Anti-Ro/SSA and anti-La/SSB antibodies are of both IgA and IgG and can be found in 33-74% and 23-52% of pSS patients respectively, depending on the identification method used [210, 216-220]. Next to being systemically present in the serum, a striking local production of anti-SSA/SSB antibodies in pSS salivary glands has been reported [195]. In addition, autoantibodies to Ro and La have been detected both in whole saliva [221] and in parotid saliva [222] from pSS patients. Anti-Ro/SSA antibodies are found either solely or together with anti-La/SSB, while sole anti-La/SS antibodies are only rarely detected. An association was reported between the presence of SSA/SSB autoantibodies and younger age at diagnosis, recurrent parotidmegaly, cutaneous vasculitis, Raynaud phenomenon, renal involvement, pulmonary involvement, peripheral neuropathy, ESR 50 mm/h, arthralgia, arthritis, leukopenia, thrombocytopenia and increased skin's sensitivity to sunlight [210, 217, 220, 223]. Finally, when women with these antibodies are pregnant, the antibodies can also cross the placenta causing in 10% of the cases neonatal lupus [224, 225]. The biggest risk of neonatal lupus is congenital heart block that occurs in 1-2% of the offspring of pregnant women positive for anti-SSA/SSB [226].

Autoantibodies not used in the everyday clinical practice, but associated with pSS are anti-α-fodrin antibodies. α-fodrin is a 240 kDa cytoskeletal protein which is cleaved during apoptosis into smaller fragments of 150 kDa and 120kDa. α-fodrin of 120 kDa is found in the salivary glands of pSS patients, but not in healthy controls (HC) and antibodies against 120 kDa α-fodrin are formed in pSS [227]. Prevalence of anti-α-fodrin is between 50% and 95%, depending on the detection method used [228]. Immunization of neonatal mice with the 120 kDa α-fodrin antigen prevents disease development in a pSS mouse model [229]. Furthermore immunization with 120 kDa α-fodrin into normal recipients induced autoimmune lesions similar to pSS, suggesting a role for the anti-α-fodrin response in the pathogenesis of pSS.

As previously mentioned, the major stimulus for saliva production is provided by acetylcholine through M3R. Autoantibodies against M3R have been reported in pSS patients and since M3Rs are expressed on salivary and lacrimal glands, it has been suggested that immune reaction to M3R plays a key role in the generation of pSS [114-117]. Interestingly, the decreased salivary flow in pSS patients does not correlate with the degree of lymphocytic infiltration in the salivary glands, suggesting that tissue destruction is not the only explanation for the sicca symptoms. In this light, anti-M3R antibodies might provide an alternative explanation for the lack of salivary flow.

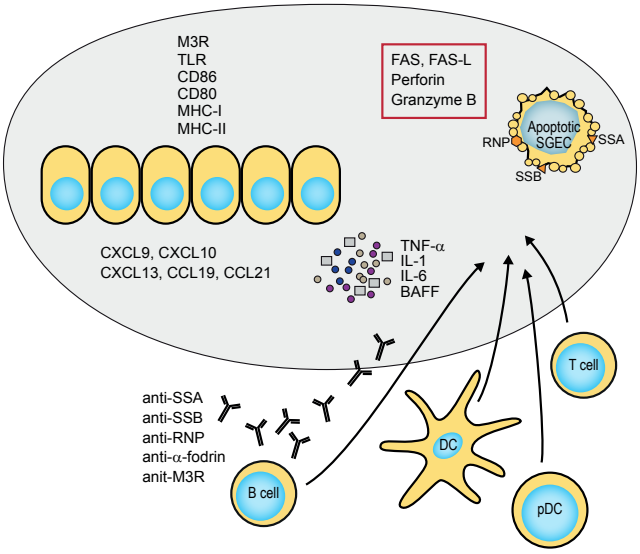

Figure 6. Hypothetical scheme on the molecular interactions in the salivary gland of pSS patients based on data from the literature. SGEC are reported to express TLRs, MHC class I and class II as well as costimulatory molecules CD80 and CD86. In addition, SGEC produce proinflammatory cytokines and a number of chemokines and may serve as a source of Ro/SSA and La/SSB autoantigens by increased apoptosis. pDCs, conventional DCs, T cells and B cells are attracted to the gland where for example autoantibody production can take place.

Interferon type I

Development of Sjögren-like symptoms has been described upon treatment with IFN type I in patients with malignancies and Hepatitis C [230-232] supporting the role for IFN type I in the pathogenesis of pSS.

Gene array studies showed an increased IFN type I activation in salivary glands of pSS patients [233, 234]. Moreover, in 2 studies with a limited number of pSS patients, pDCs were detected in salivary glands but not in healthy controls [233, 235]. Given that there are 17 different IFN type I subtypes, it is difficult to measure serum protein levels using an Enzyme-Linked Immuno Sorbent Assay (ELISA). Therefore microarray studies on peripheral blood were conducted to study the systemic effect of IFN type I in pSS. A set of IFIGs, the so-called IFN type I signature, was detected in whole blood, PBMCs and monocytes from pSS patients [236, 237]. The number of pDC was found decreased in the blood of pSS patients and the remaining pDC showed signs of increased activation, pointing to these cells as a possible source for the increased systemic IFN type I activity in pSS [237]. Interestingly, IFN type I induction was much higher in PBMCs using pSS sera that contained antibodies to RNA-binding proteins such as SSA, SSB, RNP or Sm [235]. Finally when SGECs were stimulated with an RNA virus or TLR3 ligands, production of large amounts of BAFF mRNA and protein was induced. This production could be blocked to some extent by an IFNAR antagonist, suggesting at least a contributory role for the IFN pathway in the generation of BAFF in pSS [153].

B cells

In pSS the presence of autoantibodies, hypergammaglobulinemia, development of GC-like structures in the glands and increased occurrence of lymphomas all point to B cell hyperactivity [238]. A decrease in frequencies and numbers of CD27+ memory B cells in pSS peripheral blood compared with HC and an increase in naive and CD27- memory B cells have been shown [139, 239-242]. Also a higher percentage of plasma cells has been described in the peripheral blood of pSS patients with a focus score ≥2 compared with patients with focus score ≤1 [243]. In this same study a higher ratio of CD19- plasma cells/CD19+ plasma cells was detected in pSS compared with HC. Studies on the different B cell subsets present in the salivary glands of pSS patients are few and not very definitive [238].

BAFF

The first indications that BAFF might play an important role in the pathogenesis of pSS originate from the BAFF transgenic mouse model. BAFF transgenic mice develop next to lupus-like disease also infiltrates in their salivary glands accompagnied by reduction of saliva [244]. pSS patients show an increased expression of BAFF in both serum [150, 151] and salivary glands [152]. The level of serum BAFF has been shown to correlate with the autoantibody titer in pSS [150]. The BAFF-R receptor, located on B cells and activated T cells,

is decreased in pSS and especially in patients with extraglandular involvement. In addition serum BAFF was found to correlate inversely with BAFF-R [245]. Finally, BAFF levels are found increased after Rituximab treatment [147]. It is hypothesized that a negative signal might be delivered from B cells to monocytes, thus inhibiting BAFF production. Therefore, B cell depletion could abolish this negative signal, leading to an increased transcription of BAFF mRNA.

T cells

T cells constitute more than 75% of lymphocytes infiltrating the salivary gland [246]. Activated T cells expressing MHC class II and CD38 are elevated in the glands compared to the peripheral blood T cells [247]. In many aspects, the role of T cells in pSS remains cryptic, despite recent knowledge [248]. The production of Th1 cytokines is reported in the glands [193, 249], together with increased levels in saliva from pSS patients [250]. Yet a role for Th2- derived cytokines is reported in pSS as well [251]. Several studies have shown a decrease in Treg cells in Sjögren's syndrome [252-254]. This is in contrast with other reports demonstrating an increase in Treg cells in peripheral blood and salivary gland tissue of pSS patients [255, 256].

Recent years, Th17 cells have been implicated in the pathogenesis of pSS. In the infiltrates in glands of both pSS patients and mouse models for pSS, IL-17 and IL-17 producing cells were observed, and IL-17A is thought to play an important role in the development of lymphocytic infiltrates [257-261]. In peripheral blood of pSS patients the number of IL-17 producing CD4+ T cells [259] and IL-17 plasma levels were increased [260]. Moreover, induction of IL-17A expression in salivary glands of pSS-non-susceptible C57BL/6J mice by an adenovirus resulted in pSS-like disease supporting a role for IL-17 in disease development [262]. In addition, IL-21 has been detected in the serum and within the salivary glands of pSS patients, and levels correlated with hypergammaglobulinemia and autoantibody levels [263]. Also higher serum IL-22 levels were observed, correlating with higher anti-SSA/SSB antibodies, hypergammaglobulinemia and rheumatoid factor [264].

1.6 AIM OF THE THESIS

The role of IFN type I has been implicated in the pathogenesis of generalized autoimmune diseases such as pSS. The aim of this thesis was to determine the prevalence of the increased IFN type I activity in pSS and to elucidate the relation between the increased IFN type I activity and:

a) disease manifestations
b) other immunological parameters such as BAFF and Th17 cells

Chapter 2 demonstrates a monocyte IFN type I inducible signature as a tool for assessment of systemic IFN type I activity. Moreover, the prevalence of the IFN type I signature in pSS

is described and the correlation with disease manifestations and BAFF protein and BAFF mRNA expression. Since the IFN type I activity is assessed via laborious expression profiles of multiple IFN type I inducible genes, an easy and practical assay for identifying systemic IFN type I bioactivity in pSS is described in **Chapter 3**.

IFN type I has also been implicated in diseases like SLE and systemic sclerosis. **Systemic sclerosis** (SSc) is a complex autoimmune disease with extensive fibrosis, vascular alterations and immune activation among its principal features [265]. With an incidence of 1-2 per 100.000 it is a rare disease in which chronic oxidative stress and inflammation lead to organ failure ending up in severe fibrosis of skin and internal organs including kidney, lung and heart. IFN type I activity has been observed either in the affected skin or in immune cells isolated from the circulation [266-268]. Clinically, the expression of the IFN type I induced genes Siglec-1 and IFI44 in skin biopsies correlated well with the mean Rodnan Skin Score (mRSS), currently the only available disease progression marker [269]. In addition, a positive correlation was observed between IFN type I genes and the appearance of digital ulcers and a negative correlation with the presence of anti-centromere antibodies (ACA)[270]. Increased BAFF has been detected in the skin and serum of SSc patients and correlates positively to the extent of skin fibrosis [271-273].

The prevalence of the increased IFN type I activity in SSc patients has not been established yet. In **Chapter 4** the prevalence of the monocyte IFN type I signature is assessed in SSc patients. Also the association between IFN type I and disease manifestations, BAFF expression and collagen metabolism is studied. In addition, the possible role of the IFN type I signature as a biomarker for progressive disease is investigated.

Imatinib mesylate is used as a treatment for therapy-refractory SSc with high variability in therapeutic outcomes ranging from ineffective/toxic responses to extremely encouraging clinical improvement. This high variability in treatment outcomes stresses the need for a biomarker identifying SSc patients likely to respond to Imatinib treatment. **Chapter 5** describes the IFN type I signature as a possible predictor for SSc patients responding to Imatinib treatment.

SLE is a debilitating systemic autoimmune disease characterized by the production of auto-reactive antibodies and multi- organ inflammation [274]. Also lupus like symptoms were observed after IFN type I treatment in patients with malignancies [275]. About half of the SLE patients exhibit upregulation of IFN type I induced genes which has been found to correlate with disease activity and severity as well as with the presence of autoantibodies against RNA binding proteins [25, 26, 276, 277]. IC's containing both RNA and DNA are found to be IFN type I inducers in SLE [278, 279]. This occurs through FcγR-dependent internalization of the IC's by pDCs, followed by TLR7 and 9 activation [280-282]. Increased expression of

BAFF protein has been observed in SLE patients, correlating with increased disease activity [283-285]. Finally, in SLE patients increased plasma levels of IL-17A are present and IL-17 producing cells in the peripheral blood, correlating with disease severity [286-288]. IL-17-producing cells have also been found in several effected organs of SLE patients [287, 289].

A possible co-activity between the pathogenic IFN type I and IL-17/Th17 pathway has been suggested in autoimmune diseases. Data about co-occurrence of these 2 pathways in patients has not been shown yet. In **Chapter 6** chemokine receptor defined Th17 cells have been assessed and compared between IFN+ and IFN- pSS patients. **Chapter 7** compares the IL-17A and IL-17F producing CCR6+ T memory cell percentages between IFN+ and IFN- SLE patients. Finally, **Chapter 8** discusses the significance and implications of the studies described, and provides directions for future research.

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CHAPTER 2

PREVALENCE OF INTERFERON TYPE I SIGNATURE IN CD14+ MONOCYTES OF SJÖGREN'S SYNDROME PATIENTS AND ASSOCIATION WITH DISEASE ACTIVITY AND BAFF GENE EXPRESSION

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Annals of the Rheumatic Diseases, 2013 May; 72(5):728-735.

ABSTRACT

Objective To determine the prevalence of upregulation of Interferon (IFN) type I inducible genes, the so called "IFN type I signature", in CD14+ monocytes in 69 primary Sjögren's syndrome (pSS) patients and 44 healthy controls (HC) and correlate it to disease manifestations and expression of B cell activating factor (BAFF).

Methods Expression of IFI44L, IFI44, IFIT3, LY6E and MX1 was measured using Real time Quantitative Polymerase Chain Reaction (RQ-PCR) in monocytes. Expression values were used to calculate IFN type I scores per subject. pSS patients positive for IFN type I signature (IFN score≥10) and patients negative for the signature (IFN score<10) were then compared for clinical disease manifestations and BAFF expression. A bioassay using a monocytic cell line was performed to study whether BAFF mRNA expression was inducible by IFN type I activity in serum of pSS patients.

Results IFN type I signature was present in 55% of pSS patients compared to 4.5% of HC. Patients with the IFN type I signature showed:

a) higher EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores; higher anti-Ro52, anti-Ro60 and anti-La autoantibodies; higher rheumatoid factor; higher serum IgG; lower C3, lower absolute lymphocyte and neutrophil counts
b) higher BAFF gene expression in monocytes. In addition serum of signature positive patients induced BAFF gene expression in monocytes.

Conclusions The monocyte IFN type I signature identifies a subgroup of pSS with a higher clinical disease activity together with higher BAFF mRNA expression. Such patients might benefit from treatment blocking IFN type I production and activity.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by lymphocytic infiltrates in salivary and lachrymal glands. After rheumatoid arthritis, pSS is the second most common generalized autoimmune disease [1]. Nevertheless, establishment of the diagnosis is difficult due to heterogeneity of the disease and lack of a specific diagnostic test. The diagnosis of pSS is based on 2002 American-European classification criteria [2]. Treatment is mainly symptomatic and the efficacy differs across patients. If new biomarkers based upon underlying pathogenic pathways can be identified, then more effective, evidence-based treatments for pSS might be developed.

Previously we were the first to describe a systemic upregulation of IFN type I inducible genes in CD14+ monocytes of pSS patients [3]. This was in line with described local increased activation of IFN type I in salivary glands of pSS patients [4-6] and confirmed in peripheral blood mononuclear cells (PBMCs) [7]. IFN type I plays important role in the innate immunity by inhibiting viral replication, activating natural killer cells, boosting generation and activation of dendritic cells and enhancing antibody responses [8-12]. Given that there are 17 different IFN type I subtypes, however, it is difficult to measure protein levels using an Enzyme-Linked Immuno Sorbent Assay (ELISA). For diseases as hepatitis C, IFN type I is part of the conventional treatment. Interestingly, development of Sjögren-like symptoms has been described upon treatment with IFN type I in patients with Hepatitis C [13-15] supporting the role for IFN type I in the pathogenesis of pSS.

Another factor known to be involved in the pathogenesis of pSS is B cell activating factor of the tumour necrosis factor family (BAFF). An increased expression of BAFF, which correlates with autoantibody level in both serum [16, 17] and salivary glands [18], has been described for pSS [16]. BAFF-transgenic mice develop Sjögren-like symptoms [19]. IFN type I has been shown to induce BAFF expression in cultured monocytes and salivary gland epithelial cells of pSS patients [20, 21] and a correlation between IFN type I and BAFF has been shown in IFN type I treated multiple sclerosis patients [22]. Furthermore BAFF-dependence of IFN type I functioning mechanisms has been observed in SLE-prone mice [23].

Previously we found a significant upregulation of 23 IFN type I inducible genes using whole genome analysis on pooled monocyte samples of pSS patients. Further assessment of the prevalence of the monocyte IFN type I inducible gene overexpression in pSS and correlations with disease manifestations have yet to be performed. In the current study, we therefore first measured the expression of 11 IFN type I inducible genes, which were previously detected by us, in CD14+ monocytes from 69 pSS patients and 44 healthy controls (HC). Results of factor analysis showed 5 genes (IFI44, IFI44L, IFIT3, LY6E and MX1) to explain 95% of the total variance of the 11 genes, and we therefore decided to adopt overexpression of these 5 genes as our operational definition of positivity for an "IFN type I signature". The relationship between this IFN type I signature in monocytes and various

disease manifestations was then studied. Given the data suggesting that BAFF is an IFN type I inducible factor, we further decided to measure the *in vivo* BAFF mRNA expression in monocytes of pSS patients and to correlate the expression level with the IFN type I signature.

PATIENTS AND METHODS

Patients

69 patients with a positive diagnosis for pSS according to 2002 American-European criteria [2] were recruited. Patients treated with prednisone >10 mg daily, immunosuppressants or biologicals were excluded. The level of disease activity was assessed using EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [24]. 44 healthy controls neither suffering from autoimmune diseases nor using corticosteroids were included. Characteristics of patients and controls are summarized in Table 1. Medical Ethical Review Committee of the Erasmus MC approved the study and written informed consent was obtained.

Table 1. Demographics, laboratory and clinical characteristics of participants

	Controls (n=44)	pSS (n=69)
Demographic characteristics		
Female (n)	40/44 (91%)	62/69 (90%)
Mean age (years)	51±15	58±13
Disease duration (years)	-	10.8±7.7
2002 American-European criteria	-	4.2±0.4
Sjögren manifestations (n)		
Ocular symptoms	-	69/69 (100%)
Oral symptoms	-	66/69 (96%)
Positive ocular tests	-	53/69 (77%)
Positive histopathology	-	39/69 (57%)
Positive salivary gland tests	-	2/69 (3%)
Anti-SSA	-	58/69 (84%)
Anti-Ro52	-	54/56 (96%)
Anti-Ro60	-	47/56 (84%)
Anti-SSB	-	38/69 (55%)

2002 American-European criteria: number of criteria fulfilled out of the six criteria items.
Positive histopathology: focus score≥1, defined as a number of lymphocytic foci containing more than 50 lymphocytes per 4 mm^2.
Positive salivary gland tests: sialography showing the presence of diffuse sialectasias or salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer.
pSS, primary Sjögren's syndrome

Blood collection and isolation of monocytes

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for PBMC preparation as described previously [25]. CD14 positive monocytes were isolated as described [25].

RQ-PCR

Total RNA was isolated from purified monocytes followed by cDNA preparation and RQ-PCR analysis using predesigned primer/probe sets (Applied Biosystems) [25]. For calculation of relative expression, all samples were normalized against expression of the household gene Abl [26]. Fold change values were determined from normalized CT values using 2ΔΔCT method (User Bulletin , Applied Biosystems, Foster City, California).

Measurement of complement, immunoglobulin levels and autoantibodies

C3 and C4 were measured using Immage nephelometer (Beckman Coulter, Woerden, The Netherlands).

IgG, IgA, IgM were measured via turbidimetry using an Modular P800 (Roche, Almere, The Netherlands).

Anti-SSA and anti-SSB were determined by EliA (Thermo Scientific) and confirmed with ANA profile immunoblot (EuroImmun). When discrepant, QUANTA Lite ELISA-kit from INOVA was used for confirmation.

Bio-assay for BAFF activity in serum and ELISA

To assess whether BAFF mRNA expression could be induced by IFN type I activity in serum of pSS patients, THP-1 cells were cultured in 250 µl medium as described previously [3]. 250 µl of serum from pSS patients positive or negative for the IFN type I signature, was added to THP-1 cells and incubated for 6 hours. As positive control, recombinant human IFN-alpha was added (5ng/ml, Peprotech, London, UK). HC serum was added as negative control and for blocking anti-IFN type I receptor (PBL Interferon Source, Piscataway, USA) was added (5 µg/ml).

BAFF protein in serum was assessed by ELISA (improved Quantikine Human BAFF/BLyS, R&D systems, Minneapolis, United States).

Factor analysis

Expression levels of 11 IFN type I inducible genes were submitted to a principal component analysis to identify correlated groups of genes to reduce data complexity. Kaiser-Meyer-Olkin measure of sampling adequacy was 0.833 with significant Bartlett's test of sphericity ($P< 0.001$). Eigenvalues were derived to assess the amount of variance explained by each component factor.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 package. When data were not normally distributed, values were expressed as medians with interquartile ranges (IQRs) and comparisons were made using the non-parametric Mann-Whitney U test. For normally distributed data, independent T test was used to compare means. Correlations were assessed either using Pearson correlation test for normally distributed data or Spearman's rho when data were not normally distributed. Linear regression was performed on ESSDAI components with the IFN score as the dependent variable. Differences were considered statistically significant if $p<0.05$.

RESULTS

Increased IFN type I inducible gene expression in pSS monocytes

In whole genome analyses of pooled monocytes, we previously showed 23 IFN type I inducible genes to be upregulated in pSS relative to HC [3]. On the basis of their high, intermediate or low levels of upregulation in the whole genome analysis, 11 of the 23 IFN type I inducible genes were selected for analysis in the present study using RQ-PCR in CD14+ monocytes and found to be significantly upregulated in the pSS group relative to the HC group (Figure 1A). The 11 IFN type I inducible genes analyzed in the monocytes were: IFI27, IFI44L, IFIT3, IFITM1, SERPING1, IFIT1, IFIT2, LY6E, IFI44, XAF1 and MX1.

Prevalence of "IFN type I signature" in pSS monocytes

The results of a principal component analysis showed a subset of 5 genes (IFI44, IFI44L, IFIT3, LY6E and MX1) to explain 95% of the total variance of the 11 IFN type I inducible genes. Given that the expression of these 5 IFN type I inducible genes was not normally distributed, log transformations of expression values were performed and IFN scores were calculated as described for SLE [27]. The mean and SD level of each IFN inducible gene in the HC group were used to standardize expression levels of each gene for each study subject. The standardized expression levels were subsequently summed for each patient to provide an IFN type I expression score.

The distribution of the IFN scores for the 69 patients was bimodal with an overlap at a score of 10. We therefore set the threshold for a positive IFN type I signature at 10. Adoption of this threshold showed 55% of pSS group to have an IFN type I signature and only 4.5% of the HC group (Figure 1B,1C).

To determine if the IFN type I signature possibly changes over time, we assessed IFN scores at two different time points in 24 pSS patients. Average period between the two measurement points was 3.6 ± 2.5 years. A significant difference in the scores over time was not detected (Figure 1C,1D), which shows a stability of the signature over time.

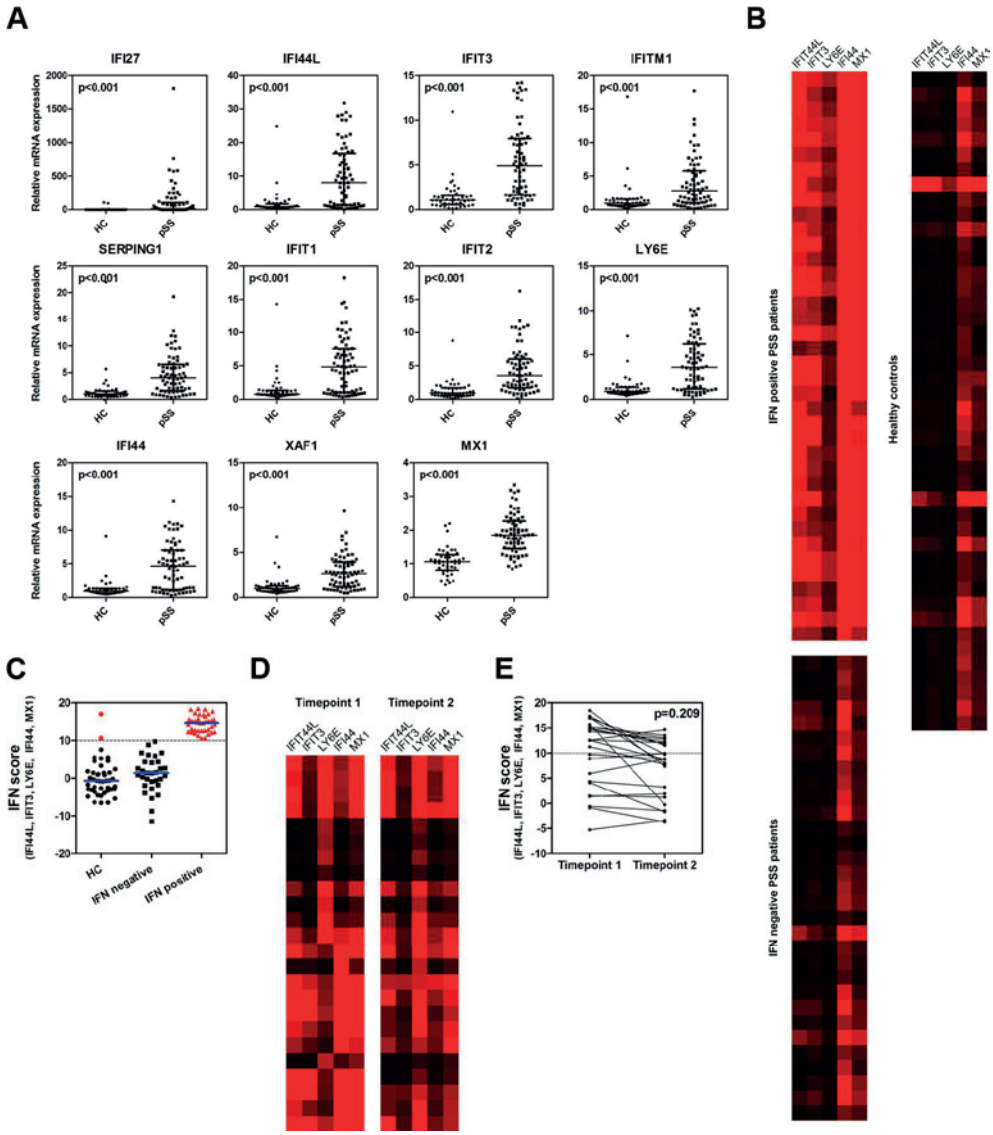

Figure 1 (A) Gene expression of 11 IFN type I inducible genes in pSS patients (n=69) and healthy controls (n=44). To compare means the Mann-Whitney U test was applied. **(B)** Heatmap showing gene expression of 5 IFN type I inducible genes in monocytes of pSS patients (n=69) and healthy controls (n=44). On the left the pSS patients are depicted and subdivided into IFN type I signature positive patients and IFN type I signature negative patients. On the right the healthy controls are depicted. Red colour indicates high gene expression **(C)** Distribution of IFN scores in IFN type I signature positive and negative patients and healthy controls. In red IFN type I positive cases are depicted. Blue lines depict medians. **(D)** Heatmap showing gene expression of IFN type I inducible genes in monocytes of pSS patients (n=24) at two different timepoints (average period between two measurements 3.6 ± 2.5 years). **(E)** No significant differences detected between two timepoints using the dependent T test.

Correlation of IFN type I signature with disease parameters

To investigate whether IFN type I activation, as reflected by a high IFN score, is associated with disease activity, we assessed ESSDAI disease activity scores in 38 pSS patients. Significant positive correlation was observed between IFN type I scores and ESSDAI scores (r=0.458, *P*=0.003). The pSS patients were next stratified according to their IFN type I signature status (IFN score<10 vs. IFN score≥10) and the disease activity scores were compared. Patients with a positive IFN type I signature (IFN score>10) showed significantly higher ESSDAI scores than those with a negative signature (Figure 2A). The high disease activity for patients with a positive IFN type I signature was mostly attributable to the presence of glandular, cutaneous and haematological manifestations (Table 2). Linear regression analysis showed glandular, cutaneous and articular manifestations - despite the nonsuggestive *P* value in the univariate analysis - to be associated with high IFN type I scores (Beta Coefficients with 95% C.I respectively: 5.90 (1.78, 10.01), 5.00 (1.66, 8.35) and 7.54 (2.38, 12.71).

Table 2. Comparison of IFN scores for pSS patients with or without different ESDDAI features

	Present		Absent		
ESSDAI feature	**No. of patients**	**Median (IQR)**	**No. of patients**	**Median (IQR)**	***P***
Constitutional	6	6.28 (-1.74,14.92)	32	5.19 (0.10,13.97)	NS
Lymphadeno-pathy	-		38	5.19 (-0.26,14.43)	ND
Glandular	9	14.7 (4.10,16.22)	29	2.65 (-1.16,12.41)	0.029
Articular	16	8.73 (1.02,14.94)	22	3.53 (-2.02,13.33)	NS
Cutaneous	6	15.26 (11.85,15.90)	32	3.53 (-2.02,13.33)	<0.001
Pulmonary	-	-	38	5.19 (-0.26-14.43)	ND
Renal	-	-	38	5.19 (-0.26-14.43)	ND
Muscular	-	-	38	5.19 (-0.26-14.43)	ND
PNS	3	15.20 (-5.27-15.58)	35	4.40 (-0.16,12.87)	NS
CNS	-	-	38	5.19 (-0.26-14.43)	ND
Hematological	2	16.09 (15.32,16.85)	36	4.40 (-0.46,12.83)	<0.001
Biological	20	10.21 (1.80,14.94)	18	3.97 (-3.21,12.88)	NS

CNS, central nervous system; PNS, peripheral nervous sytem, NS, nonsignificant; ND, not determined

In addition to the ESSDAI, demographic, laboratory and clinical parameters were collected. Comparison of IFN type I signature positive patients with IFN type I signature negative patients revealed significant differences in anti-SSA autoantibodies (anti-Ro52 and anti-Ro60), anti-SSB autoantibodies, rheumatoid factor, C3, IgG, lymphocyte and neutrophil count (Table 3).

Table 3. Comparison of pSS patients with or without a positive IFN type I signature

		IFN type I signature category		
Variable	**n**	**Positive**	**Negative**	***P***
Demographics				
Female gender (n)	69	34/38 (89%)	28/31 (90%)	0.908
Age (years)	69	56.7±12.5	59.5±13.3	0.376
Disease duration (years)	67	11.2±8.2	10.3±7.1	0.631
Laboratory parameters				
Anti-SSA (n)	69	37/38 (97%)	21/31 (68%)	**0.001**
Anti-Ro52 (n)	69	35/38 (92%)	19/31 (61%)	**0.002**
Anti-Ro60 (n)	69	34/38 (89%)	13/31 (42%)	**<0.001**
Anti-SSB (n)	69	32/38 (84%)	6/31 (19%)	**<0.001**
Rheumatoid factor (IE/ml)	50	25 (0,100)	0 (0,44)	**0.048**
C3 (g/l)	46	1.06±0.20	1.27±0.33	**0.012**
C4 (g/l)	46	0.18 (0.13,0.20)	0.18 (0.14,0.25)	0.261
IgG (g/l)	68	16.10 (13.15,18.55)	11.60 (10.6,15.00)	**0.001**
IgA (g/l)	54	3.20 (2.18,4.25)	2.44 (1.69,4.20)	0.179
IgM (g/l)	54	1.22 (0.78,1.97)	1.36 (1.07,1.67)	0.876
CRP (mg/l)	61	1.0 (0.0,5.5)	3.0 (1.0,7.0)	0.068
Hb (mmol/l)	60	7.95 (7.58,8.33)	8.25 (7.78,8.80)	0.159
Thrombocytes (*10E9/l)	51	219.5 (199.3,284.8)	257 (221,298)	0.190
Lymphocytes (*10E9/l)	42	1.32±0.55	1.85±0.57	**0.005**
Neutrophiles (*10E9/l)	41	3.09±1.18	4.42±1.78	**0.014**
Medical therapy				
Pilocarpine (n)	69	15/38 (39%)	12/31 (39%)	0.949
Plaquenil (n)	69	23/38 (61%)	17/31 (55%)	0.636
Corticosteroids (n)	69	3/38 (8%)	1/31 (3%)	0.412

Values are the mean ± SD, median (25% quartile-75% quartile), or number (%) of patients, depending on whether the data are normally distributed or not, and whether the data are continuous or dichotomous. When the data followed normal distribution, the independent t-test was conducted; otherwise the Mann-Whitney U test was conducted. CRP, C-reactive protein; Hb, haemoglobin

When patients were next stratified according to their autoantibody status (autoantibody positive vs. autoantibody negative) for comparison of their IFN scores, patients with autoantibodies showed higher IFN scores than patients without autoantibodies (Figure 2B). Rheumatoid factor and higher IgG levels were more often present in the IFN signature positive patients compared to IFN type I signature negative patients (Figure 2C, 2D). C3, lymphocytes and neutrophils were lower in IFN type I signature positive pSS patients (Figure 2E, 2F and 2G). No differences were found with respect to demographic characteristics or medication status (Table 3).

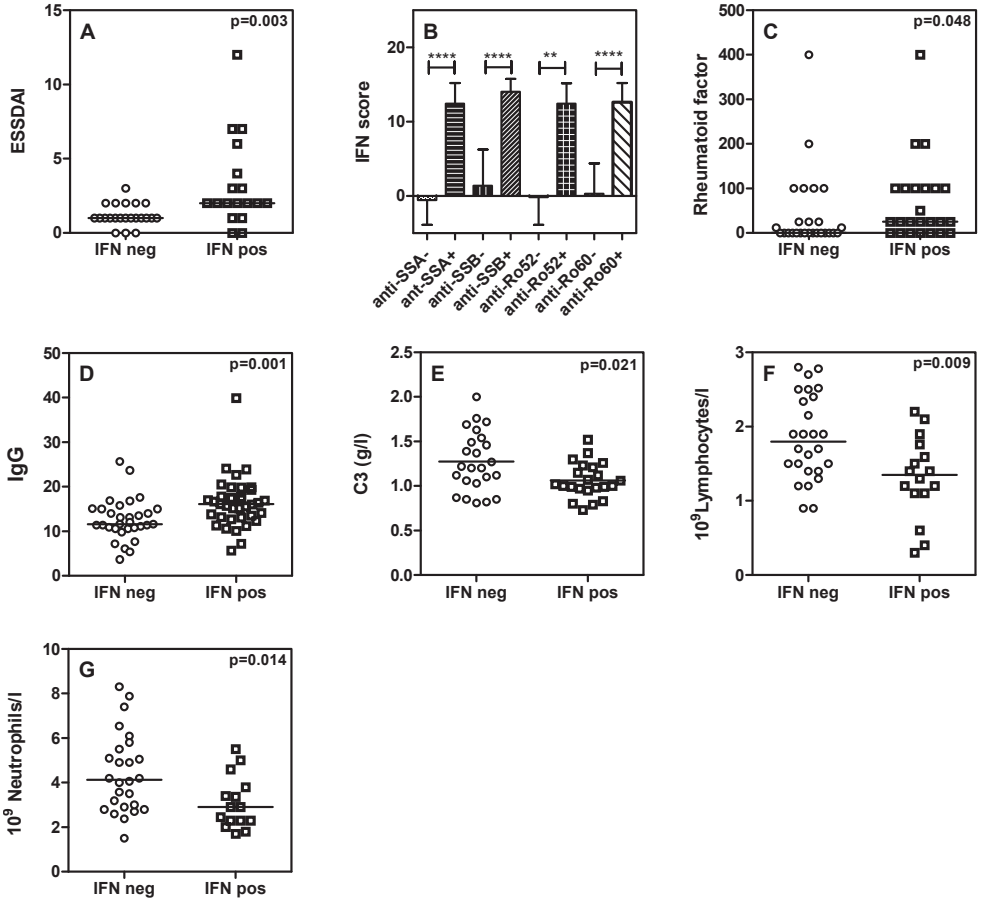

Figure 2 (A) ESSDAI scores in IFN type I signature positive and negative pSS patients (n=38). **(B)** IFN scores in pSS patients positive or negative for anti-SSA (both Ro52 and Ro60) and anti-SSB (n=69). **(C)** Rheumatoid factor levels in IFN type I signature positive and negative pSS patients (n=50). **(D)** IgG levels in IFN type I signature positive and negative pSS patients (n=68). **(E)** C3 levels in IFN type I signature positive and negative pSS patients (n=46). **(F)** Absolute lymphocyte levels in IFN type I signature positive and negative pSS patients (n=42). **(G)** Absolute neutrophil levels in IFN type I signature positive and negative pSS patients (n=41). Independent T-test was used to compare means in E-G where horizontal bars represent the means. Mann-Whitney U test was used to compare means in A-D where horizontal bars represent the medians. In B medians and interquartile range (IQR) are depicted; ** represents *P* value<0.01 and **** represents *P* value <0.0001.

Correlation between BAFF expression and IFN type I signature

As already mentioned, a factor also known to be involved in the pathogenesis of pSS and found to correlate with elevated serum levels of IgG, anti-SSA and anti-SSB is BAFF [16]. BAFF mRNA expression was increased in monocytes of the pSS group relative to the HC group (Figure 3A). Significant positive correlation between IFN score and BAFF mRNA expression was also thus found (Figure 3B).

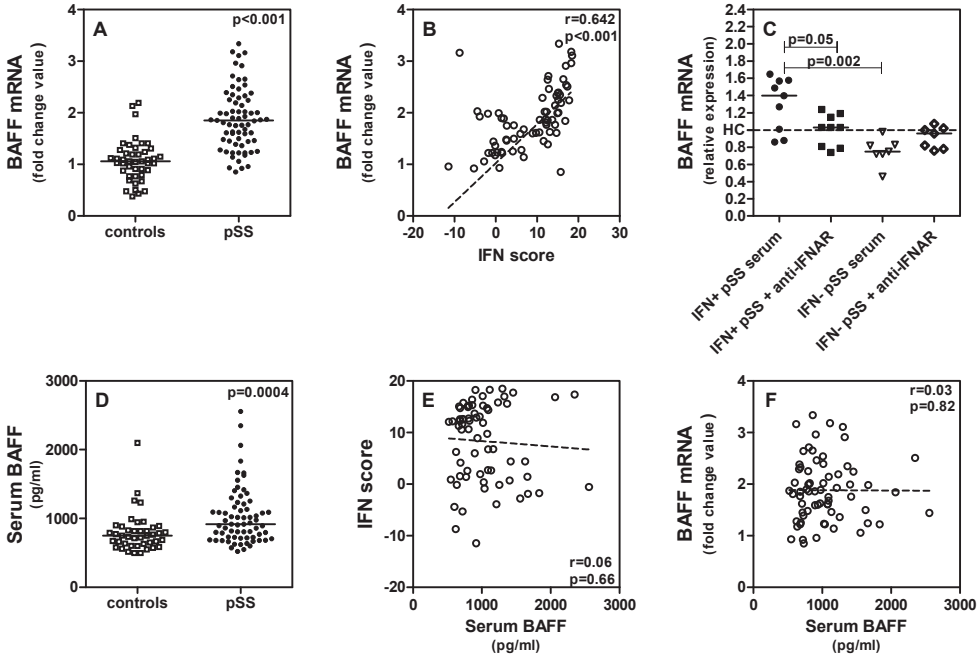

Figure 3 (A) BAFF mRNA expression in monocytes in pSS patients (n=69) and healthy controls (n=44). BAFF mRNA expression was determined by RQ-PCR. **(B)** Correlation between IFN type I score and BAFF mRNA expression in monocytes in pSS patients (n=69). **(C)** Induction of BAFF mRNA expression in THP-1 cells by incubation with 50% serum of IFN type I signature positive pSS patients (n=9) and IFN type I signature negative pSS patients (n=7) in the presence of a blocking IFN type I receptor antibody. Expression is relative compared to the mean expression in healthy controls (n=8) **(D)** BAFF protein levels in serum of pSS patients (n=68) and healthy controls (n=42) measured by ELISA. **(E)** Correlation between serum BAFF protein and IFN scores in pSS patients (n=68). **(F)** Correlation between serum BAFF protein and BAFF mRNA expression in monocytes in pSS patients (n=68). The correlation coefficients (r) and *P* values are shown. For correlations Spearman's rho correlation test was used and to compare means the Mann-Whitney U test was used.

We therefore next investigated if BAFF mRNA expression could be induced in monocytes by IFN type I serum activity. A bioassay was performed for this purpose using THP-1 monocytic cell line exposed to 50% by volume serum of pSS patients who were either positive or negative for the IFN type I signature with and without a blocking antibody against the IFN type I receptor. After incubation with serum from IFN type I signature positive patients (n=9), BAFF mRNA expression was indeed induced in THP-1 cells. Blocking IFN type I receptor diminished BAFF mRNA expression in THP-1 (Figure 3C). Serum of IFN type I signature negative patients did not induce higher BAFF mRNA expression relative to that found for the HC group.

To assess BAFF protein in serum, we performed an ELISA on 68 pSS samples and 42 HC. A statistical difference between pSS and HC was observed (Figure 3D). After stratification for positive versus negative IFN signature, however, no differences and no correlations between the IFN scores and serum BAFF protein were found (Figure 3E). Noteworthy serum BAFF

protein does not correlate with BAFF mRNA in monocytes (Figure 3F) and we also found in a smaller series that BAFF mRNA in monocytes does not correlate to BAFF mRNA in PBMC (see online supplementary Figure S1). This suggests that production of BAFF is differently regulated in different circulating leucocytes, however this needs further investigation.

DISCUSSION

This study shows increased IFN type I activity for 55% of a group of pSS patients versus to 4.5% of a HC group. The presence of such an "IFN type I signature" in monocytes of pSS patients was further shown to be associated with the ESSDAI, biological markers of activity and BAFF mRNA in monocytes. BAFF expression could also be induced in cultured monocytes by serum from pSS patients with a positive IFN signature.

Taken together, these results suggest the following scenario: A raised level of IFN type I, present in the serum of a significant number of pSS patients, induces in monocytes overexpression of IFN type I inducible genes amongst which is BAFF. After production, this cytokine can induce polyclonal B cell stimulation which results in higher autoantibody production and enhanced autoantigen-autoantibody reaction with complement consumption. This scenario fits with our observation of a correlation between the presence of a positive IFN type I signature in monocytes and cutaneous manifestations in the ESSDAI index. Moreover the biological domain of the ESSDAI includes markers for B cell activation such as IgG, cryoglobulinemia, decrease of complement. Thus part of the association between the IFN signature and the ESSDAI may be related to the role of IFN type I in B cell activation.

A similar scenario resulting in vasculitis has been described for SLE, systemic sclerosis and dermatomyositis. For subgroups of these diseases overexpression of IFN type I induced genes has also been observed. In SLE the overexpressed genes were associated – similar to the findings of our studies – with nuclear autoantibodies, glomerulonephritis and higher disease activity [28-30], again indicating the relationship of IFN activity to B cell activation and immune complex vasculitis. While these studies used PBMCs, we examined monocytes, as our focus of previous work was on the role of monocytes and dendritic cells in pSS. The IFN type I signature which we report for pSS monocytes is similar to the IFN inducible gene expression profile observed for pSS PBMCs [7]. Furthermore, we assessed the IFN type I signature in PBMCs of 12 pSS and 6 HC in supplementary analyses and found a high correlation with the IFN type I signature identified for monocytes in the same sample (see online supplementary Figure S2).

There was surprisingly no correlation of monocyte activation related to an IFN type I signature with other hallmarks of pSS (i.e. sicca symptoms and fatigue) (unpublished results). This is surprising as there are different reports of sicca syndrome and fatigue being induced

by Interferon type I therapy in patients with Hepatitis C and cancer [31, 32]. With regard to the sicca syndrome all patients scored positive and these symptoms are not quantified in the ESSDAI. Quantification using a Schirmer test or analogous test is therefore merited in future studies. With regard to fatigue this might be explained by the fact that we assessed fatigue using the VAS-score and not by the Multidimensional Fatigue Inventory, which covers different dimensions of fatigue. There are also reports that immune activation is not the only determinant of fatigue in IFN type I treated patients, but also the induction of IDO (indoleamine 2,3-dioxygenase) and the catabolism of tryptophan to quinolinic acid at the expense of serotonin [33]. More in-depth research into the relations between fatigue and IFN type I signature in pSS is thus essential.

Previously, we showed that although the number of plasmacytoid dendritic cells (pDC) was lowered in the blood of pSS patients - possibly due to migration into the glands - the remaining pDC showed increased activation [3]. There are indications that pDC are the source of the high IFN type I serum levels in SLE [34]. pDC are the most powerful producers of IFN type I. Stimuli for the pDC to produce IFN type I can be either exogenous (viral DNA/RNA) or endogenous, such as immune complexes of nuclear antigens and antibodies (hallmarks of pSS) which bind to FcγRIIa on the pDC, followed by internalization and binding to intracellular TLR7 and TLR9 [35]. This IFN type I production by pDC upon immune complex binding could be a potential explanation for the association we found between IFN type I scores and autoantibodies, IgG and lowered C3. The absence of modification of the IFN type I signature in patients treated with Plaquenil is surprising as Plaquenil is known to inhibit the activation of intracellular TLR. This could be explained by the fact that patients in our cohort are using Plaquenil for years. Assessment of IFN scores in patients before and after initiation of Plaquenil treatment in future research could therefore be informative.

At this point 2 limitations on our study should be mentioned. First, the patient group did not completely match the HC group with regard to age. In the statistical analysis we took that into account and could not find a significant influence of age on outcomes. A second possible limitation is the use of multiple parameters for our correlations between the presence of an IFN type I signature and clinical manifestations. Although it is questionable whether a Bonferroni correction is needed, when we applied such correction for n=22 (resulting in a *P* value of 0.002), the differences in rheumatoid factor, C3 and lymphocyte and neutrophil counts lost significance. In fact to reach significance after such correction for C3 for example, 88 patients would have to be studied. Our study of 69 pSS patients is relatively large, but new validation studies with larger numbers of participants are called for — also in light of the fact that the ESSDAI was measured in only 38 patients and laboratory parameters were not available for all patients.

To conclude, the findings of the present study suggest that determining the presence of an "IFN type I signature" in monocytes of pSS patients can be done to identify subgroups

of patients for specific treatment. Patients identified in such a manner may benefit from treatment aimed at blocking the IFN type I activity and thereby counteracting B cell activation and autoantibody production.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. J.J. Luime and Dr. G.J.J.M. Borsboom (Erasmus MC Rotterdam, The Netherlands) for their help with the epidemiological and statistical analyses.

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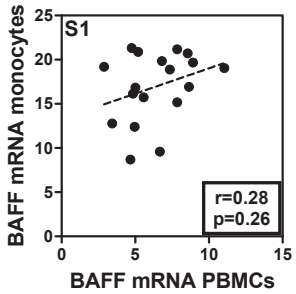
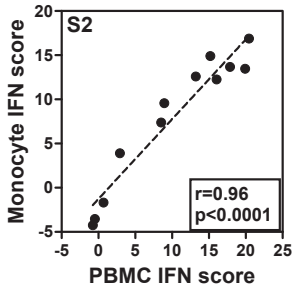


Supplemental Figure 1 BAFF mRNA expression was assessed in PBMCs of 6 IFN signature+ pSS patients (monocyte data), 6 IFN signature- pSS patients (monocyte data) and 6 HC. BAFF mRNA in PBMCs of these cases does not correlate to BAFF mRNA in monocytes of the same cases. Spearman's rho correlation test was used and correlation coefficient (r) and *P* value are shown.



Supplemental Figure 2 Expression of the 11 IFN type I inducible genes was assessed in PBMCs of 6 IFN signature+ pSS patients (monocyte data), 6 IFN signature- pSS patients (monocyte data) and 6 HC. The results of a principal component analysis showed a subset of 4 genes (IFI44L, IFIT1, IFI44 and MX1) to explain 98% of the total variance of the 11 IFN type I inducible genes. The IFN scores based on these 4 genes in PBMCs correlate with the IFN scores in monocytes of the same cases. Spearman's rho correlation test was used and correlation coefficient (r) and *P* value are shown.

CHAPTER 3

MXA AS A CLINICALLY APPLICABLE BIOMARKER FOR IDENTIFYING SYSTEMIC INTERFERON TYPE I IN PRIMARY SJÖGREN'S SYNDROME

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Annals of the Rheumatic Diseases. 2013 Jul 6. 10.1136/annrheumdis-2012-202552. [Epub ahead of print]

ABSTRACT

Objective To establish an easy and practical assay for identifying systemic Interferon (IFN) type I bioactivity in primary Sjögren's syndrome (pSS). The IFN type I signature is present in over half of pSS patients and identifies a subgroup with higher disease activity. This signature is currently assessed via laborious expression profiles of multiple IFN type I inducible genes.

Methods In a cohort of 35 pSS patients, Myxovirus resistance protein A (MxA) was assessed as a potential biomarker for IFN type I activity, using an enzyme immunoassay (EIA) on whole blood and flow cytometric analyses (FACS) of isolated $CD14^+$ monocytes. In addition, potential biomarkers CD64, CD169 and BAFF were simultaneously analysed in $CD14^+$ monocytes using FACS. The IFNscore, a measure for total IFN type I bioactivity, was calculated using expression values of the IFN type I signature genes – IFI44, IFI44L, IFIT3, LY6E, MX1 – in $CD14^+$ monocytes, determined by real-time quantitative PCR.

Results IFNscores correlated strongest with monocyte MxA-protein ($r=0.741$, $p<0.001$) and whole blood MxA-levels ($r=0.764$, $p<0.001$), weaker with CD169 ($r=0.495$, $p<0.001$) and CD64 ($r=0.436$, $p=0.007$), and not at all with BAFF protein. In particular, whole blood MxA-levels correlated with ESSDAI scores and numerous clinical pSS parameters. Interestingly, patients on hydroxychloroquine showed reduced MxA-levels (EIA, $p=0.04$; FACS $p=0.001$).

Conclusion The MxA-assays were excellent tools to assess IFN type I activity in pSS, MxA-EIA being the most practical. MxA-levels associate with features of active disease and are reduced in hydroxychloroquine-treated patients, suggesting the clinical applicability of MxA in stratifying patients according to IFN-positivity.

INTRODUCTION

Primary Sjögren's syndrome (pSS), the second most common systemic autoimmune disease, is characterized by lymphocytic infiltrates in the salivary and lachrymal glands. Clinical manifestations range amongst others from ocular and oral dryness to vasculitis and severe fatigue. To date, effective therapy is not available and treatment has been mainly symptomatic. The etiology of pSS is largely unknown, but evidence for a role of Interferon (IFN) type I in the pathogenesis of pSS has been emerging [1-12]. Further unraveling the complex pathophysiology of pSS is essential for finding disease-related biomarkers and identifying new treatment targets.

Previously, we described systemic upregulation of IFN type I inducible genes (IFIGs) in $CD14^+$ monocytes of pSS patients, the so-called IFN type I signature [7]. The signature is not restricted to monocytes, as a similar IFN type I signature has been observed in pSS peripheral blood mononuclear cells (PBMCs) [12, 13]. Assessment of the IFN type I signature is however a laborious real-time-PCR technique for the expression of multiple IFIGs and at present, another reliable immunoassay for detection of type I IFNs in blood or serum is not available. The latter is mainly due to the presence of multiple subtypes of IFN type I [5, 14, 15]. An easy and functional assay to determine the presence of IFN type I activity in the circulation would facilitate the identification of pSS patients with an IFN type I signature and further contribute to unraveling the role of IFN type I in pSS.

The IFN type I signature, as defined by us, resulted from a factor analysis of multiple tested IFIGs and showed five genes – IFI44L, IFI44, IFIT3, LY6E and MX1– to predict 95% of the total variance of a larger set of IFIGs. These 5 genes were used to calculate an IFN type I expression score (IFNscore) per subject. Recently, we showed this IFN type I signature in pSS monocytes to be associated with higher EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores as well as higher auto-antibody production, rheumatoid factor and serum IgG, lower C3 complement levels, and higher B cell activating factor (BAFF) *gene* expression [13].

The IFN type I signature is also displayed by subgroups of patients with systemic sclerosis (SSc), systemic lupus erythematosus (SLE), dermatomyositis and rheumatoid arthritis (RA), and several biomarkers for IFN type I activity have been proposed, amongst others MxA, CD64 and CD169 [15-24]. However, these protein markers have never been tested systematically and simultaneously in a single (pSS) patient group.

MxA (Myxovirus-resistance protein 1) is a key mediator of the IFN-induced antiviral response and is tightly regulated by type I IFNs [25-27]. In SSc, MxA gene expression was proposed as biomarker for IFN type I bioactivity and found to correlate with disease activity [15]. Several methods for detection of MxA have been previously described [15, 19, 28, 29]. Vallittu et al. evaluated their enzyme immunoassay (EIA) for MxA in whole blood for monitoring IFN type I bioactivity in multiple sclerosis (MS) patients treated with IFN-β. The MxA-EIA was considered by the authors to be a faster and more reliable method compared

to flow cytometric analysis of MxA in PBMCs [19]. To our knowledge, MxA-EIA has never been used for the detection of IFN type I activity in systemic autoimmune diseases.

In this study, we tested MxA as a candidate biomarker for systemic IFN type I activity in pSS using EIA and flow cytometry in whole blood lysates and $CD14^+$ monocytes, respectively. In addition, the expression of CD64 (FcγRI), CD169 (Siglec-1), and BAFF proteins were simultaneously assessed in $CD14^+$ monocytes, as these markers have also been proposed as biomarkers for IFN type I bioactivity in systemic autoimmunity [21-23, 30].

PATIENTS AND METHODS

Patients

Thirty-five patients positively diagnosed with pSS, according to the 2002 American-European criteria were recruited [2]. Patients treated with high doses of prednisone (>10mg daily), immunosuppressants, or biologicals were excluded. Level of disease activity was assessed using EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [31]. Twenty-seven HC, neither suffering from autoimmune diseases nor using corticosteroids, were included. Study subjects were screened to be free of symptoms of underlying viral infections at inclusion (Table 1). The Medical Ethical Review Board of the Erasmus MC Rotterdam approved the study and written informed consent was obtained.

Blood collection, preparation and monocytes isolation

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes (Greiner Bio-One, Germany). From heparinized blood, PBMCs and consecutively $CD14^+$ peripheral blood monocytes, were isolated as previously described [32].

Measurement of complement, immunoglobulin levels and autoantibodies

C3 and C4 complement, IgG, IgM and IgA immunoglobulin and anti-SSA/B autoantibody levels were measured as described previously [13].

Real-time quantitative PCR

Total RNA was isolated from purified $CD14^+$ monocytes using RNAeasy columns Qiagen, Hilden, Germany), subsequently reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit and RQ-PCR analysis using predesigned primer/probe sets (Applied Biosystems, Foster City, California, USA) [32]. For calculation of relative expression, all samples were normalized to expression of the household gene Abl [33]. Fold change values were determined from normalized CT values using $2^{-}\Delta\Delta CT$ method (User Bulletin, Applied Biosystems).

Table 1. Patient and control characteristics

		pSS	
	Controls (n=27)	**IFNpos (n=21)**	**IFNneg (n=14)**
Demographics			
Female (%)	24/27 (89)	16/21 (76)	12/14 (86)
Mean age (years)*	53 ± 11	57(30-85)	55 (37-76)
Disease duration (years)*	NA	13.8 ± 6.4	10.9 ± 7.7
pSS manifestations (%)			
Ocular symptoms	NA	20/21 (95)	14/14 (100)
Oral symptoms	NA	21/21 (100)	13/14 (93)
Positive ocular tests	NA	15/21 (71)	12/14 (86)
Positive histopathology	NA	11/21 (52)	7/14 (50)
Positive salivary gland tests	NA	1/21 (5)	1/14 (7)
Anti-SSA	NA	20/21 (95)	10/14 (71)
Anti-SSB	NA	17/21 (81)	6/14 (43)
Medication status (%)			
Pilocarpine	NA	5/21 (24)	4/14 (29)
Hydroxychloroquine	NA	13/21 (62)	13/14 (93)
Corticosteroids	NA	1/21 (5)	1/14 (7)

*Data are presented as means with corresponding range or SD.
IFNpos, IFN type I signature positive; IFNneg, IFN type I signature negative; NA, not applicable; pSS, primary Sjögren's syndrome.

Flow Cytometry

FACS (fluorescence-activated cell sorting) analysis was used to determine surface expression and intracellular cytokine content in purified $CD14^+$ monocytes. Membrane staining was performed with fluorescently labeled antibodies: anti-CD14 (APC/Cy7; BD Biosciences, CA, USA), and either anti-CD64 (PE; Serotech, Toronto, Canada) or CD169 (AF; Serotech), 20 min in the dark. Subsequently cells were fixed (paraformaldehyde), permeabilized (0.5% saponin), and stained with either anti-BAFF (FITC; R&D Systems, PHL, USA) or unconjugated anti-MxA (ProteinTech group, IL, USA), incubated in the dark 30 min on ice; secondary antibody goat-anti rabbit-FITC (Supertech). Unstained cells and appropriate isotype-matched controls (BD Biosciences) were used to confirm antibody-specificity. Fluorescence was assayed using four-colour flow cytometry (FACS® Calibur, Beckon&Dickinson, USA) and analysed using FlowJo Software (TreeStar Inc., USA).

MxA Enzyme immunoassay (EIA)

MxA-EIA was executed as previously described [19]. Heparinised blood (25µl) was lysed 1:20 and stored at -70°C until assayed [19]. Briefly (see online supplementary Figure S1 for full description), lysed whole blood samples and biotinylated detector-monoclonal antibody (MAb) were loaded onto MAb-coated micortiter strips and incubated overnight at 8°C, color-reaction was stopped, absorbance$_{450}$ was measured and [MxA] read from a master standard-curve. Detection-limit was 10µg/l, determined as three times the standard deviations (SD) of 8 negative control replicates [26].

IFN score

Monocyte IFN type I signature was defined by the relative expression of 5 IFIGs – IFI44L, IFI44, IFIT3, LY6E and MxA [13]. Mean$_{HC}$ and SD$_{HC}$ of each gene in the HC-group were used to standardize expression levels. IFNscores per subject represent the sum of these standardized scores, calculated as previously described [34, 35]. IFNscore-distribution for the 35 patients was bimodal, with an overlap at a score of 10. Furthermore HC only occasionally show IFNscore≥10; consequently values≥10 represent IFN type I signature positivity [13].

Statistical analysis

Comparisons were analyzed using the non-parametric Mann-Whitney U test to compare medians, as an independent T-test was used to compare means (for normally distributed data). For correlation studies the Spearman's rho (r_s) or Pearson correlation coefficient (r_p) were calculated. Values of $p<0.05$ were considered statistically significant. For multiple variable testing, values of $p<0.01$ ($p<0.05/5$) were considered statistically significant after applying a Bonferroni correction (n=5). Multiple group comparisons were analyzed using the Kruskal-Wallis test; Statistical analysis performed using SPSS17.0.

RESULTS

Stratification of pSS patients into IFN type I signature positive and negative patients

We utilized our previously described definition for the IFN type I signature [13] and reconfirmed the presence of the signature in the current cohort – using the 5 indicator genes IFI44L, IFI44, IFIT3, LY6E and MX1 (Figure 1A). We stratified the patient group as previously described in patients positive for the IFN type I signature (*IFNpos*; IFNscore≥10) and patients negative for the signature (*IFNneg*; IFNscore<10). Using this paradigm, none of the HC scored above 10 whereas 21 out of 35 pSS patients were IFNpos – a prevalence of 60% (Figure 1B). In Figure 1C gene expression for the 5 signature genes is depicted in a heatmap.

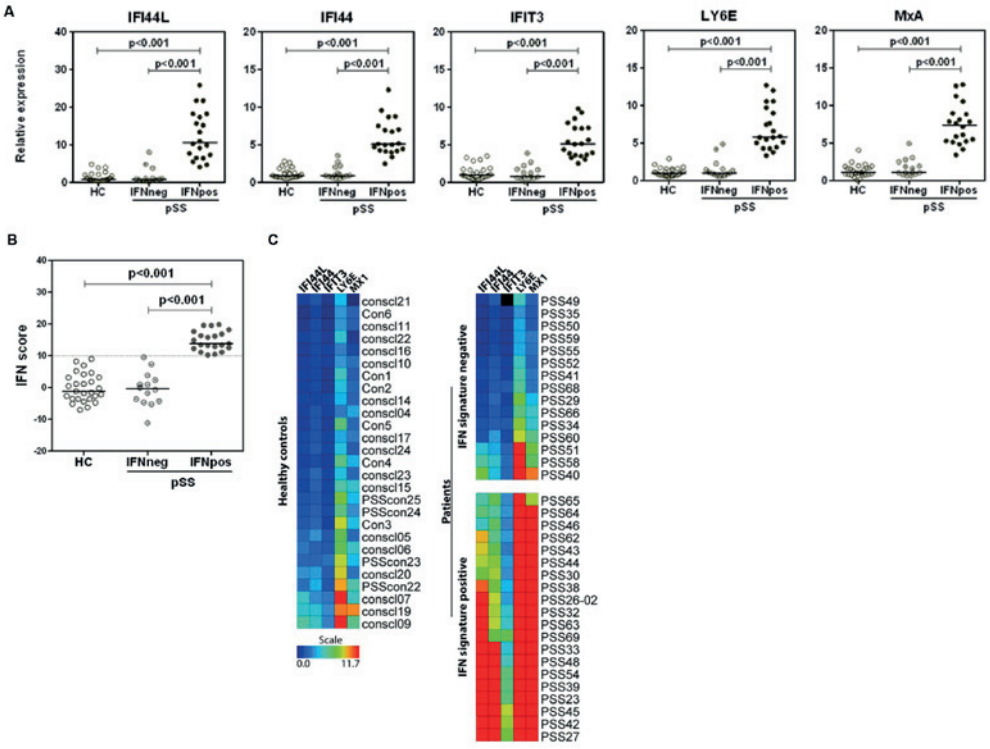

Figure 1 (A) Relative mRNA gene expression is shown for the 5 IFN type I inducible genes of the IFN type I signature in CD14⁺ monocytes of pSS patients (n=35) and healthy controls (HC) (n=27). Patient group is stratified in patients negative for the IFN type I signature (IFNneg) (n=14) and patients positive for signature (IFNpos) (n=21). **(B)** The IFNscore is shown for HC and pSS patients. A cutoff value of 10 is used as threshold: IFNneg (IFNscore <10) and IFNpos (IFNscore≥10) pSS patients. Each symbol represents an individual sample; horizontal lines represent the median. *P* values are shown, and to compare medians the Mann-Whitney U test was used. **(C)** Heatmap depicting gene expression of IFN type I inducible genes. Individual study subjects sorted according to increasing IFNscore (scale from blue to red) and stratified in HC (left), IFNneg (right upper panel) and IFNpos pSS patients (lower panel).

MxA correlates strongly to IFN type I bioactivity in pSS

To assess MxA protein as a potential biomarker for the IFN type I signature, intracellular MxA was measured in CD14⁺ monocytes using flow cytometry and in whole blood lysates using MxA-EIA.

Significant positive correlation was observed between the IFNscore and intracellular MxA-levels, as assessed by flow cytometry (Figure 2B). Relative mean fluorescence intensity (MFI) scores of MxA were next compared between HC, IFNneg and IFNpos pSS patients. Quantification of CD14⁺ monocyte MxA-levels showed a significant increase in IFNpos patients compared to both IFNneg patients and HC, whereas IFNneg patients showed MxA-levels indistinguishable from HC (Figure 2C,D).

Utilizing the MxA enzyme immunoassay (EIA), MxA protein-levels (µg/l) were assessed in lysed whole blood of the same study subjects. Correlating whole blood MxA-levels to IFNscores showed a significant positive correlation (Figure 2E). IFNpos patients showed significantly elevated MxA protein-levels, compared to both IFNneg patients and HC. An increase was not observed in IFNneg patients, again showing MxA-levels equal to HC (Figure 2F).

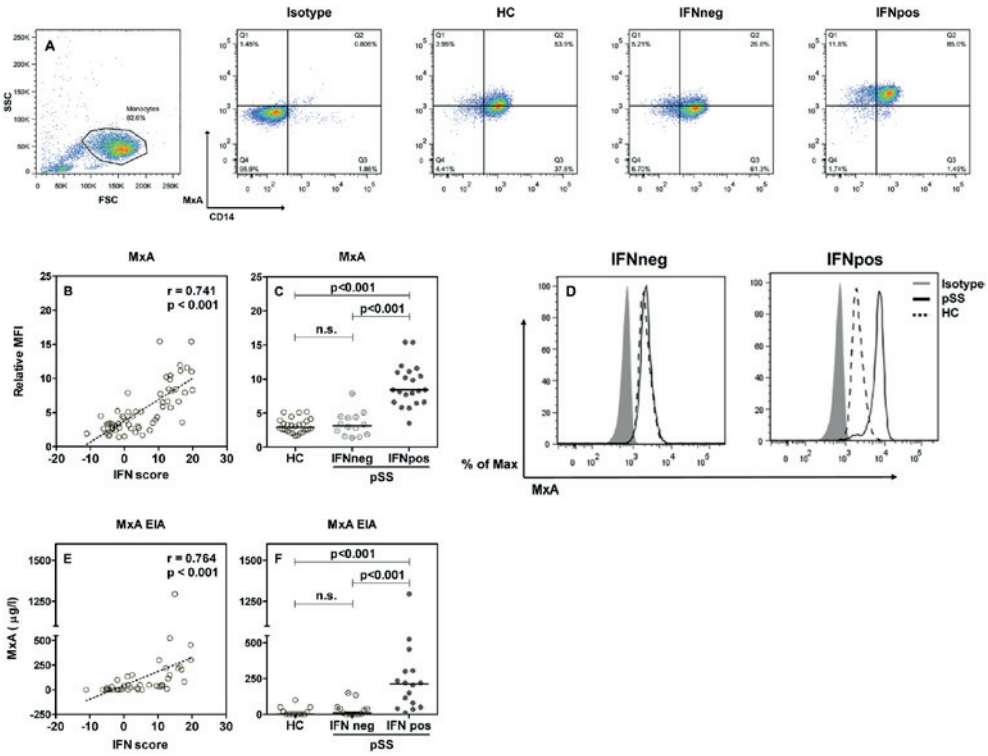

Figure 2 Flow cytometric analysis of MxA protein expression in CD14⁺ monocytes from HC (n=27) and pSS patients stratified in IFNneg (n=14) and IFNpos (n=21) patients. **(A)** Representative dot plots show CD14 and MxA protein expression on monocytes from study subjects. **(B)** Correlation plot shown between IFN scores for all study subjects and relative mean fluorescent intensity (MFI) of MxA. **(C)** Relative MFI of MxA is shown for HC, IFNneg and IFNpos pSS patients. **(D)** MxA levels are shown for IFNneg (left) and IFNpos (right) pSS patients. Representative histograms are depicted; Shaded histogram represents the isotype control, dotted line represents the HC and the solid line the pSS patients. Relative expression was calculated as MxA-specific staining (MFI)/ isotype control (MFI).
MxA levels (µg/l) determined by MxA-enzyme immunoassay (EIA) in whole blood lysates of HC and pSS patients. **(E)** Correlation plot between IFN scores for all study subjects and intracellular MxA-EIA (µg/l) levels. **(F)** MxA-EIA (µg/l) levels shown for HC and pSS patients, stratified in IFNneg and IFNpos patients. Each symbol represents an individual sample (duplicates of 2 separate EIA-measurements per study subject); horizontal lines represent the median. The correlation coefficients (r) and *P* values are shown. For correlations Spearman's rho correlation test was used and to compare medians the Mann-Whitney U test was used.

CD64 and CD169 correlate weakly to IFN type I bioactivity in pSS

We also assessed surface protein levels of CD64 and CD169 (other proposed biomarkers of IFN activity) on CD14⁺ monocytes using flow cytometry (see online supplementary Figure S2). A significant positive correlation was observed between the IFNscore and surface expression of both CD64 (Figure 3A) and CD169 (Figure 3B) respectively, though with considerably lower correlation coefficients as found for MxA (Fig.2).

Figure 3D shows CD64 upregulation on monocytes of pSS patients irrespective of their IFN positivity. For CD169 surface expression this was not the case as the marker was solely upregulated in IFNpos pSS patients (Figure 3E).

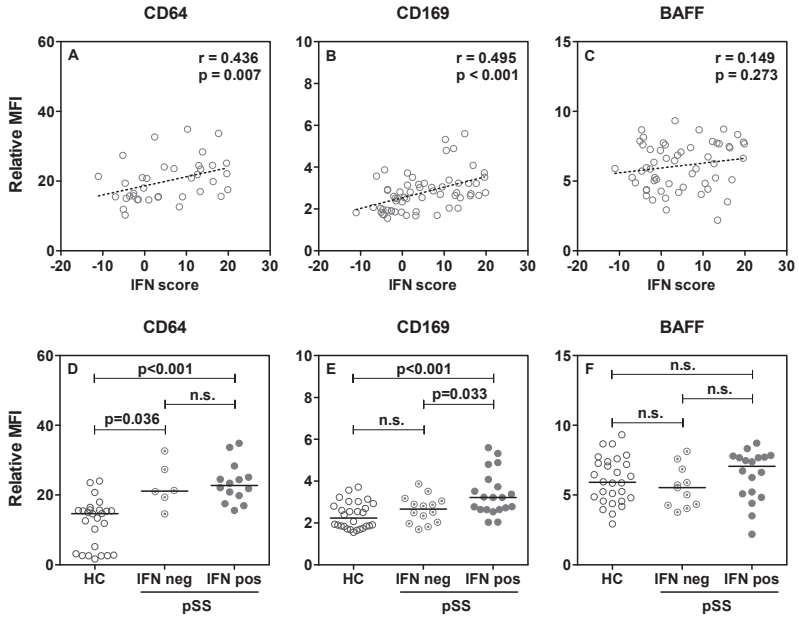

Figure 3 Flow cytometric analysis of CD14⁺ monocytes from HC and pSS patients. Correlation plots shown between IFN scores for all study subjects and **(A)** CD64, **(B)** CD169 and **(C)** BAFF protein levels, respectively. Relative mean fluorescent intensity (MFI) for **(D)** CD64 **(E)** CD169 and **(F)** BAFF protein levels shown for CD14⁺ monocytes from HC (n=27) and pSS patients, stratified in IFNneg (n=14) and IFNpos (n=21) patients. Each symbol represents an individual sample; horizontal lines represent the median. Relative expression was calculated as protein-specific staining (MFI)/ isotype control (MFI). The correlation coefficients (r) and *P* values are shown. For correlations on CD64 and CD169, Spearman's rho correlation test was used and to compare medians the Mann-Whitney U test was used. For BAFF a Pearson correlation test was used and to compare means the independent T-test.

BAFF does not correlate to IFN type I bioactivity in pSS

Previously we established a positive correlation for the presence of the IFN type I signature and BAFF mRNA *gene* expression in CD14⁺ monocytes[13]. This observation was confirmed in our current cohort (Figure S3). We therefore assessed intracellular BAFF *protein* in CD14⁺ monocytes as a candidate biomarker for IFN type I, using flow cytometry. Intracellular BAFF

levels showed no correlation with the IFNscore (Figure 3C, S2). Moreover, BAFF protein levels were not significantly elevated in pSS patients compared to HC, neither in IFNpos nor IFNneg patients (Figure 3F).

MxA correlates with disease manifestations of pSS

Previously observed association of the IFN type I signature with higher ESSDAI disease activity scores [13] (reconfirmed in this study (Figure 4A)), gave rise to the question whether this also holds true for the here described potential biomarkers MxA, CD64 and CD169. We were able to assess ESSDAI disease activity scores in 23 pSS patients of this cohort. Significant correlation was observed between ESSDAI scores and MxA-levels measured by either assay, though the correlation was strongest when the EIA was used (Table 2, Figure 4B,C). For the other biomarkers no correlation was found (Table S1).

Table 2. Comparisons of the MxA biomarker – assessed by MxA-EIA and MxA-FACS - with clinical and laboratory parameters of pSS patients.

		MxA-EIA			MxA-Facs	
Variable	**N**	**r**	**p value**	**N**	**r**	**p value**
ESSDAI score	24	**0.65**	**0.00**	28	0.45	0.02
Laboratory parameters						
Rf (IE/ml)	22	0.57	0.01	26	0.33	n.s.
C3 (g/l)[a]	27	0.03	n.s.	33	-0.17	n.s.
C4 (g/l)	27	-0.07	n.s.	33	-0.43	n.s.
IgG (g/l)[a]	29	**0.52**	**0.00**	35	0.40	0.02
IgA (g/l)	27	0.44	0.02	33	0.41	0.02
IgM (g/l)	27	0.46	0.02	33	0.18	n.s.
CRP (mg/l)	26	0.09	n.s.	31	0.22	n.s.
Hb (mmol/l)	29	-0.45	0.02	35	-0.61	n.s.
Thrombocytes (*10E9/l)[a]	28	0.20	n.s.	34	0.03	n.s.
Lymphocytes (*10E9/l)[a]	26	0.03	n.s.	32	-0.11	n.s.
Neutrophiles (*10E9/l)	26	-0.40	0.05	32	-0.18	n.s.
Variable	**N (%)**	**Z**	**p value**	**N (%)**	**Z**	**p value**
Auto-antibodies						
Anti-SSA	24/29 (83)	**-3.07**	**0.00**	30/35 (86)	-2.40	0.02
Anti-Ro52	16/22 (73)	-2.26	0.02	21/27 (78)	-1.98	0.05
Anti-Ro60	15/22 (68)	**-3.02**	**0.00**	19/27 (70)	-2.76	0.01
Anti-SSB	19/29 (66)	**-3.20**	**0.00**	23/35 (66)	-1.63	n.s.
Medical therapy						
Pilocarpine	7/29 (24)	-1.36	n.s.	9/35 (26)	-1.47	n.s.
Plaquenil	23/29 (79)	-2.05	0.04	26/35 (74)	**-3.25**	**0.00**
Corticosteroids	2/29 (7)	-1.64	n.s.	3/35 (9)	-0.59	n.s.

Data are presented as Spearman's rho (r) or according to κ2 test (Z), unless otherwise mentioned
[a]Data normally distributed are presented as Pearson's rho (r)
Rf, Rheumatoid factor; CRP, C-reactive protein; Hb, Hemoglobin; ns, not significant

We subsequently determined whether the potential biomarkers were associated with classical aberrant immune parameters of pSS, such as anti-SSA and anti-SSB autoantibodies, rheumatoid factor, immunoglobulin levels and neutrophil counts (Table 2). In particular whole blood MxA-levels showed the strongest and most significant correlations with many of these parameters; patients with autoantibodies showed higher whole blood MxA-levels compared to patients free of autoantibodies (Figure 4D). Significant positive correlations were also found between whole blood MxA and rheumatoid factor, and immunoglobulin levels (IgG, IgA and IgM) (Table 2, Figure 4E). Significant negative correlations were found between whole blood MxA, hemoglobin levels and neutrophil counts. Monocyte MxA(-Facs) and CD169-levels performed less well in these comparisons (Table 2, Table S1).

Patients on hydroxychloroquine (Plaquenil®) treatment had significantly reduced biomarkers of IFN activity (Figure 4F,G); Table 2 and Figure 4G show patients on hydroxychloroquine treatment to have reduced levels of MxA, measured by both EIA and FACS. Hydroxychloroquine was however found to have no significant effect on ESSDAI levels (Figure 4H).

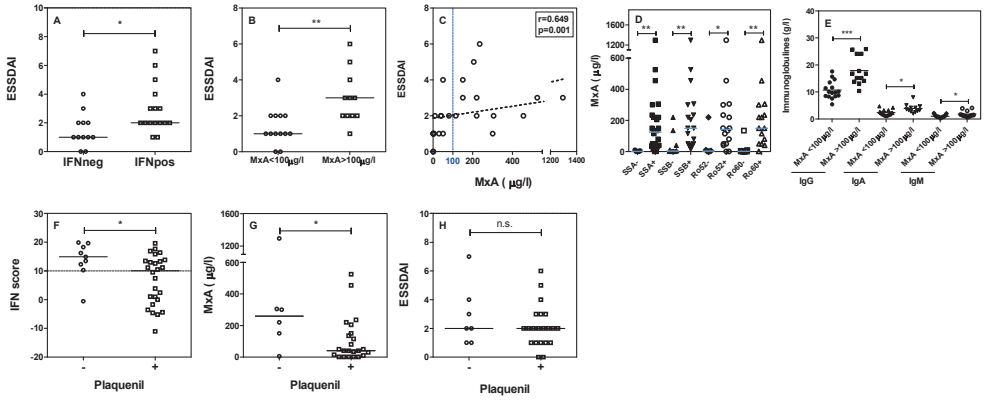

Figure 4 EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores stratified in **(A)** IFNneg and IFNpos pSS patients **(B)** MxA (µg/l) levels as measured by the MxA-EIA; MxA<100µg/l (low) and MxA>100µg/l (high). Cut-off determined as mean$_{HC}$ + 2 x SD$_{HC}$ **(C)** Correlation plot shown between ESSDAI scores and MxA (µg/l) levels. **(D)** MxA (µg/l) levels in pSS patients in presence (+) or absence (-) of auto-antibodies; anti-SSA (divided in Ro52 and Ro60) and anti-SSB **(E)** Immunoglobulin - IgG, IgA and IgM - levels (g/l) in pSS patients stratified in MxA<100µg/l and MxA>100µg/l. Comparisons for pSS patients in presence (+) or absence (-) of hydroxychloroquine (Plaquenil®) treatment plotted for **(F)** the IFNscore; line represents IFNscore cut-off ≥10, **(G)** MxA (µg/l), as measured by the MxA-EIA and **(H)** ESSDAI scores. Each symbol represents an individual sample; horizontal lines represent the median. The correlation coefficient (r) and *P* values (*p>0.05; **p>0.01; ***p>0.001) are shown, Spearman's rho correlation test was used and to compare medians the Mann-Whitney U test was used.

DISCUSSION

Our study is the first to simultaneously assess protein levels of MxA, CD64, CD169 and BAFF as biomarkers for IFN type I detection. Herein MxA was distinguished as the best functional biomarker for systemic IFN type I activity in pSS.

MxA protein is considered an important mediator of early innate immune defense and its expression has been used as a marker for IFN bioactivity in experimental as well as clinical settings [19, 27]. The MxA gene, in contrast to other IFIGs, is not directly inducible by viruses and depends strictly on IFN signaling [27]. Furthermore, the stability of MxA protein in cells was observed to be higher than corresponding mRNA levels [26]. We assessed intracellular MxA protein using an EIA-method on whole blood and flow cytometric analysis on circulating $CD14^+$ monocytes. In our patients, flow cytometric detection of MxA in monocytes showed a slightly better distinction between IFNpos and IFNneg patients within this pSS group, relative to the EIA in whole blood. Nevertheless, the MxA-EIA was a more practical assay as it requires only 25µl of whole blood – obtained during routine blood collection and easily stored at -70°C to the time of assessment – without necessity of laborious PBMC- and subsequent monocyte-isolation. Vallittu *et al.*, who previously validated the MxA-EIA for monitoring MS patients treated with IFN-β, concluded the EIA favourable for large scale clinical application due to its high level of standardization and reproducibility [19]. We also experienced the EIA to be less variable than FACS (data not shown). This report shows whole blood MxA-levels measured by EIA to have the strongest correlation with features of active pSS: i.e with higher ESSDAI scores, anti-SSA/SSB antibodies, rheumatoid factor, immunoglobulin levels, lower hemoglobulin levels and neutrophil counts. We found no association with complement consumption, as previously found for the IFNscore [13]. On the basis of these observations, we propose to use the MxA-EIA for identification of IFN type I bioactivity in pSS.

Alongside MxA, we also considered CD64, CD169 and BAFF as candidates, observing a significant correlation between CD64 and CD169 and the IFNscore, be it with a relatively low correlation-coefficient.

CD64, the high affinity Fc-gamma receptor I (FcγRI) recognizing Fc portion of IgG, is exclusively expressed on myeloid cells and found to correlate to IFIG-expression in SLE [21, 23]. Levels of CD64 on monocytes did neither correlate with IFN-activity, nor with the clinical and laboratory parameters. In contrast, the surface marker was overexpressed in all pSS patients. We therefore consider it a marker of systemic autoimmunity in general [36].

CD169, also termed sialoadhesin (Siglec-1), previously identified as a biomarker for IFN type I activation in SLE, was found to correlate with disease severity [16]. Also in SSc, elevated CD169 levels were found on monocytes [17]. In our hands, CD169 was elevated in IFNpos pSS patients and correlated to several clinical and laboratory parameters, although not as strongly as the MxA assays.

BAFF overexpression has been observed in serum, salivary glands, and monocytes in pSS [18, 20, 37, 38]. We previously observed a strong correlation between the IFN type I signature and BAFF mRNA in pSS monocytes [13]. Moreover we found BAFF serum protein increased in pSS compared to HC but did not find a correlation with the IFNscore, additionally BAFF serum protein did not correlate with BAFF mRNA. We here report that BAFF protein levels showed a slight but not significant increase in IFNpos patients, while BAFF mRNA was indeed significantly increased in IFNpos patients. This again indicates the lack of correlation between BAFF mRNA and protein.

Some limitations of our study are for one, the relatively small sample size for CD64. Assessing this surface marker in a larger set of patients is warranted to better understand its role in systemic autoimmunity. Secondly, MxA-EIA levels were not assessed for all subjects due to whole blood necessity. Furthermore, absence of viral infections was defined as 'no symptoms of underlying viral infections' at inclusion, whereas no viral screening was done to confirm. In HC with relatively high MxA-levels however, ENA-profiles were run to rule out autoantibody-presence. Third, the ESSDAI as well as some clinical and laboratory parameters were not assessed in all pSS patients.

In this study we reconfirmed a monocyte IFN type I signature prevalence in over half of pSS patients, a subgroup with higher disease activity [13]. We proposed identification of these subgroups useful in establishing patient-eligibility for specific treatments. Indeed, an interesting finding here was patients on hydroxychloroquine (HCQ)-treatment to have considerably reduced MxA-levels, indicating HCQ to perhaps interfere with IFN-production. A recent report shows HCQ to impair systemic IFNα-production [39]. Our data suggests that identifying IFN type I signature by performing MxA-assays might prove useful in predicting HCQ-treatment responsiveness. In this cohort there are more HCQ-users in the IFNneg group compared to IFNpos. However it is not likely that increased ESSDAI levels in the IFNpos group are an effect of less HCQ-use in this group, since in IFNpos group still 62% of patients use HCQ and are nevertheless IFNpos. Moreover, we previously showed 61% (23/38) of IFNpos patients to use HCQ compared to 55% (17/31) of IFNneg patients. In addition HCQ-use did not significantly affect ESSDAI scores (Figure 4H). Larger studies are however warranted to fully elucidate the effect of HCQ on IFN type I, thus on MxA-levels.

The association between the IFN type I signature, MxA protein and disease activity levels in pSS needs to be further assessed, by for one looking longitudinally, in order to validate MxA as a disease activity marker. The same holds true for association between IFN type I polymorphisms and MxA, as these polymorphisms have been linked to increased IFNα-sensitivity [40, 41].

To determine applicability of the MxA-EIA in other IFN type I related-diseases, MxA-levels were assessed in a small SLE-cohort (see online supplemental Figure S4) and showed results similar to pSS – a significant correlation between IFNscores and MxA-levels (r=0.885,

p<0.0001) – suggesting a broad applicability of MxA-EIA in systemic autoimmunity.

In conclusion, we identify MxA protein expression by EIA and FACS analysis as practical and rapid approaches for assessment of IFN type I bioactivity in pSS. MxA-EIA is the simplest and most reliable of these assays and indicates clinical applicability in stratifying pSS patients according to IFN-positivity. Use of MxA as a biomarker for identifying patients eligible for therapy in pSS clearly requires further research.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Ms. M. Kivivirta (University of Turku, Finland) for technical assistance in running the MxA-EIAs, and Msc. W. Beumer (Erasmus MC Rotterdam, The Netherlands) for his scientific perspective as well as aid in figure-design.

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Table S1

Variable	CD169			CD64		
	N	r	p value	N	r	p value
ESSDAI score	28	0.28	n.s.	18	0.03	n.s.
Laboratory parameters						
Rf (IE/ml)	26	0.19	n.s.	18	0.39	n.s.
C3 (g/l)[a]	33	0.13	n.s.	20	0.14	n.s.
C4 (g/l)	33	0.07	n.s.	20	-0.161	n.s.
IgG (g/l)[a]	34	0.40	0.02	20	0.18	n.s.
IgA (g/l)	33	0.10	n.s.	20	0.37	n.s.
IgM (g/l)	33	0.36	0.04	20	0.18	n.s.
CRP (mg/l)	31	0.14	n.s.	19	0.16	n.s.
Hb (mmol/l)	34	-0.29	n.s.	20	-0.04	n.s.
Thrombocytes (*10E9/l)[a]	33	-0.01	n.s.	19	-0.31	n.s.
Lymphocytes (*10E9/l)[a]	31	0.00	n.s.	19	0.23	n.s.
Neutrophiles (*10E9/l)	31	-0.24	n.s.	19	-0.11	n.s.
Variable	**N (%)**	**Z**	**p value**	**N (%)**	**Z**	**p value**
Auto-antibodies						
Anti-SSA	29/34 (85)	-2.16	0.03	16/20 (80)	-0.95	n.s.
Anti-Ro52	21/27 (78)	-0.99	n.s.	15/19 (79)	-1.20	n.s.
Anti-Ro60	19/27 (70)	-1.97	0.05	14/19 (74)	-1.94	n.s.
Anti-SSB	22/34 (65)	-2.05	0.04	13/20 (65)	-0.83	n.s.
Medical therapy						
Pilocarpine	9/34 (26)	-2.25	0.03	6/20 (30)	-0.17	n.s.
Plaquenil	25/34 (74)	-1.93	n.s.	13/20 (65)	-0.20	n.s.
Corticosteroids	3/34 (9)	-1.12	n.s.	2/20 (10)	-0.25	n.s.

Data are presented as Spearman's rho (r) or according to κ2 test (Z), unless otherwise mentioned
[a]Data normally distributed are presented as Pearson's rho (r)
Rf, Rheumatoid factor; CRP, C-reactive protein; Hb, Hemoglobin

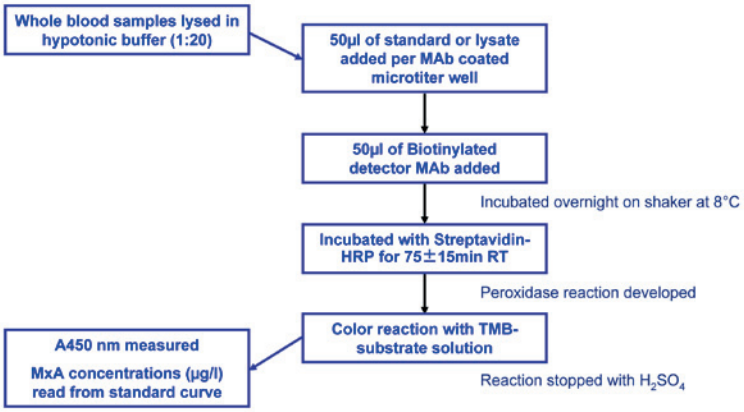

Figure S1. MxA Enzyme immunoassay (EIA)

MxA-EIA was executed as previously described with new monoclonal antibodies and recombinant MxA as standard, subsequent to validation for corresponding results [19]. Mouse MAbs AFI-7B3 and AFI-10G10 (Institute of Clinical Medicine, University of Eastern Finland) were used as capture and biotinylated detector-antibody, respectively. MAbs were used as IgG, purified from cell culture supernatants by using FPLC with CIM protein G monolithic column (BIA separations, Ljubljana, Slovenia). Detector antibody was biotinylated with NHS-PEO_4-Biotin reagent (Pierce, Rockford, IL) according to manufacturer's instructions. Recombinant MxA, produced with baculovirus expression system, was kindly provided by Ilkka Julkunen and Krister Melen (National Institute of Health and Welfare, Helsinki, Finland) [26]. The heparinized whole blood samples (25µl) were lysed by diluting 1:20 in hypotonic buffer containing 1.5% bovine serum albumin (BSA), 1% ascorbic acid, 0.5% $NaHCO_3$ and 0.05% NaN3 and stored at -70°C until assayed. Standard dilutions for the assay were prepared in hypotonic buffer. Microtitre strip wells were coated by overnight incubation with 0.4 µg per well of AFI-7B3 IgG in 0.1 M carbonate, pH 9.6. The strips were washed twice with 5 mm Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.75 and saturated for 1 h with the assay buffer containing 0.5% BSA, 0.05% Tween 20 and 0.1 mM merthiolate in PBS. Strips were washed four times, and 50 µl each of the sample and 0.8 µg/ml biotinylated AFI-10G10 IgG, in the assay buffer, were added into the duplicate wells. After overnight incubation with shaking at 8°C, the strips were washed six times, and 100 µl of streptavidin–peroxidise-HRP (Pierce, #21127) diluted 1:15.000 in assay buffer was added into the wells. Strips were shaken for 75±15 min, washed eight times and incubated with 100 µl of tetramethylbenzidine (TMB) peroxidise substrate solution (Ani Labsystems, Vantaa, Finland) for 10–30 min in the dark. Color development was stopped by adding 100µl of 0.5 N H_2SO_4 and absorbance values were measured at 450 nM using Victor^3 multilabel counter (PerkinElmer, Turku, Finland). MxA concentrations (µg/l) of the specimens were read from a master curve (50-800µg/l) plotted with the standard values using Mutlicalc software with spline smoothed fitting algorithm. Hypotonic buffer was used as the negative control. The limit of detection of the MxA EIA assay was 10µg/l, which was determined as three times the standard deviations (SD) of eight negative control replicates. One negative and one positive control sample were also included in each assay.

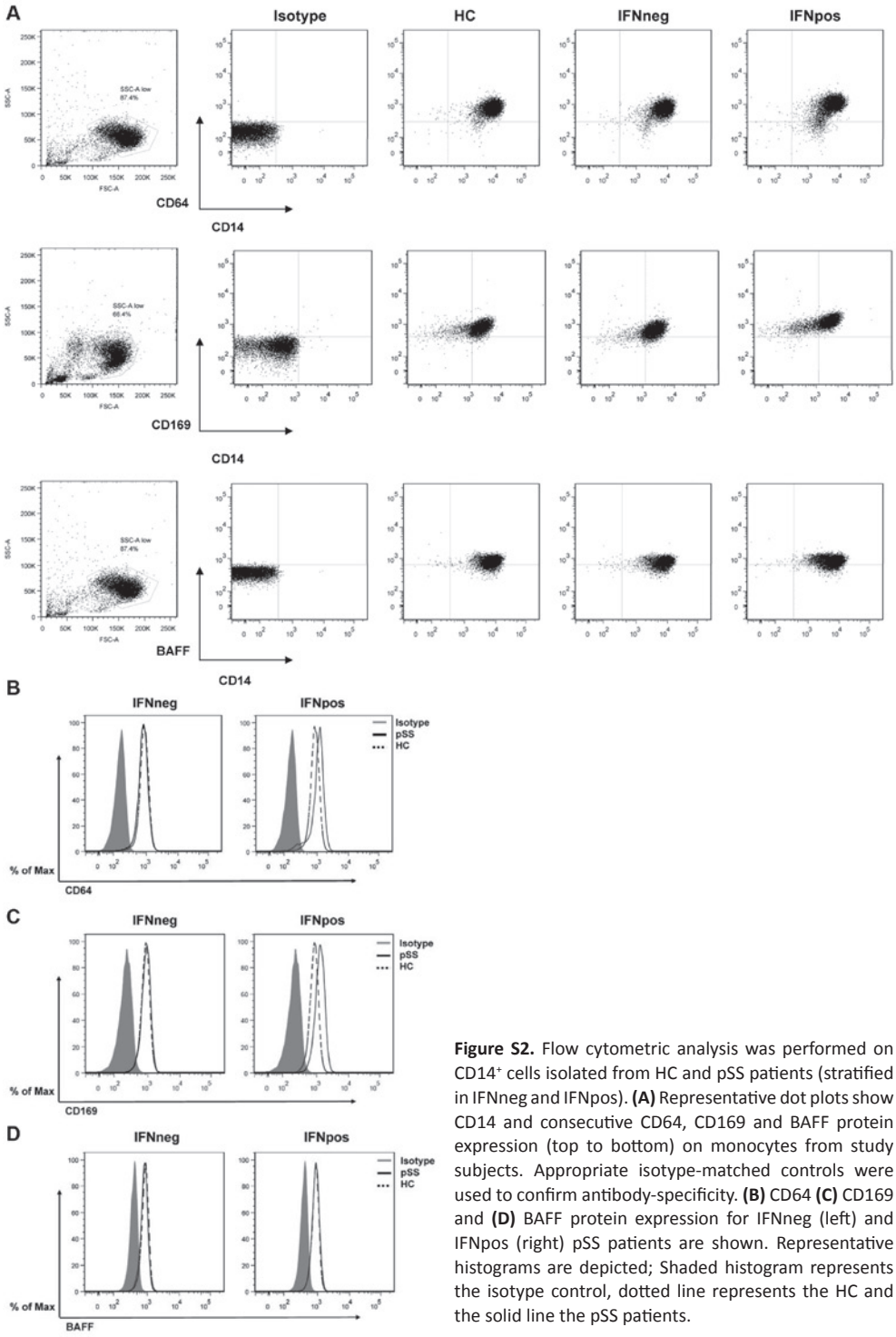

Figure S2. Flow cytometric analysis was performed on CD14^{+} cells isolated from HC and pSS patients (stratified in IFNneg and IFNpos). **(A)** Representative dot plots show CD14 and consecutive CD64, CD169 and BAFF protein expression (top to bottom) on monocytes from study subjects. Appropriate isotype-matched controls were used to confirm antibody-specificity. **(B)** CD64 **(C)** CD169 and **(D)** BAFF protein expression for IFNneg (left) and IFNpos (right) pSS patients are shown. Representative histograms are depicted; Shaded histogram represents the isotype control, dotted line represents the HC and the solid line the pSS patients.

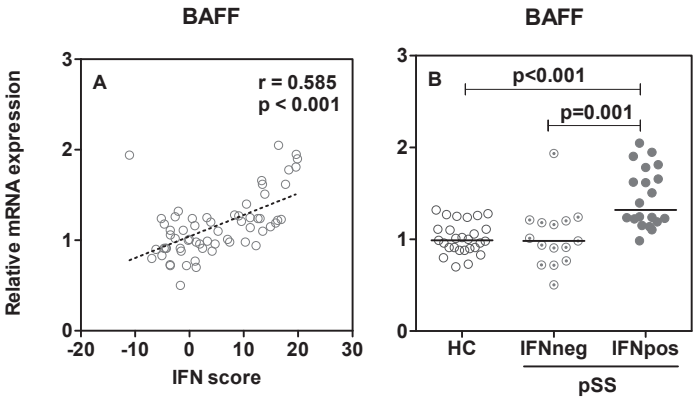

Figure S3. For relative BAFF mRNA gene expression **(A)** a correlation plot shown with IFNscores for all study subjects and **(B)** BAFF mRNA gene expression levels stratified in HC (n=27), IFNneg (n=14) and IFNpos (n=21) pSS patients. Each symbol represents an individual sample; horizontal lines represent the mean. The correlation coefficient (r) and *P* values are shown. For BAFF a Pearson correlation was used and to compare means the independent T-test was used.

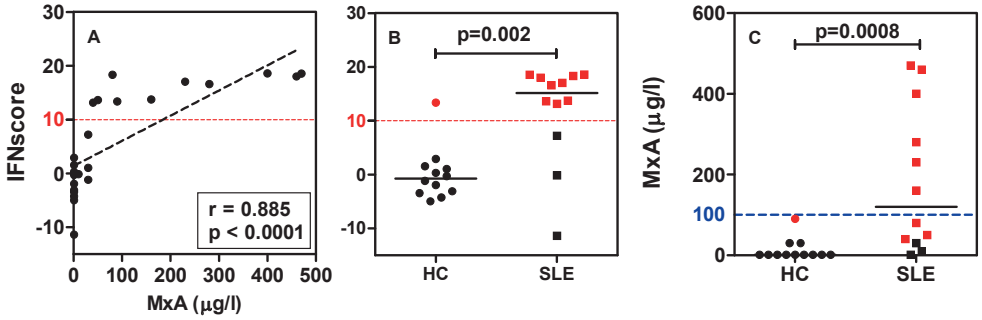

Figure S4. In a small cohort of systemic lupus erythemathosis (SLE) patients **(A)** a correlation plot is shown between IFNscores (for all age and gender matched study subjects) and intracellular MxA (µg/l) levels, as assessed by the MxA-EIA. **(B)** IFNscores shown for HC (n=12) and SLE patients (n=12), stratified in IFNneg and IFNpos by red dotted line at 10; Red dots represent IFNpositivity (IFNscore≥10). **(C)** MxA-EIA (µg/l) levels shown for HC (n=12) and SLE patients (n=12). Each symbol represents an individual sample; horizontal lines represent the median. The correlation coefficients (r) and *P* values are shown. For correlations Spearman's rho correlation test was used and to compare medians the Mann-Whitney U test was used.

CHAPTER 4

SYSTEMIC SCLEROSIS PATIENTS WITH THE INTERFERON TYPE I SIGNATURE SHOW FASTER DISEASE DEVELOPMENT AND HIGH BAFF GENE EXPRESSION AND COLLAGEN SYNTHESIS

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Submitted for publication

ABSTRACT

Objective

To determine the prevalence of the monocyte Interferon (IFN) type I inducible signature in 58 systemic sclerosis (SSc) patients and 27 healthy controls (HC) and to relate the IFN type I activity to disease manifestations.

Methods

IFN type I scores were calculated based on the mRNA expression levels of IFI44L, IFIT3, IFITM1, IFIT1 and MXA in monocytes. SSc patients with IFN type I activity, as reflected by a positive IFN type I signature (IFN score≥10) were compared with patients negative for the IFN type I signature (IFN score<10) for clinical phenotype, auto-antibody profile, mRNA expression of B cell activating factor (BAFF) and type III procollagen N-terminal propeptide (PIIINP) in serum.

Results

IFN type I signature was expressed in 29.3% of SSc patients. No significant differences could be found between limited and diffuse SSc. SSc patients with the IFN type I signature were characterized by the presence of anti-RNP autoantibodies and absence of anti-centromere antibodies. Moreover, in these IFN type I positive patients the time between the onset of Raynaud's phenomenon and development of the first non-Raynaud symptom was markedly shorter. Along with these observations, monocyte BAFF mRNA levels and PIIINP levels in serum were increased in the IFN type I signature positive SSc patients.

Conclusions

Increased IFN type I activity is present in monocytes of about one third of SSc patients. The presence of the IFN type I signature might be useful as a marker for predicting progression of patients with Raynaud's phenomenon to SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune disease with extensive fibrosis, vascular alterations and immune activation among its principal features [1]. With an incidence of 1-2 per 100.000 it is a rare disease in which chronic oxidative stress and inflammation lead to organ failure ending up in severe fibrosis of skin and internal organs including kidney, lung and heart. Ultimately, these disease hallmarks culminate in profound decline in the quality of life and premature death. SSc represents a particularly difficult disease to unravel, partly due to the intricacies of the genetics and partly by virtue of complicated nature of the disease process. Despite extensive efforts to understand the underpinnings of this devastating disease, our current knowledge of the causal pathways driving SSc and related mechanisms involved in fibrosis and accelerated ageing is still limited. A unique feature of SSc that distinguishes it from other fibrotic disorders is that inflammation, autoimmunity and vasculopathy characteristically precede the development of tissue remodeling and fibrosis. This latter implicates a window of opportunity in which patients with more progressive disease and/or worse prognosis can be selected for early and aggressive therapy. In line with this, in those patients with less severe prognosis, side-effects and therapy induced co-morbidity might be prevented.

In SSc accumulating evidence suggests the implication of an aberrant immune system in the development of the disease. This is supported by studies on genetic risk factors for SSc that revealed genes involved in the immune system [2], and amongst others STAT4 and IRF5, which are both involved in the Interferon (IFN) type I signalling pathway [3-6]. A substantial part of the SSc patients display IFN type I activation either in the affected skin or in immune cells isolated from the circulation [7-9]. Clinically, the expression of the IFN type I induced genes Siglec-1 and IFI44 in skin biopsies correlated well with the mean Rodnan Skin Score (mRSS), currently the only available disease progression marker [10]. It is widely accepted that plasmacytoid dendritic cells (pDCs) are the main source of IFN type I. Upon activation of toll-like receptors (TLRs) by so-called danger signals, pDCs start to secrete marked amounts of IFN type I, which in turn probably is reflected by the IFN type I induced gene expression pattern, the so called IFN type I signature, observed now in multiple autoimmune diseases [11, 12].

Previously, microarray studies showed increased expression of IFN type I inducible genes in peripheral blood mononuclear cells (PBMCs), monocytes and skin biopsies from SSc patients [7-9, 13, 14]. Some studies showed clinical associations with the IFN type I expression, such as a positive correlation between IFN type I genes and the appearance of digital ulcers and a negative correlation with the presence of anti-centromere antibodies (ACA) [13]. However, there is discrepancy when it comes to other autoantibodies such as anti-topoisomerase I (ATA), anti-RNP and anti-SSA antibodies. Recently Wuttge *et al.* showed that 27% of early SSc sera were able to induce increased IFN type I expression in a reporter

cell line. This increased IFN type I activity correlated with higher IgG levels, lower leucocytes and higher amount of antibodies against extractable nuclear antigens [14].

One of the most important cell types affected by IFN type I are the monocytes. Monocytes stimulated by IFN type I differentiate into activated DCs and macrophages and in combination with other triggers will direct the immune system into potentially harmful conditions like autoimmune diseases. Previously we found in 55% of primary Sjögren's syndrome (pSS) patients a monocyte IFN type I signature compared to 4.5% of healthy controls (HC) [15]. One of the genes that strongly correlated to the presence of the IFN type I signature was B cell activating factor of the tumour necrosis factor family (BAFF) [15].

Taken together, further unravelling the clinical implications of the IFN type I gene signature is highly justified. In this study we replicated findings from the literature and analyzed for the first time the prevalence of the monocyte IFN type I signature in two independent cohorts of SSc patients. The presence of the IFN type I signature was correlated to disease manifestations, BAFF expression and collagen metabolism and thus supports a direct role for IFN type I in the pathogenesis of SSc.

PATIENTS AND METHODS

Patient collection

Twenty-eight patients presenting at the department of Rheumatology, Radboud University Nijmegen Medical Center (RUNMC) and thirty patients visiting the Erasmus Medical Center, Rotterdam were included in the study. All patients met the American College of Rheumatology preliminary criteria for the classification of SSc [16]. For analyses, patients were subdivided as having limited cutaneous SSc (lcSSc, n=42) or diffuse cutaneous SSc (dcSSc, n=16) on the basis of the extent of their skin involvement [17]. A further subdivision was made between early and late disease based upon the duration of disease: early SSc is defined as disease duration <2 years and late as SSc longer than 2 years. The presence of SSc specific autoantibodies, anti-topoisomerase I (ATA) and anti-centromere (ACA), were tested according to local laboratory standards. Pulmonary fibrosis was defined as the presence of significant fibrosis on high-resolution computed tomography scan combined with a restrictive pulmonary function pattern. Pulmonary artery hypertension was diagnosed by right heart catheterization and considered present if the mean pulmonary artery pressure was greater than 25 mmHg at rest in the presence of a normal wedge pressure. As a comparator group 27 healthy controls were studied. Blood samples were obtained with informed consent under local Institutional Review Board approval. Characteristics of patients and controls are summarized in Table 1.

Table 1. Demographics, laboratory and clinical characteristics of participants

Variable	Controls (n=44)	SSc		IFN type I signature	
		Limited (n=42)	Diffuse (n=16)	Positive (n=17)	Negative (n=41)
Demographic s					
N females (%)	19/27 (70%)	34/42 (81%)	10/16 (63%)	12/17 (78%)	32/41 (71%)
Age (years)	46.0±15	52.0±14	54.0±10	48.8±11.7	54.6±12.8
Disease duration (years)	-	6.5±4.9	3.9±5.5	5.2±4.5	6.1±5.4
Rodnan skin score	-	3.6±3.0	11.5±7.6	6.0 (1.0,10.5)	4.5 (2.0,9.75)
Digital ulcers	-	12/42 (29%)	5/15 (33%)	3/17 (18%)	14/40 (35%)
Digital loss	-	2/39 (5%)	0/3 (0%)	0/12 (0%)	2/30 (7%)
Pulmonary hypertension	-	8/40 (20%)	2/16 (13%)	1/16 (6%)	9/40 (23%)
Lung fibrosis	-	14/40 (35%)	11/16 (69%)	8/16 (50%)	17/40 (43%)
ACA positivity	-	17/40 (43%)	2/16 (13%)	1/16 (6%)	18/40 (45%)
ATA positivity	-	9/37 (24%)	4/16 (25%)	5/15 (33%)	8/38 (21%)
Anti-RNP positivity	-	2/39 (5%)	2/13 (15%)	4/13 (31%)	0/39 (0%)
Anti-SSA positivity	-	11/39 (28%)	2/13 (15%)	3/14 (21%)	10/38 (26%)
Medical therapies					
Mycophenolate mofetil	-	2/42 (5%)	2/15 (13%)	1/17 (6%)	3/40 (8%)
Cyclophosphamide	-	0/42 (0%)	1/15 (7%)	1/17 (6%)	0/40 (0%)
Prednisolone	-	5/42 (12%)	1/15 (7%)	2/17 (11.8%)	4/40 (10%)
Hydroxychloroquine	-	1/42 (2%)	0/15 (0%)	0/17 (0%)	1/40 (3%)
Methotrexate	-	8/42 (19%)	2/15 (13%)	7/17 (41%)	3/40 (8%)
Azathioprine	-	2/42 (5%)	0/15 (0%)	1/17 (6%)	1/40 (3%)

Values are the mean (± SD), median (25% quartile,75% quartile), or number (%) of patients, depending on whether the data are dichotomous or continuous following a normal distribution or not.

Blood collection and isolation of monocytes

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for PBMC preparation as described previously [18]. CD14 positive monocytes were isolated as described [18].

Real Time Quantitative Polymerase Chain Reaction (RQ-PCR)

Total RNA was isolated from purified monocytes followed by cDNA preparation and RQ-PCR analysis using predesigned primer/probe sets (Applied Biosystems, Foster City, California) [18]. For calculation of relative expression, all samples were normalized against expression of the household gene Abl [19]. Fold change values were determined from normalized CT values using $2^{-}\Delta\Delta CT$ method (User Bulletin , Applied Biosystems, Foster City, California).

Measurement of autoantibodies

Anti-SSA and anti-SSB were determined by EliA (Thermo Scientific, Uppsala, Sweden) and confirmed with ANA profile immunoblot (EuroImmun, Lubeck, Germany). When the results showed a discrepancy, a QUANTA Lite ELISA-kit from INOVA (San Diego, USA) was used for confirmation.

Bio-assay for IFN type I activity in serum

To assess whether the expression of IFN type I inducible genes could be induced by serum of SSc patients, THP-1 cells were cultured in 250 µl medium as described previously [15]. 250 µl of serum from SSc patients positive or negative for the IFN type I signature, was added to THP-1 cells and incubated for 6 hours. HC serum was added as negative control. For blocking purposes anti-IFN type I receptor (anti-IFNAR) (PBL Interferon Source, Piscataway, USA) was added (5 µg/ml) and anti-IgG2a (Santa Cruz Biotechnology, Dallas, USA) as an isotype control. IFN type I gene expression induced by serum from IFN type I positive patients was compared with expression induced by serum from IFN type I negative patients, both relative to the expression induced by HC serum.

Radioimmunoassay for quantitative N-terminal propeptide III

For quantitative assessment of intact N-terminal propeptide of type III (PIIINP) procollagen, a Radioimmunoassay (RIA-) kit was used according to manufacturer's protocol (UniQ, Orion Diagnostica, Espoo, Finland). Previously frozen sera (stored at -80°C), collected at the time of patient-inclusion were analyzed. The kit is based on the competitive radioimmunoassay technique. A known amount of labeled PIIINP and an unknown amount of unlabeled PIIINP in the sample compete for the limited number of high affinity binding sites of the antibody. After separating the free antigen, the amount of labeled PIIINP in the sample tube is inversely proportional to the amount of PIIINP in the sample. The concentrations in unknown samples are obtained from a calibration curve. Measurement range: 1.0-50µg/l, detection limit: ±0.3µg/l, defined as twice the standard deviation (SD) of the 0-binding value.

Factor analysis

Expression levels of 11 IFN type I inducible genes were submitted to a
principal component analysis to identify correlated groups of genes to reduce data complexity. Kaiser-Meyer-Olkin measure of sampling adequacy was 0.843 with significant Bartlett's test of sphericity ($P< 0.001$). Eigenvalues were derived to assess the amount of variance explained by each component factor.

Statistical analyses

Statistical analyses were performed using SPSS 20.0 package. When data were not normally distributed, values were expressed as medians with interquartile ranges (IQRs) and comparisons were made using the non-parametric Mann-Whitney U test. For normally

distributed data, independent T test was used to compare means. In case of more than two samples one-way ANOVA was performed or the non-parametric Kruskal-Wallis test. For dichotomous data the Fisher's Exact Test was performed. To compare survival curves the Gehan-Breslow-Wilcoxon Test was used. For correlations Spearman's Rho correlation test was used. Differences were considered statistically significant if $p<0.05$.

For multiple variable testing (Rodnan skin score, vascular digital complications, pulmonary hyperstension, lung fibrosis, auto-antibodies, time to first non-Raynaud sytmptom, BAFF mRNA and PIIINP), values of $p\leq0.006$ ($p\leq0.05/8$) were considered statistically significant after applying a Bonferroni correction (n=8).

RESULTS

The prevalence of the IFN type I signature in monocytes of SSc patients

In 58 SSc patients and 27 HCs we assessed the expression levels of 11 IFN type I inducible genes previously assessed in monocytes (IFI27, IFI44L, IFIT3, IFITM1, SERPING1, IFIT1, IFIT2, LY6E, IFI44, XAF1 and MXA) [15]. To reduce data complexity, expression levels of the 11 genes were submitted to a principal component analysis to identify correlated groups of genes. The results of the principal component analysis identified a subset of 5 genes (IFI44L, IFIT3, IFITM1, IFIT1 and MXA) to explain 96% of the total variance of the 11 IFN type I inducible genes within the SSc cohort. Given that the expression of these 5 IFN type I inducible genes was not normally distributed, log transformations of expression values were performed and IFN scores were calculated as described for pSS and SLE [15, 20]. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN scores per subject represent the sum of these standardized scores. When we set the threshold for a positive IFN type I signature at 10 [15], 29.3% of SSc patients displayed an IFN type I signature, while none of the subjects in the HC group (Figure 1A, B).

To investigate whether the increased IFN type I activity represents a systemic effect, we performed a bioassay using THP-1 monocytic cell line exposed to 50% by volume serum from SSc patients who were either positive or negative for the IFN type I signature (Figure S1). Serum from IFN type I signature positive patients (n=11) enhanced the expression of IFN type I signature genes in THP-1 monocytes, while this was not the case for serum from IFN type I signature negative patients (n=9). Blocking IFN type I receptor diminished significantly the mRNA expression of IFN type I signature genes in THP-1 monocytes.

To determine if the IFN type I production is higher in diffuse SSc compared to limited SSc as suggested by Kim *et al*. [21], we stratified the patients according to the extent of their skin involvement and compared the IFN scores. A trend for higher IFN scores was observed in dcSSc group in comparison to lcSSc , but this was not significant (Figure 1C). The same holds true when we stratified patients according to the duration of disease, defining patients as early SSc when having a disease duration <2 years and late when having SSc longer than 2 years (Figure 1D, E).

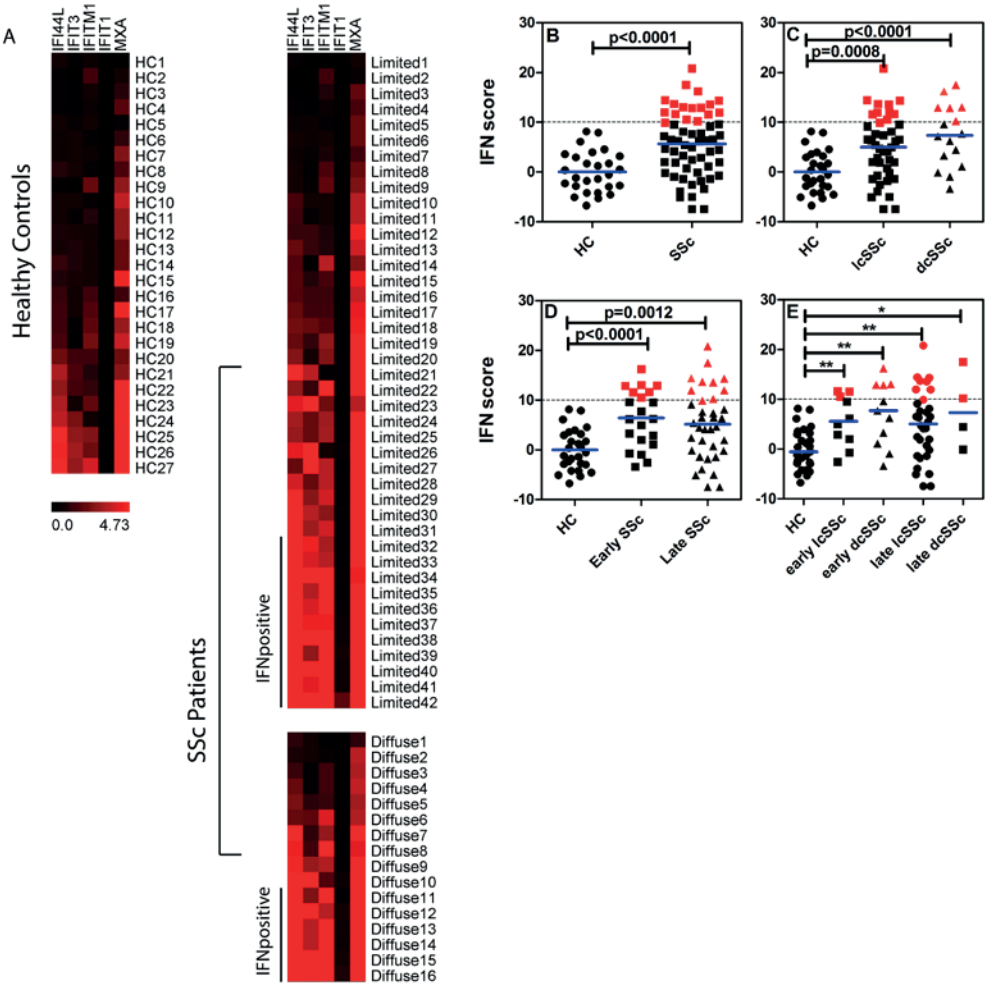

Figure 1 (A) Heatmap showing gene expression of 5 IFN type I inducible genes in monocytes of lcSSc patients (n=42), dcSSc (n=16) and HC (n=27). On the left the HC are depticted and on the right the SSc patients are depticted and subdivided into lcSSc and dcSSC. Red colour indicates high gene expression and cases are depicted according to ascending IFN scores.

Distribution of IFN scores in **(B)** SSc patients and HC, **(C)** limited and diffuse SSc patients and HC, **(D)** early and late SSc patients and HC, **(E)** early limited and diffuse SSc, late limited and diffuse SSc and HC. IFN type I positive cases are depicted in red. In case of more than two samples one-way ANOVA was performed (C-D) or the non-parametric Kruskal-Wallis test (E). Independent T-test was used to compare means in B-D where blue lines represent means. Mann-Whitney U test was used in E where blue lines represent medians.

* respresents p-values <0.05

** respresents p-values <0.01

IFN type I signature in SSc is associated with the presence of anti-RNP autoantibodies and absence of anti-centromere antibodies

To determine whether IFN type I activity, as reflected by a positive IFN type I signature (IFN score≥10), is associated with specific autoantibodies, we stratified patients according to their autoantibody profile and compared the IFN scores. Even despite the fact that it is a small group, all patients positive for anti-RNP antibodies displayed the IFN type I signature and showed significantly higher IFN scores compared to patients without anti-RNP antibodies (Fisher's Exact test p=0.003) (Figure 2A). Interestingly, almost all patients with circulating anti-centromere antibodies were negative for the IFN type I signature (Fisher's Exact test p=0.005) (Figure 2B). There were no significant differences in IFN type I gene expression between patients positive or negative for ATA and anti-SSA (Figure 2C, D).

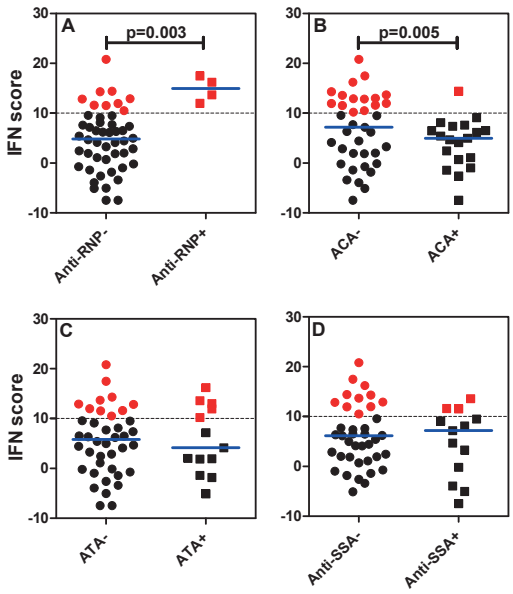

Figure 2 IFN scores in SSc patients positive and negative for **(A)** anti-RNP antibodies (n=52), **(B)** anti-centromeric antibodies (ACA) (n=56), **(C)** anti-topoisomerase I antibodies (ATA) (n=53), **(D)** anti-SSA antibodies (n=52). IFN type I positive cases are depicted in red. Fisther's Exact Test was performed. Only significant differences are shown.

IFN type I signature correlates positively with BAFF mRNA and PIIINP levels and faster disease development

Next we investigated whether patients positive for the IFN type I signature differ in clinical manifestations compared to SSc patients negative for the IFN type I signature. We did not observe differences between the clinical variables investigated (Table 2). Intriguingly however, the time between the onset of Raynaud's phenomenon and development of the first non-Raynaud symptom, was markedly shorter in those individuals with a positive IFN

type I signature (p= 0.003) (Figure 3A). This suggests that IFN type I gene signature is a marker for accelerated disease development.

Table 2. Comparison of SSc patients with or without a positive IFN type I signature

		IFN type I signature category		
Variable	**n**	**Positive**	**Negative**	***P***
				ns
Rodnan skin score	33	6.0 (1.0,10.5)	4.5 (2.0,9.75)	ns
Vascular digital complications				
Digital ulcers (n)	57	3/17 (18%)	14/40 (35%)	ns
Digital loss (n)	42	0/12 (0%)	2/30 (7%)	ns
Pulmonary hypertension (n)	56	1/15 (7%)	9/40 (23%)	ns
DLCO (%)	54	70.3±19.3	63.5±17.3	ns
Lung fibrosis (n)	56	8/16 (50%)	17/40 (43%)	ns
FVC (%)	56	88.9±24.9	91.2±21.0	ns
Auto-antibodies				
ACA positivity (n)	56	1/16 (6%)	18/40 (45%)	**0.005**
ATA positivity (n)	53	5/15 (33%)	8/38 (21%)	ns
Anti-RNP positivity (n)	52	4/13 (31%)	0/39 (0%)	**0.003**
Anti-SSA positivity (n)	52	3/14 (21%)	10/38 (26%)	ns
Time to first non-Raynaud symptom *	46	15.0 (3.0,24.0)	38.5 (11.0,117.8)	**0.003**
BAFF mRNA monocytes	58	22.4±6.2	16.4±4.1	**<0.0001**
PIIINP	42	6.5 (5.8,52.5)	5.0 (3.8, 6.7)	0.007

Values are the mean (± SD), median (25% quartile,75% quartile), or number (%) of patients, depending on whether the data are dichotomous or continuous following a normal distribution or not. For dichotomous data the Fisher's Exact Test was performed. When continuous data followed normal distribution, the independent T-test was conducted; otherwise the Mann-Whitney U test was conducted. For comparison of survival curves the Gehan-Breslow-Wilcoxon Test was used (*). For multiple variable testing, values of p≤0.006 (p≤0.05/8) were considered statistically significant after applying a Bonferroni correction (n=8).

Previously, we observed that BAFF levels were positively associated with IFN type I signature in patients with Sjögren's syndrome [15]. In line with these observations, we observed that BAFF mRNA levels were significantly higher in dcSSc compared with HC (p=0.006) and significantly higher in IFN type I signature positive SSc patients compared with the signature negative patients (p<0.0001) (Figure 3B, C).

BAFF serum levels were found to correlate with the extent of skin fibrosis in SSc [22]. We therefore next investigated whether an indicator of *de novo* collagen type III synthesis, namely N-terminal propeptide of type III collagen (PIIINP), is increased in SSc patients

positive for the IFN type I signature. We found increased PIIINP levels in SSc compared to HC (p=0.005) and by far the highest levels in the patients positive for the IFN type I signature (p=0.007) (Figure 3D, E). After applying a Bonferroni correction for multiple variable testing (significance < 0.006 (0.05/8)) the differences in PIIINP lost significance. However when looking more in depth to our data, we observed that IFN type I negative patients with high PIIINP levels displayed also high IFN scores. IFN scores correlated significantly positive with the PIIINP levels (r= 0.502, p=0.0007) (Figure 3F). Together with the increased BAFF mRNA levels, these data indicate more extensive fibrosis in the IFN type I positive SSc patient group.

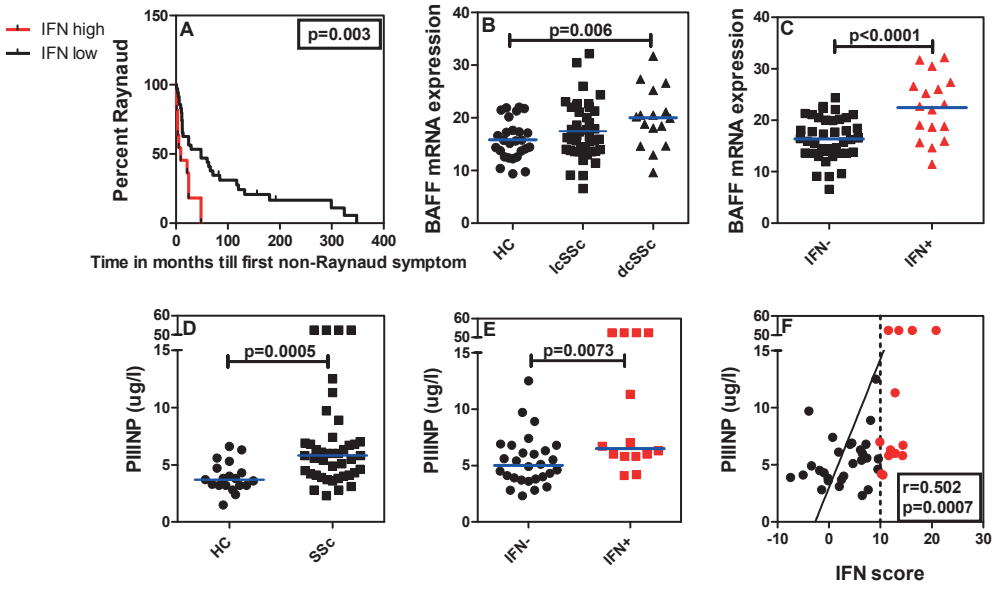

Figure 3 (A) Survival diagram depicting the time in months between Raynaud's phenomenon and development of the first non-Raynaud symptom. Red line represents SSc patients positive for the IFN type I signature (n=12) and the black line represents SSc patients negative for the signature (n=34). **(B)** BAFF mRNA expression in HC (n=27), limited (n=42) and diffuse SSc patients (n=16). **(C)** BAFF mRNA expression in monocytes from SSc patients positive for the IFN type I signature (n=17) and patients negative for the signature (n=41). **(D)** N-terminal propeptide III (PIIINP) (µg/l) in serum from HC (n=19) and SSc patients (n=42). **(E)** N-terminal propeptide III (PIIINP) (µg/l) in serum from SSc patients positive (n=14) and negative (n=28) for the IFN type I signature. **(F)**, Correlation between IFN scores and N-terminal propeptide III (PIIINP) levels in serum.
IFN type I positive cases are depicted in red. The correlation coefficient (r) and p values are shown. For comparison of survival curves the Gehan-Breslow-Wilcoxon Test was used (A). Independent T-test was used to compare means in (B-C) where horizontal lines represent means. Mann-Whitney U test was used in (D-E) where horizontal lines represent medians. Spearman's Rho correlation test was used in (F).

DISCUSSION

In the current study we describe the prevalence of the monocyte IFN type I signature in SSc patients. As main producers of IFN type I, pDCs play a role in the pathogenesis of diseases with the IFN type I signature. In Systemic Lupus Erythematosus (SLE) for example, the IFN type I signature has been associated with pDC activation either by CpG-DNA containing complexes [11] and/or neutrophil extracellular traps (NETS) [23]. In this light, the observation that IFNα is able to induce the expression of TLR3 in SSc dermal fibroblasts, leading to a self-perpetuating inflammatory loop might directly link pDC activation, IFN type I and the onset of fibrotic events [24, 25]. Indeed the number of pDCs has been shown to be markedly increased in the skin of SSc patients, mainly in those with diffuse cutaneous disease. This is in line with our observations that this SSc clinical subset demonstrate a trend for higher IFN type I scores (this paper and unpublished data).

SSc can be difficult to identify in the general clinic both due to its rarity and heterogeneity, which may give rise to marked delayed diagnosis. The onset of SSc is often heralded by Raynaud's phenomenon, but Raynaud's phenomenon is far from specific for SSc. In addition, primary Raynaud's phenomenon is more common among women than men [26], and thus may be less likely to raise suspicion of SSc in women. Therefore, possibly the greatest unmet need in SSc is the lack of easy access biomarkers that would identify those Raynaud's phenomenon patients at risk for disease onset and/or progression. It is generally well accepted that patients which present with their first non-Raynaud's phenomenon early after onset of Raynaud's have faster disease progression and worse outcome compared with those who have a long lag-time between these signs [27]. Therefore, markers that predict the development of SSc have high clinical relevance for early recognition, intensive clinical monitoring and possibly therapeutic interventions. Our data indicate that the presence of an IFN type I signature is associated with rapid progression from primary Raynaud's phenomenon to full blown SSc. This justifies a role for IFN type I signature determination as novel biomarker alongside capillaroscopy in assessment of early disease. Taken into consideration that type I interferons stimulate B-cells in the generation of autoantibodies and in situations of hypoxic stress where there is a danger of immune complex formation containing self-DNA of RNA, our correlation with BAFF expression suggests a pathogenic pathway in the early phase of disease development. This is further supported by the elevated PIIINP levels in SSc patients [28, 29] and by our finding that the PIIINP levels are higher in IFN type I signature positive patients compared to IFN type I negative patients.

When considering autoantibody profiles, we show a pattern of IFN type I positive patients being anti-RNP positive [14] and ACA negative [13]. In contrast to earlier association and mechanistic studies [21], we did not find an association between ATA and the IFN type I signature. This might be related to the limited ATA positivity in our study. However, in support of our findings Eloranta *et al.* reported that ATA did not correlate with the presence of IFN type

I in functional in vitro testing [30]. Furthermore, earlier literature on this subject showed a correlation between a positive IFN type I signature and disease severity/progression [31]. To quantify disease severity and activity the mRSS is the most performed assessment but also shows a disappointing correlation with progression towards organ involvement [27] and a significant interobserver variability [32]. Our present study comprised patients included in two different centers where the mRSS was performed by different, but a limited number of clinicians. This might explain the lack of correlation between the IFN type I signature and the mRSS in our study. In our study, the actual mRSS was noted, but the correlation between the maximum mRSS and IFN type I signature was not studied. In line with our findings the paper of York *et all.* showed that the expression of IFN type I inducible gene Siglec-1, does not correlate with the mRSS in dSSc patients [8]. We further pursued the question if the IFN type I signature is more frequent in dSSc, which is denied by our data in line with earlier studies [9, 31]. Interestingly the signature is not confined to the early phase of the disease but remains present in a part of all subtypes as recently shown with an IFN type I induced chemokine profile [31].

In summary we show that an increased IFN type I activity is present in monocytes of about one third of SSc patients. Moreover the IFN type I signature is associated with the presence of anti-RNP autoantibodies and absence of ACA, high BAFF mRNA expression, high serum PIIINP levels and markedly shorter time of disease development. Therefore the presence of the IFN type I signature might be useful as a marker for prediction of progression of patients with Raynaud's phenomenon to SSc. The lack of a clear correlation between the IFN type I signature and most clinical parameters leaves the intriguing question what defines the IFN type I positive patients unresolved.

ACKNOWLEDGEMENTS

The authors would like to thank Kim Heesen for practical assistance.

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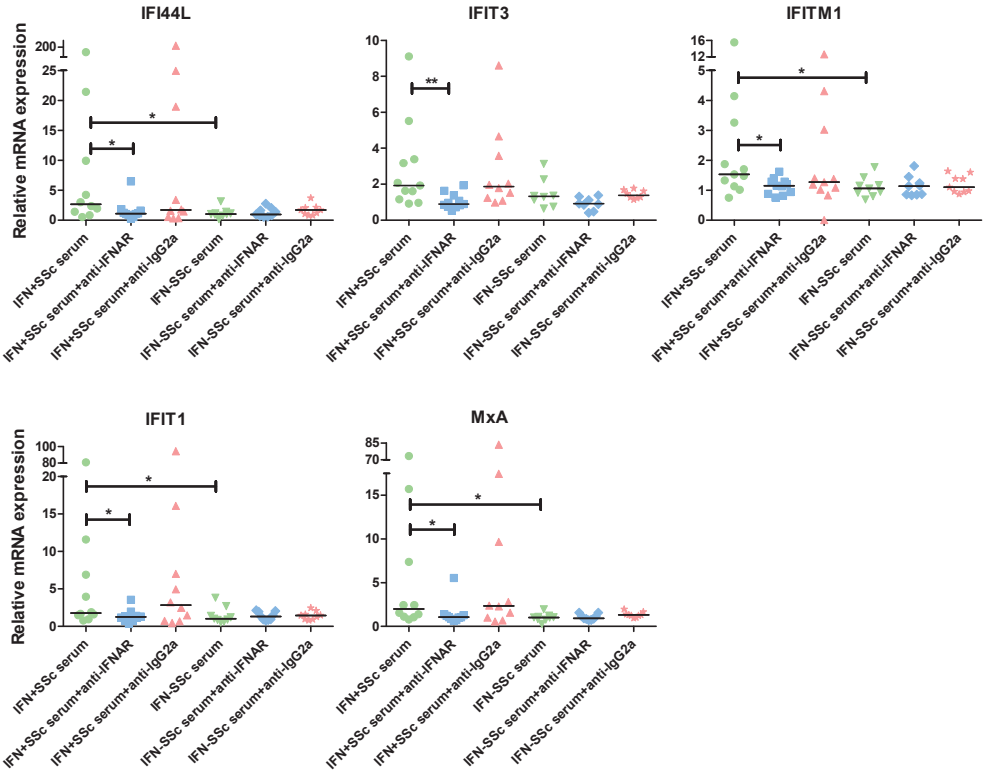

Figure S1 Induction of mRNA expression of IFN type I signature genes in THP-1 monocytes by incubation with 50% serum from IFN type I signature positive pSS patients (n=11) and IFN type I signature negative pSS patients (n=9) in the presence of a blocking IFN type I receptor antibody (IFNAR) and istotype control (IgG2a). Expression is relative compared to the mean expression induced by serum from healthy controls (n=10). Kruskal-Wallis test was performed followed by the Mann-Whitney U test.

* respresents p-values <0.05

** respresents p-values <0.01

CHAPTER 5

INTERFERON TYPE I ACTIVATION AND TYPE III PROCOLLAGEN N-TERMINAL PROPEPTIDE AS PREDICTORS FOR RESPONSIVENESS TO IMATINIB MESYLATE IN SYSTEMIC SCLEROSIS

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Submitted for publication

Systemic sclerosis (SSc) is a difficult to treat, severely debilitating, autoimmune disease characterized by vasculopathy, immune activation and fibrosis of the skin and internal organs. Imatinib mesylate (IM), is used as a treatment for therapy-refractory SSc with high variability in therapeutic outcomes ranging from ineffective/toxic responses to extremely encouraging clinical improvement [1]. This high variability in treatment outcomes stresses the need for a biomarker identifying SSc patients likely to respond to IM treatment.

In this study we treated 10 therapy-refractory SSc patients with IM 400mg/day for one year and assessed whether gene expression patterns and serological characteristics could function as biomarkers for beneficial response to IM treatment. All patients included in this open-label trial met the American College of Rheumatology criteria [2]. Patient characteristics are summarized in Table 1.

Table 1. Patient characteristics

Variable	Non-responders	Responder 1	Responder 2
Gender	5/8 female	female	female
Age (years)	54 ±12.7	43	52
MRSS baseline	11 ±9.0	24	23
MRSS 12 months	10 ±8.3	16	9
TLC baseline %	78 ±22.9	77	82
TLC 12 monhts %	74 ±22.1	92	86
DLCO baseline %	66 ±13.8	74	87
DLCO 12 monhts %	59 ±11.3	88	94

MRSS, mean Modified Rodnan Skin Score; TLC, total lung capacity; DLCO, diffusing capacity for carbon monoxide
Values for non-responders are the mean±SD

The primary endpoints for response to IM were defined as significant decrease in mean Modified Rodnan Skin Score (MRSS) and significant increases in total lung capacity (TLC) and diffusing capacity for carbon monoxide (DLCO) during treatment compared to baseline. In addition to these parameters, blood and serum were collected from 8 of the SSc patients prior to IM treatment and at 3, 6, 9 en 12 months of treatment. In terms of the primary endpoints, 2 of the 10 patients were identified as responders to IM (Table 1).

Subsets of patients with various generalized autoimmune diseases have been found to display an IFN type I signature that correlates with increased disease activity [3-6]. We isolated monocytes from 8 of the SSc patients included in our study and analysed the IFN type I signature expression. Remarkably only the monocytes from the 2 patients who responded well to IM displayed a positive IFN type I signature at baseline (Figure 1A). The IFN type I signature remained the same during IM treatment (Figure 1A-B).

Previously, we found B cell activating factor (BAFF) mRNA to strongly correlate with the presence of the IFN type I signature in Sjögren's syndrome patients [3] and BAFF serum levels were found to correlate with the extent of SSc skin fibrosis [7]. We therefore assessed BAFF mRNA expression in monocytes from the SSc patients in our study. The two responders showed the highest levels of BAFF mRNA at baseline, which decreased significantly upon IM treatment (Figure 1C-D).

An indicator of *de novo* collagen type III synthesis, namely N-terminal propeptide of type III collagen (PIIINP), has been reported increased in the skin and serum of SSc patients [8, 9]. We assessed PIIINP in serum of 7 of the SSc patients. The 2 responders showed much higher baseline PIIINP levels than the other SSc patients, decreasing drastically upon 6 months of IM treatment (Figure 1E). In line with this, a beneficial clinical response to IM has previously been reported when the SSc skin displays excessive deposition of collagen type III [10].

In our study, we identified monocyte IFN type I signature and associated BAFF mRNA levels as well as serum PIIINP as potential biomarkers for the selection of SSc patients who are likely to respond to treatment with IM.

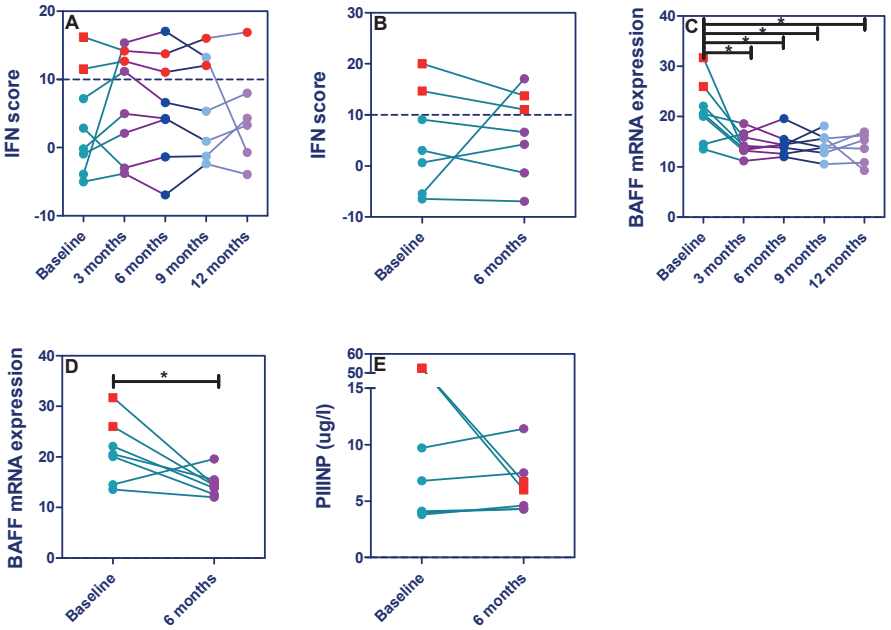

Figure 1. (A) IFN scores in monocytes from IM treated SSc patients (n=8) at baseline, 3 months, 6 months, 9 months and 12 months of treatment. **(B)** IFN scores in IM treated SSc patients (n=8) at baseline and 6 months of treatment. **(C)** BAFF mRNA expression levels in monocytes of IM treated SSc patients (n=8) at baseline, 3 months, 6 months, 9 months and 12 months of treatment. **(D)** BAFF mRNA expression levels in monocytes of IM treated SSc patients (n=8) at baseline and 6 months of treatment. **(E)** PIIINP levels in serum from IM treated SSc patients (n=7) at baseline and 6 months of treatment.
Data from the two patients who responded to IM is displayed in red. In A and C the Kruskal-Wallis test was performed followed by Mann Whitney U test in A-E. Only significant differences are shown where (*) represents *P*-values <0.05.

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CHAPTER 6

INTERFERON TYPE I AND TH17 CELLS: A DANGEROUS LIAISON IN PRIMARY SJÖGREN'S SYNDROME

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Submitted for publication

ABSTRACT

Introduction The novel T helper 17 subset (Th17 cells), which produces IL-17A, IL-17F, IL-22 and IL-21, has been implicated in the pathogenesis of primary Sjögren's syndrome (pSS). Interferon (IFN) type I is yet another key player in the pathogenesis of pSS. The aim of this study was to determine whether Th17 cells are increased in patients with high IFN type I activity, as reflected by the presence of IFN type I signature.

Methods IFN type I signature was assessed by mRNA expression of IFN type I inducible genes in monocytes. T helper memory (CD4+CD45RO+) cell populations defined by chemokine receptor expression (Th17: CCR6+CCR4+CXCR3-CCR10-; Th22: CCR6+CCR4+CXCR3-CCR10+; Th1: CCR6-CXCR3+CCR4-; and Th2: CCR6-CXCR3-CCR4+; Treg: CD25hiFoxP3+) were analyzed by flow cytometry in peripheral blood of 12 pSS patients positive (IFN+) and 12 patients negative (IFN-) for the IFN type I signature and 12 healthy controls (HC).

Results Th17 and Treg percentages were significantly increased in IFN+ patients compared with IFN- patients and HC. This was accompanied by a significant decrease in Th1 cell percentages in IFN+ patients compared with HC. Interestingly, Th17 cell percentages were significantly higher in patients with anti-SSA antibodies, hypergammaglobulinemia and elevated expression of monocyte B cell activating factor (BAFF), all three previously found in IFN+ patients.

Conclusions Here we show for the first time increased Th17 cell percentages in IFN+ pSS patients together with increased Treg and decreased Th1 cell percentages. The co-acting potential of IFN type I and Th17 pathway in the pathogenesis of pSS warrants further investigation.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a generalized autoimmune disease characterized by lymphocytic infiltrates in salivary and lacrimal glands. Recently, a novel T helper cell subset has been identified, named Th17. Naive CD4+ T cells differentiate into Th17 cells under the influence of IL-6 and TGF-β [1]. Expansion and stability of the Th17 population is induced by IL-21 and IL-23, respectively [2, 3]. Th17 cells and their cytokines IL-17A, IL-17F, IL-21 and IL-22, have been implicated in several autoimmune diseases, including pSS [4, 5].

In the infiltrates in glands of both pSS patients and mouse models for pSS, IL-17 and IL-17 producing cells were observed, and IL-17A is thought to play an important role in the development of these infiltrates [6-10]. In peripheral blood of pSS patients the number of IL-17 producing CD4+ T cells [8] and IL-17 plasma levels were increased [9]. Moreover, overexpression of IL-17A in salivary glands of pSS-non-susceptible C57BL/6J mice resulted in induction of pSS-like disease, supporting a role for IL-17 in disease development [11].

Several lines of evidence support a key role for interferon (IFN) type I in the pathogenesis of pSS [12]. We recently showed that IFN type I induced gene expression, named the IFN type I signature, is present in 55% of pSS patients and correlates with higher disease activity, anti-SSA/anti-SSB antibodies, IgG levels and B cell activating factor (BAFF) gene expression [13]. Our observation was in line with the described local and systemic increased IFN type I expression in pSS [14-18].

Interestingly, co-activity between IFN type I and Th17 pathway has been suggested in autoimmune diseases [19, 20]. IFN type I treatment was effective in EAE, a mouse model for MS, if the disease was Th1 driven, but caused exacerbation if the disease was Th17 driven [20]. In line with this, MS patients that did not respond to IFN type I therapy had higher serum levels of IL-17A before therapy onset [20]. These two observations suggest additional effects of the IFN type I and Th17 system to co-act in the pathogenesis of autoimmune diseases. Further evidence strengthening the co-activity between the Th17 and IFN type I pathways was provided by the Ro52/TRIM21-/- mouse model. Ro52/TRIM21 is involved in the ubiquitination of interferon regulatory factors (IRFs), a process which limits the IFN type I response [21]. Antibodies directed against Ro52 are present in about 75% of pSS patients [22]. After ear tagging Ro52/TRIM21-/- mice develop an SLE like phenotype [23]. Interestingly, when these mice are crossed on an IL-23p19-/- mouse line, they do not develop SLE, indicating that the development of an SLE phenotype through enhanced IFN type I production in these mice is dependent on the IL-23/Th17 pathway.

In pSS patients higher serum IL-22 levels were observed, correlating with higher anti-SSA/SSB antibodies, hypergammaglobulinemia and rheumatoid factor [24]. Interestingly we found higher anti-SSA/SSB antibodies, hypergammaglobulinemia and rheumatoid factor in IFN type I signature positive patients compared with IFN type I negative patients [13]. Moreover, BAFF — a factor strongly correlating with the presence of the IFN type I signature

in our cohort study — is involved in dendritic cell (DC) maturation and DC driven Th17 cell differentiation *in vitro* [25]. BAFF gene silencing ameliorated joint pathology and inhibited the generation of Th17 cells in the joints of a collagen induced arthritis (CIA) mouse model. IL-17 was furthermore shown to be involved in BAFF-mediated exacerbation of CIA.

In summary, a role for Th17 cells and IL-17A has been found in pSS [5-11]. In addition, the evidence for the involvement of IFN type I in pSS is increasing [12-18]. Several animal models suggest an association between IFN type I and Th17 cells [19-21]. So far, no studies on co-occurrence of these two key players in pSS patients have been performed. Here we show, for the first time, an increase in Th17 cells in pSS patients that are positive for the IFN type I signature, reflecting an increased IFN type I activity. Furthermore, we investigated the relationship between Th17 cell frequency and various disease manifestations and BAFF gene expression.

PATIENTS AND METHODS

Patients

24 patients with a positive diagnosis for pSS according to 2002 American-European criteria [26] were recruited at the outpatient clinic of the Immunology department of the Erasmus Medical Center Rotterdam. Patients treated with prednisone >10 mg daily, immunosuppressants or biologicals were excluded. The level of disease activity was assessed using EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) [27]. 12 age and gender matched healthy controls neither suffering from autoimmune diseases nor using corticosteroids were included. Characteristics of patients and controls are summarized in Table 1. Medical Ethical Review Committee of the Erasmus MC approved the study and written informed consent was obtained.

Blood collection and isolation of monocytes

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for peripheral blood mononuclear cell (PBMC) preparation as described previously [28]. CD14 positive monocytes were isolated as described[28].

RQ-PCR

Total RNA was isolated from purified monocytes followed by cDNA preparation and RQ-PCR analysis using predesigned primer/probe sets (Applied Biosystems) [28]. For calculation of relative expression, all samples were normalized against expression of the household gene Abl [29]. Fold change values were determined from normalized CT values using 2ΔΔCT method (User Bulletin, Applied Biosystems, Foster City, California).

Table 1. Demographics and clinical characteristics of participants

Variable	pSS patients (n=24)		Healthy controls (n=12)
	IFN type I negative (n=12)	IFN type I positive (n=12)	
Demographics			
N females (n)	12/12 (100%)	12/12 (100%)	12/12 (100%)
Age (years)	63.2±10.7	62.3±11.4	54.8±14.2
Disease duration (years)	12.3±7.4	14.0±7.9	-
ESSDAI	1.0 (1.0,1.25)	2.0 (1.0,3.0)	-
Laboratory parameters			
Anti-SSA (n)	4/12 (33%)	12/12 (100%)	-
Anti-Ro52 (n)	4/12 (33%)	12/12 (100%)	-
Anti-Ro60 (n)	2/12 (17%)	11/12 (92%)	-
Anti-SSB (n)	1/12 (8%)	10/12 (83%)	-
IgG (g/l)	9.25 (6.10,13.10)	16.55 (10.48,20.05)	-
IgA (g/l)	1.84 (1.31,2.87)	3.20 (1.61,4.45)	-
IgM (g/l)	1.09 (0.66,2.68)	1.20 (0.90,3.90)	-
Medical therapy			
Pilocarpine (n)	6/11 (55%)	4/12 (33%)	-
Hydroxychloroquine (n)	6/11 (55%)	8/12 (67%)	-
Corticosteroids (n)	0/11 (0%)	1/12 (8%)	-

Values are the mean ± SD, median (25% quartile,75% quartile) or number (%) of patients, depending on whether the data are continuous or dichotomous, and whether the data are normally distributed or not.

Flow cytometry and antibodies

Monoclonal antibody stainings, intracellular cytokine detection and flow cytometry were performed as described previously [30]. For intracellular cytokine detection by flow cytometry, cells were stimulated for 4 hours with 50 ng/ml PMA, 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO) and Golgistop (BD Biosciences, San Diego, CA). Detection of the transcription factor Foxp3 was performed using the transcription factor staining buffer set, according to the manufacturer's instructions (eBioscience, San Diego, CA). The following monoclonal antibodies (MoAb) were purchased from BD Biosciences: CXCR3, CCR4, CD45RO, CCR6 and CD4. Furthermore, Foxp3 MoAb was purchased from eBioscience (San Diego, CA). CCR6, and CD25 MoAb were obtained from BioLegend (San Diego, CA). CCR10 was purchased from R&D systems (Minneapolis, MN). Samples were acquired on a FACSCantoII flow cytometer and analyzed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR). Cells were gated on the lymphocyte fraction.

Measurement of immunoglobulin levels and autoantibodies

IgG, IgA, IgM were measured via turbidimetry using an Modular P800 (Roche, Almere, The Netherlands).

Anti-SSA and anti-SSB were determined by EliA (Thermo Scientific, Waltham, MA) and confirmed with ANA profile immunoblot (EuroImmun, Luebeck, Germany). When discrepant, QUANTA Lite ELISA-kit from INOVA (San Diego, CA) was used for confirmation.

Calculation of IFN scores

IFN scores were calculated as described before [13]. The standardized
expression levels of IFI44, IFI44L, IFIT3, LY6E and MX1 were summed for each subject to provide an IFN type I expression score. The mean and SD level of each IFN inducible gene in the HC group were used to standardize expression levels of each gene for each study subject. As previously described we set the threshold for a positive IFN type I signature at IFN score of 10. pSS patients were stratified according to their IFN type I signature status (IFN score<10 vs. IFN score ≥10).

Statistical analyses

Statistical analyses were performed using SPSS 20.0 package. When data were not normally distributed, values were expressed as medians with interquartile ranges (IQRs) and comparisons were made using the non-parametric Mann-Whitney U test. For normally distributed data, independent T test was used to compare means. In case of more than two samples one-way ANOVA was performed or the non-parametric Kruskal-Wallis test. Correlations were assessed either using Pearson correlation test for normally distributed data or Spearman's rho when data were not normally distributed. Differences were considered statistically significant if $p<0.05$.

RESULTS

Stratification of pSS patients into IFN type I signature positive and negative patients

Previously we described the prevalence of the IFN type I signature in pSS based on the mRNA expression levels of 5 interferon type I inducible genes (IFIGs) in monocytes: IFI44, IFI44L, IFIT3, LY6E and MX1 [13]. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN scores per subject represent the sum of these standardized scores. In this cohort the threshold for a positive IFN type I signature was set at an IFN score of 10, as described before [13]. pSS patients were stratified in IFN+ and IFN- patients according to their IFN type I scores (IFN score<10 vs. IFN score ≥10).

Increased Th17, Treg percentages and decreased Th1 percentages in pSS patients positive for the IFN type I signature

We hypothesized that the frequency of Th17 cells would be increased in IFN+ patients. To test this hypothesis, T helper (Th) cell populations were analyzed in peripheral blood of 12 IFN+ patients, 12 IFN- patients and 12 HCs by chemokine receptor expression. The gating strategy is shown in figure 1. To distinguish Th1, Th2, Th17 and Th22 cells a gating strategy was used as previously described [31]. In short, after gating on lymphocytes, we first excluded CD25+ cells in order to omit regulatory T cells (Treg), which were analyzed in a separate staining. Subsequently, we gated on CD4+CD45RO+ cells to obtain memory Th cells. This Th memory pool was divided into a CCR6- and a CCR6+ fraction. In the CCR6- fraction, we distinguished Th1 and Th2 cells based on the expression CXCR3 and CCR4, since Th1 cells are CXCR3+CCR4- and Th2 are CXCR3-CCR4+ [32]. In the CCR6+ fraction, we first selected CXCR3-CCR4+ cells. This fraction contains both Th17 and Th22 cells. They were separated based on CCR10 expression, as Th17 cells do not express CCR10 and Th22 cells do [32-34]. The Treg cells were studied in a separate staining. After gating on lymphocytes, we selected CD4+CD45RO+ cells to obtain memory Th cells. Subsequently, by gating on CD25+ we enriched for Tregs. To analyze the Treg population, we included expression of Foxp3, the Treg-specific transcription factor.

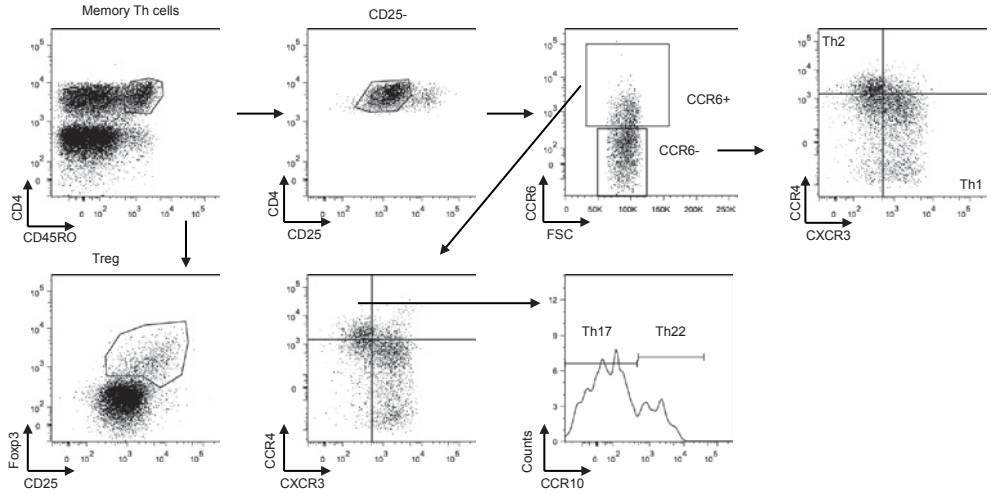

Figure 1. Gating strategy for the T helper cell populations

T helper memory (CD4+CD45RO+) cell populations were defined by chemokine receptor expression by flow cytometry in peripheral blood. Th17: CCR6+CCR4+CXCR3-CCR10-; Th22: CCR6+CCR4+CXCR3-CCR10+; Th1: CCR6-CXCR3+CCR4-; and Th2: CCR6-CXCR3-CCR4+; Treg: CD25hiFoxP3+.

By applying this gating strategy we found that chemokine receptor defined Th17 and Treg cell percentages were significantly increased in IFN+ versus IFN- patients (p=0.005 and p=0.020 respectively) and HC (p=0.029 and p=0.015 respectively). In contrast, Th1 cell percentages were decreased in IFN+ patients compared with HC (p=0.004). Percentages of Th22 and Th2 cells were unchanged between groups (Figure 2A).

Th17 cell percentages positively correlated significantly with Th22 cell percentages (r=0.458, p=0.024) (Figure 2B) and with Treg percentages (r=0.523, p=0.037) (Figure 2C). Moreover, a significant negative correlation was observed between Th17 cell populations and Th1 cell populations (r=-0.424, p=0.039) (Figure 2D).

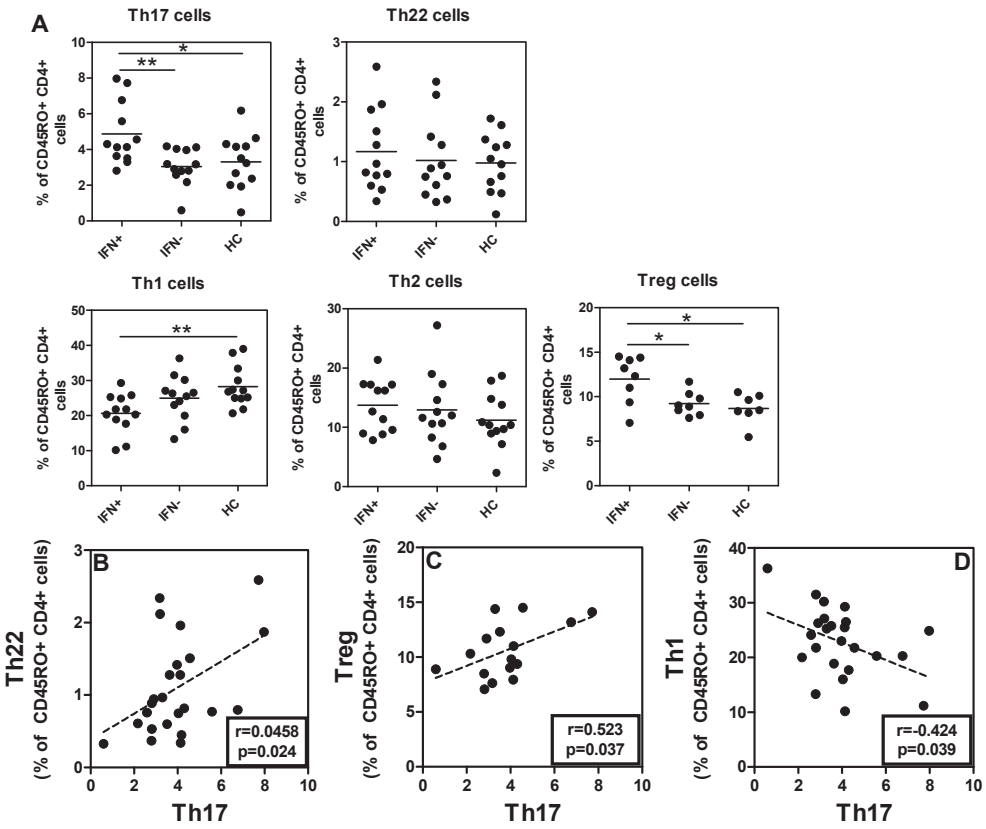

Figure 2. Th17 and Treg cell proportions are increased in IFN+ patients compared with IFN- patients and healthy controls and Th1 percentages are decreased in IFN+ patients

(A) FACS analysis of T helper subset populations, obtained as described under (Figure S1). Correlation between Th17 population and: **(B)** Th22 population (n=22). **(C)** Treg population (n=22). **(D)** Th1 population (n=22). The correlation coefficients (r) and *P* values are shown. Horizontal line indicates mean for 8-12 IFN+ patients, 8-12 IFN- patients and 8-12 healthy controls (HC).

* $p<0.05$; ** $p<0.01$; to compare means one-way ANOVA was performed followed by the independent T test; for correlations Pearson correlation test was used.

Th17 cell percentages correlate with IFN scores and are increased in patients with anti-SSA antibodies and higher serum IgA levels

A significant positive correlation was found between the expression of IFIGs, as reflected by the IFN scores and Th17 cell percentages (r=0.553, p=0.005) (Figure 3A). When patients were stratified according to their autoantibody status (anti-SSA positive vs. anti-SSA negative) for comparison of their Th17 percentages, patients with anti-SSA showed higher Th17 percentages than patients without anti-SSA (p=0.025) (Figure 3B). This increase was observed for both anti-Ro52 (=0.025) (Figure 3C) and anti-Ro60 antibodies (p=0.01) (Figure 3D). Interestingly, we previously described pSS patients with anti-SSA antibodies to have higher IFN scores [13]. No significant differences in Th17 percentages were found with respect to anti-SSB autoantibody status (Figure 3E).

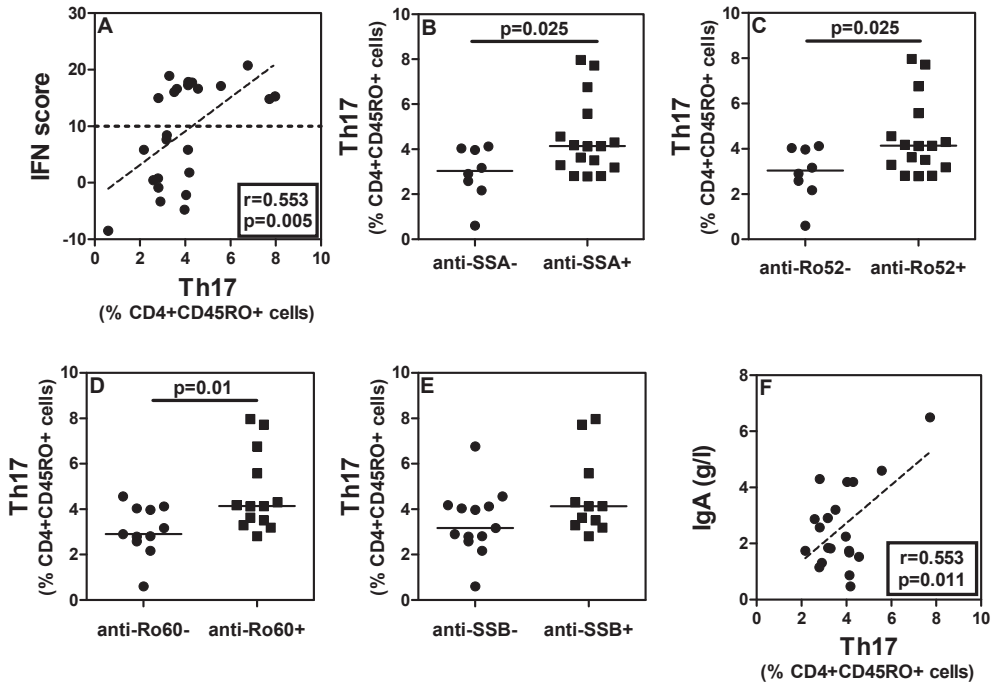

Figure 3. Th17 cell percentages correlate with IFN scores and are increased in patients with anti-SSA antibodies and with higher IgA levels

(A) Correlation between IFN scores and Th17 percentages. **(B)** Th17 percentages in pSS patients negative (n=8) or positive (n=16) for anti-SSA. **(C)** Th17 percentages in pSS patients negative (n=8) or positive (n=16) for anti-Ro52. **(D)** Th17 percentages in pSS patients negative (n=11) or positive (n=13) for anti-Ro60. **(E)** Th17 percentages in pSS patients negative (n=13) or positive (n=11) for anti-SSB. **(F)** Correlation between Th17 percentages and serum IgA levels. The correlation coefficients (r) and *P* values are shown. Horizontal line indicates median. For correlations Pearson correlation test was used and to compare means the Mann-Whitney U test was used.

Next to the presence of auto-antibodies, we also observed higher IgG serum levels in pSS patients positive for the IFN type I signature [13]. In this cohort no correlation was found between IgG or IgM serum levels and Th17 percentages (data not shown), but a significant correlation between IgA serum levels and Th17 percentages was observed (r=0.553, p=0.011) (Figure 3F).

Since we previously found higher ESSDAI disease activity scores in patients with higher IFN scores [13], we next investigated whether Th17 cell percentages are also associated with the ESSDAI disease activity scores. No significant correlation was observed between the ESSDAI disease activity scores and Th17 percentages (data not shown).

BAFF mRNA expression is positively correlated with Th17 percentages and negatively correlated with Th1 percentages

As already mentioned, a factor known to be involved in the pathogenesis of pSS and found to correlate strongly with the IFN type I signature is BAFF. In this pSS cohort, again a strong positive correlation between IFN scores and monocyte BAFF mRNA expression in pSS patients was found (r=0.780, p<0.0001) (Figure 4A). Furthermore, a positive significant correlation was present between BAFF mRNA expression and Th17 cell percentages (r=0.412, p=0.046) (Figure 4B). A negative significant correlation was found between BAFF mRNA and Th1 cell percentages (r=-0.478, p=0.018) (Figure 4C).

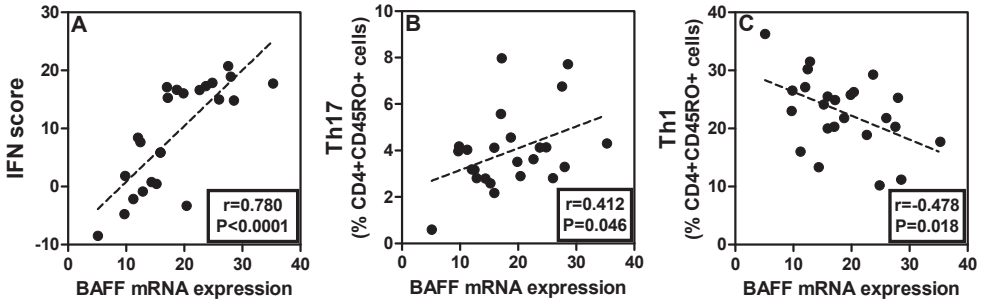

Figure 4. BAFF mRNA expression is positively correlated with Th17 percentages and negatively correlated with Th1 percentages

Correlation between monocyte BAFF mRNA expression and **(A)** IFN score, **(B)** Th17 percentages, **(C)** Th1 percentages in pSS patients (n=24). The correlation coefficients (r) and *P* values are shown. For correlations Pearson correlation test was used.

DISCUSSION

The present study demonstrated that chemokine receptor defined Th17 cell percentages are increased in peripheral blood from pSS patients positive for the IFN type I signature. Moreover, Th17 cell percentages were significantly correlated with anti-SSA antibodies, hypergammaglobulinemia and monocyte BAFF mRNA expression, all three previously found

in IFN type I signature positive patients. These findings strengthen the hypothesis that IFN type I and Th17 cells act in concert to sustain and amplify the autoimmune processes in Sjögren's syndrome [13].

A possible mechanism explaining the co-occurrence of increased IFN type I activity and increased Th17 percentages might be that both IFN type I and DC production of Th17 inducing cytokines such as IL-6 and IL-23 are simultaneously regulated through TLR signalling. Indeed TLR7 activation in plasmacytoid DCs, the main producers of IFN type I, promotes and modifies Th17 cell differentiation and function [35]. IFN type I itself is also able to promote Th17 differentiation and IL-17 production through induction of STAT3 in T cells and IL-6 induction in DCs [36, 37]. In addition Santini *et al.* showed that IFN type I conditioned monocytes differentiate into DCs driving the development of Th17 cells from autologous naive CD4 T cells [38].

Next to the direct effect of IFN type I on Th17 cells, IFN type I may also act indirectly through the production of BAFF. BAFF gene silencing in a CIA mouse model showed inhibited generation of Th17 cells in the arthritic joint [25]. In addition IL-17A can induce the formation of neutrophil extracellular traps (NETs) [39], which could potentially provide new auto-antigens to activate TLRs on DCs, thereby forming a pro-inflammatory loop.

IL-17 and Th17 cells are also increased in the salivary glands of pSS patients. Salivary gland epithelial cells of pSS patients showed a skewed cytokine production, with increased IL-6 and decreased TGF-ß concentrations [40]. Moreover, salivary gland epithelial cells are able to produce IFN type I upon TLR3 stimulation with poly (I:C) [41]. Thus, a scenario with the pSS salivary gland epithelial cells contributing to an environment which is enriched in IFN type I and Th17 cells leading to a chronic inflammatory loop can be envisaged.

The increase in chemokine receptor defined Th17 cell percentages in IFN+ patients compared with IFN- patients and HCs was accompanied by a significant decrease in Th1 cell percentages in IFN+ patients versus HCs. Furthermore, there was a significant negative correlation between Th1 fractions and Th17 fractions (Fig 1). A possible explanation for this reciprocal relationship might be found in STAT3 signaling. In mice, STAT3 activation in T lymphocytes and other hematopoietic cells was shown to inhibit Th1 differentiation through inhibition of IL-12, necessary for Th1 differentiation, and T-bet, the Th1 lineage master transcription factor. At the same time, STAT3 signaling stimulates Th17 development by increasing IL-23 gene transcription and ROR-γt expression [42, 43]

Interestingly, we observed an increase in Treg cell percentages in IFN+ patients versus IFN- patients and HCs, and a significant correlation between Th17 and Treg cell percentages (Fig 1). This is in concordance with previous research showing an increase in Treg cells (defined as CD4+CD25high cells) in peripheral blood and salivary gland tissue in Sjögren's syndrome patients [44, 45]. However, others have shown a decrease in Treg cells in pSS [46-48]. The discrepancies between these studies cannot readily be explained, but different

medication might play a role. Immunosuppressive drugs including biologicals have an inducing or a reducing effect on Treg cell numbers (reviewed in [49]). In this study, no patients were receiving biologicals and no effect of medication on different Th subsets or cytokine expression was detected (data not shown). A limitation of our study is that no functional analysis of the Treg population has been performed.

Recently CD3+CD4-CD8- DN T cells were found as major producers of IL-17 in pSS [50]. DN T cells displayed a Th17 phenotype, as supported by their ROR-γt expression. Moreover, these cells produced IL-17 in the infiltrated salivary glands. Active involvement of DN T cells in the pathogenesis of pSS has been suggested. Whether these DN T cells are linked to IFN type I pathway as well, remains a matter for future research.

Although we have shown in this study an increase in chemokine receptor defined Th17 cells (CD4+CD45RO+CCR6+CXCR3-CCR4+CCR10-cells) in IFN+ patients versus IFN- patients and healthy controls, due to limitations in current antibody-fluorochrome availability we were not able to analyze cytokine expression in these chemokine defined Th17 cells. Future studies where both chemokine receptor expression and cytokine expression can simultaneously be assessed, are called for.

In conclusion, our study shows for the first time a co-occurrence of increased IFN type I activity and increased Th17 cell percentages in pSS. This was accompanied by higher Treg percentages and reduced percentages of Th1 cells in IFN+ patients. These findings provide support for the concept that IFN type I acts in concert with Th17 cells in pSS. This observation might have implications for future treatment of pSS and other systemic autoimmune diseases where IFN type I plays a role. Up till now there is mainly symptomatic treatment for pSS and no evidence-based therapies yet. Our data demonstrate that Th17 cell percentages are increased in IFN+ pSS patients, paving the way for combination therapies possibly resulting in more significant clinical effects in the future.

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CHAPTER 7

TH17 CYTOKINES AND INTERFERON TYPE I: PARTNERS IN CRIME IN SLE?

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Submitted for publication

ABSTRACT

Introduction A hallmark of systemic autoimmune diseases like systemic lupus erythematosus (SLE) is the increased expression of Interferon (IFN) type I inducible genes, so-called IFN type I signature. Recently, T helper 17 subset (Th17 cells), which produces IL-17A, IL-17F, IL-21 and IL-22, has been implicated in SLE. As CCR6 enriches for Th17 cells, we used this approach to investigate whether CCR6+ memory T helper cells producing IL-17A, IL-17F, IL-21 and/or IL-22 are increased in SLE patients and whether this increase is related to the presence of IFN type I signature.

Methods 25 SLE patients and 15 healthy controls (HC) were included. SLE patients were divided into IFN type I signature positive (IFN+) (n=16) and negative (IFN-) (n=9) patients as assessed by mRNA expression of IFN inducible genes (IFIGs) in monocytes. Expression of IL-17A, IL-17F, IL-21 and IL-22 by CD4+CD45RO+CCR6+ T cells (CCR6+ cells) was measured by flow cytometry and compared between IFN+, IFN- patients and HC.

Results Increased percentages of IL-17A and IL-17A/IL-17F double producing CCR6+ cells were observed in IFN+ patients compared with IFN- patients and HC. IL-17A and IL-17F expression within CCR6+ cells correlated significantly with IFIG expression. In addition, we found significant correlation between BAFF – a factor strongly correlating with IFN type I – and IL-21 producing CCR6+ cells.

Conclusions We show for the first time higher percentages of IL-17A and IL-17A/IL-17F double producing CCR6+ memory T helper cells in IFN+ SLE patients, supporting the hypothesis that IFN type I co-acts with Th17 cytokines in SLE pathogenesis.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a debilitating systemic autoimmune disease characterized by the production of auto-reactive antibodies and multi-organ inflammation [1]. A hallmark of systemic autoimmune diseases is the increased expression of Interferon (IFN) type I in both blood and disease-affected tissues [2]. About half of the SLE patients exhibit an IFN type I signature or upregulation of IFN type I induced genes (IFIGs) which have been found to correlate with disease activity and severity [3-5].

Another key factor in the pathogenesis of SLE apart from IFN type I, is interleukin-17A (IL-17A). IL-17A is produced by several immune cell types, including CD4+ T cells (Th17 cells), CD8+ T cells, CD4-CD8-CD3+ (double negative, DN) T cells, NK cells, γδ-T cells and mast cells. Naïve CD4+ T cells differentiate to Th17 cells under the influence of IL-6 and TGFβ [6]. The expansion and stability of the Th17 population is regulated by IL-21 and IL-23, respectively [7, 8]. C57BL/6-lpr/lpr mice that lack IL-23 receptor signalling are protected for SLE development [9]. In SLE patients increased plasma levels of IL-17A correlated with disease severity (SLEDAI) [10]. In addition, in peripheral blood of SLE patients an increased number of IL-17 producing cells was observed. These cells correlated with disease activity and decreased upon treatment [11, 12]. IL-17-producing cells have also been found in several affected organs of SLE patients [11, 13].

Co-activity between IFN type I and IL-17/Th17 cells has been suggested in autoimmune diseases [14, 15]. In EAE, a mouse model for MS, IFN type I treatment caused exacerbation if the disease was Th17 driven but was effective if the disease was Th1 driven [15]. In the same study, MS patients that did not respond to IFN type I therapy had higher serum levels of IL-17A before therapy onset [15]. These two observations suggest additional effects of the IFN type I and Th17 system co-acting in the pathogenesis of autoimmune diseases.

Co-activity of IFN type I and Th17 pathways has also been suggested for SLE by the Ro52/TRIM21-/- mouse model. Ro52/TRIM21 is involved in the ubiquitination of interferon regulatory factors (IRFs), a process which limits the IFN type I response [16]. After ear tagging Ro52/TRIM21-/- mice develop an SLE like phenotype [17]. Interestingly, when these mice are crossed on an IL-23p19-/- mouse line, they do not develop SLE, indicating that the development of a SLE phenotype through enhanced IFN type I production in these mice is dependent on the IL-17/Th17 pathway.

Yet another important factor involved in the pathogenesis of SLE is B cell activating factor of the tumor necrosis factor family (BAFF). BAFF transgenic mice develop lupus-like disease [18] and increased expression of BAFF protein has been found in SLE patients, correlating with increased disease activity [19-21]. We previously described a strong correlation between BAFF mRNA expression in monocytes and the IFN type I signature in primary Sjögren's syndrome (pSS) patients [22]. Interestingly, IL-21, a cytokine produced by Th17 cells, in combination with BAFF has been reported to synergistically induce the

differentiation of human memory B cells into antibody-producing plasma cells in absence of further co-stimulation [23]. BAFF is known to be involved in germinal center formation [24], a process in which IL-17 is also involved [25].

The above mentioned literature suggests an association between the pathogenic IFN type I and Th17 pathway. So far, no studies have been performed on the co-occurrence of these pathogenic pathways in SLE patients. In this study, we report for the first time a higher percentage of IL-17A and IL-17A/F producing CCR6+ T memory cells in IFN type I positive SLE patients. Moreover, BAFF gene expression in monocytes correlates significantly with IL-21 expression in these CCR6+ cells, supporting the concept of co-activity of IFN type I, Th17 and BAFF in the pathogenesis of SLE.

PATIENTS AND METHODS

Patients

25 patients fulfilling the American College of Rheumatology revised criteria for SLE [26] were recruited at the outpatient clinic of the Immunology department and the Rheumatology department of the Erasmus Medical Center Rotterdam. The level of disease activity was assessed using the SLEDAI [27]. 15 healthy controls (HC) neither suffering from autoimmune diseases nor using corticosteroids were included. Characteristics of patients and controls are summarized in Table 1. Medical Ethical Review Committee of the Erasmus MC approved the study and written informed consent was obtained.

Table 1. Demographics and clinical characteristics of participants

Variable	SLE patients (n=25)		Healthy controls (n=15)
	IFN type I negative (n=9)	IFN type I positive (n=16)	
Demographics			
N females (%)	9/9 (100%)	14/16 (88%)	15/15 (100%)
Age (years)	41.3±17.5	39.8±15.7	41.0±14.0
Disease duration (years)	12.1±8.0	14.4±11.3	-
SLEDAI	4.0 (1.0,17.0)	4.50 (2.0,10.0)	-
Treatment			-
Hydroxychloroquine	7/9 (78%)	11/16 (69%)	-
Corticosteroids	5/9 (56%)	10/16 (63 %)	-
Mycophenolate mofetil	0/9 (0%)	4/16 (25%)	-
Azathioprine	2/9 (22%)	3/16 (19%)	-
Cyclophosphamide	0/9 (0%)	1/16 (6%)	-

Values are the mean ± SD, median (25% quartile,75% quartile) or number (%) of patients, depending on whether the data are continuous or dichotomous, and whether the data are normally distributed or not

Blood collection and isolation of monocytes

Blood was collected in clotting tubes for serum preparation (stored at -80°C) and in sodium-heparin tubes for peripheral blood mononuclear cell (PBMC) preparation as described previously [28]. CD14 positive monocytes were isolated as described [28].

RQ-PCR

Total RNA was isolated from purified monocytes followed by cDNA preparation and RQ-PCR analysis using predesigned primer/probe sets (Applied Biosystems) [28]. For calculation of relative expression, all samples were normalized against expression of the household gene Abl[29]. Fold change values were determined from normalized CT values using $2^{-}\Delta\Delta CT$ method (User Bulletin, Applied Biosystems, Foster City, California).

Flow cytometry

PBMCs were restimulated, stained and measured by flow cytometry as previously described [30]. For extracellular staining, CD4, CD45RO and CCR6 monoclonal antibodies were obtained from BD Biosciences (San Diego, CA), and CD25 antibodies from Biolegend Inc. (San Diego, CA). For intracellular staining, FoxP3, IL-17A, IL-17F and IL-22 monoclonal antibodies were obtained from eBioscience (San Diego, CA), and IL-17A monoclonal antibodies from Biolegend Inc. Samples were measured on a FACScantoII flow cytometer (BD Biosciences). Analysis was performed using FlowJo v7.6 research software (Tree Star Inc. Ashland, OR).

Factor analysis

Expression levels of 11 IFN type I inducible genes were submitted to a principal component analysis to identify correlated groups of genes to reduce data complexity. Kaiser-Meyer-Olkin measure of sampling adequacy was 0.839 with significant Bartlett's test of sphericity ($P< 0.001$). Eigenvalues were derived to assess the amount of variance explained by each component factor.

Statistical analyses

Statistical analyses were performed using SPSS 20.0 package. When data were not normally distributed, values were expressed as medians with interquartile ranges (IQRs) and comparisons were made using the non-parametric Mann-Whitney U test. In case of more than two samples the non-parametric Kruskal-Wallis test was performed. Correlations were assessed either using Pearson correlation test for normally distributed data or Spearman's rho when data were not normally distributed. Differences were considered statistically significant if $p<0.05$.

RESULTS

Prevalence of the IFN type I signature in SLE patients

In monocytes of 25 SLE patients and 15 HCs we assessed the expression levels of 11 IFIGs previously assessed in monocytes from patients with primary Sjögren's syndrome (pSS) (IFI27, IFI44L, IFIT3, IFITM1, SERPING1, IFIT1, IFIT2, LY6E, IFI44, XAF1 and MXA) [22]. To reduce data complexity, expression levels of the 11 genes were submitted to a principal component analysis to identify correlated groups of genes. The results of the principal component analysis identified a subset of 4 genes (IFI44L, IFITM1, SERPING1 and LY6E) to explain 95% of the total variance of the 11 IFN type I inducible genes within the SLE cohort.

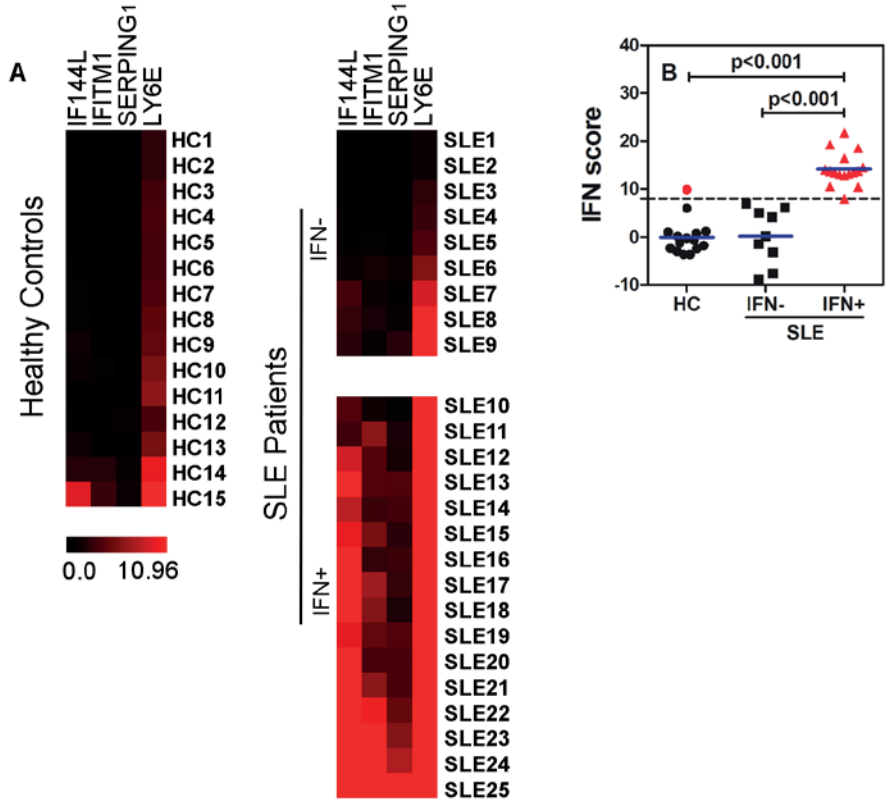

Figure 1. Prevalence of IFN type I signature in SLE patients

(A) Heatmap showing gene expression of 4 IFN type I inducible genes in monocytes of SLE patients (n=25) and HC (n=15). On the left the HC are depicted and on the right the SLE patients are depicted and subdivided into IFN type I signature positive and negative patients. Red colour indicates high gene expression and cases are depicted according to ascending IFN scores. **(B)** Distribution of IFN scores in IFN type I signature positive and negative patients and HC. In red IFN type I positive cases are depicted. Blue lines represent medians.

Given that the expression of these 4 IFN type I inducible genes was not normally distributed, log transformations of expression values were performed and IFN scores were calculated as described for pSS [22]. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN scores per subject represent the sum of these standardized scores. When we set the threshold for a positive IFN type I signature at IFN score of 8 [22], 64% of SLE patients displayed an IFN type I signature and one of the 15 HC subjects (7%) (Figure 1A and 1B).

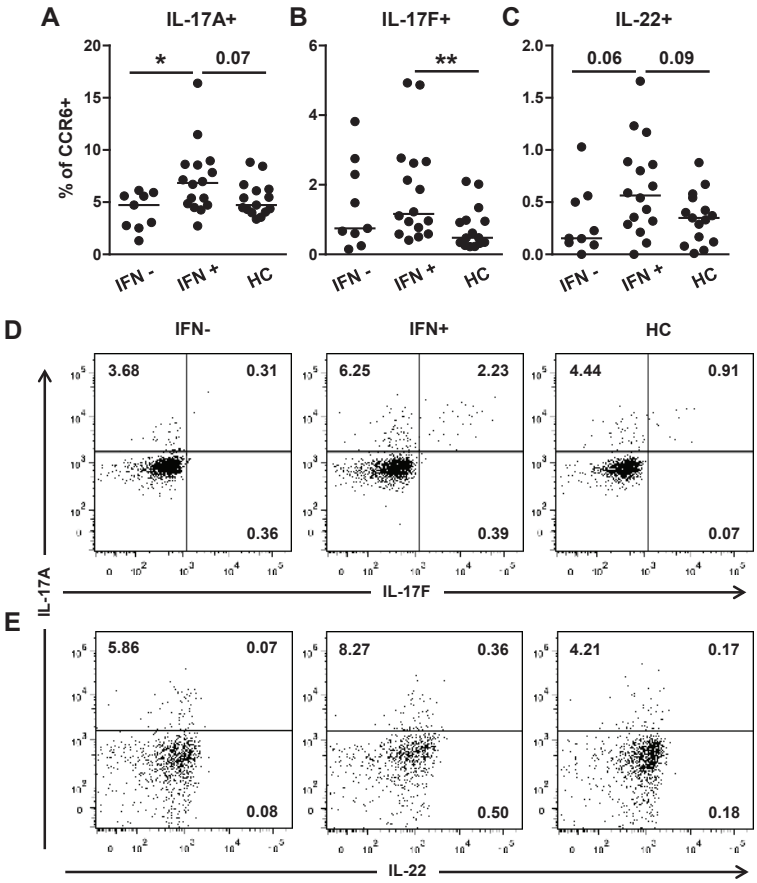

Figure 2. SLE patients with IFN type I signature show higher percentages of IL-17A and IL-17A/IL-17F producing CCR6+ cells

(A) Representative graphs of proportions of IL-17A and IL-17F expressing cells within CCR6+ memory T cell population (defined as CD4+CD45RO+CD25-CCR6+) in PBMCs of IFN negative (IFN-) and IFN positive (IFN+) patients and healthy controls (HC). **(B)** Representative graphs of proportions of IL-17A and IL-22 expressing cells within CCR6+ memory T cell population in PBMCs of IFN- and IFN+ patients and HC. **(C-E)**, Proportions of IL-17A [C], IL-17F [D] and IL-22 [E] expressing cells within CCR6+ memory T cell population in PBMCs of IFN- (n=9) and IFN+ (n=16) patients and HC (n=15). All proportions were measured by intracellular flow cytometry. Horizontal line indicates median. *p<0,05; **p<0,01; to compare means Kruskal-Wallis test was used followed by Mann-Whitney U test.

SLE patients with IFN type I signature show higher percentages of IL-17A and IL-17A/IL-17F producing CCR6+ cells

Because CCR6 enriches for Th17 cells [31-33], CCR6+ cells were selected after gating on lymphocytes and memory Th cells (CD4+CD45RO+ cells) within PBMCs and after CD25high cells were excluded. To investigate whether the IFN type I signature is associated with an increase in Th17 cytokines expressed by memory CCR6+ T cells, we measured the percentages of IL-17A, IL-17F, IL-22 and IL-21 producing CCR6+ T memory cells in SLE patients positive for the IFN type I signature (IFN+) and patients negative for the signature (IFN-) and HC. Interestingly, the percentages of CCR6+IL-17A+ cells were significantly increased in IFN+ patients as compared with IFN- patients (p=0.03) and a higher trend was observed compared with HC (p=0.07) (Figure 2A, D, E). The percentages of CCR6+IL-17F+ and in particular the IL-17A/IL-17F double producers were significantly increased in the IFN+ group compared with HC (p=0.009) (Figure 2B and D). The percentages of CCR6+IL-22+ cells showed a higher trend for IFN+ vs. IFN- patients (p=0.06) and IFN+ patients vs. HC (p=0.09) (Figure 2C and E). These data suggest an association between the presence of the IFN type I signature and the expression of Th17 cytokines IL-17A and IL-17F in SLE. In addition, we investigated whether the Th17 cytokine production is associated with disease activity as assessed by SLEDAI scores. No significant correlations were observed between the SLEDAI scores and IL-17A and/or IL-17F expression (data not shown).

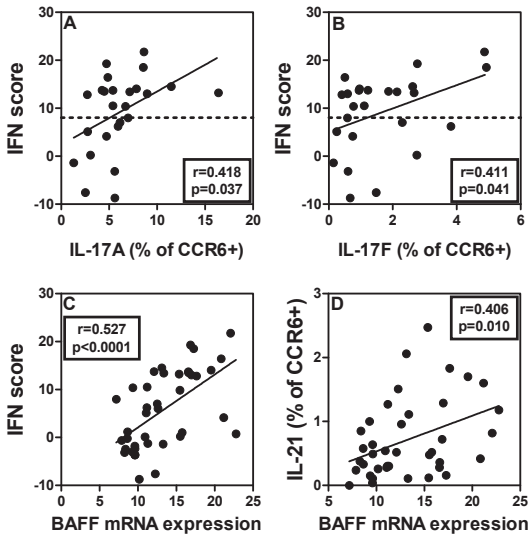

Figure 3. BAFF mRNA expression is correlated with IL-21 expression within CCR6+ memory T cells
(A) Correlation between IFN score and IL-17A expression within CCR6+ memory T cells in SLE patients (n=25). **(B)** Correlation between IFN score and IL-17F expression within CCR6+ memory T cells in SLE patients (n=25). **(C)** Correlation between monocyte BAFF mRNA expression and IFN score in SLE patients and HC (n=40). **(D)** Correlation between monocyte BAFF mRNA expression and IL-21 expression within CCR6+ cells in SLE patients and HC (n=40). The correlation coefficients (r) and *P* values are shown. For correlations Spearman's rho correlation test was used in A, B and D and Pearson correlation test was used in C.

BAFF mRNA expression is correlated with IL-21 expression within CCR6+ memory T cells
Correlating the expression of IFIGs (as reflected by the total IFN score) with other parameters assessed in this study, we observed a significant positive correlation between the expression of IL-17A and IL-17F within CCR6+ cells and IFIG expression (Figure 3A and B). Also in this SLE cohort, IFIG expression correlated strongly with the BAFF mRNA expression in monocytes ($r=0.527$, $p<0.0001$) (Figure 3C). No correlation was observed between BAFF and IL-17A and/or IL-17F expression. However we did find a significant correlation between BAFF mRNA and the percentages of IL-21 producing CCR6+ cells ($r=0.406$, $p=0.010$) (Figure 3D). Both BAFF and IL-21 are involved in the selection and activation of B cells, which is crucial in the pathogenesis of SLE, indicating that downstream factors of the IFN type I and Th17 pathways might also be associated.

DISCUSSION

Here we show for the first time a co-occurrence of increased IFN type I activity and increased IL-17/Th17 system in SLE patients. We found increased percentages of IL-17A and IL-17F producing CCR6+ T memory cells in IFN type I positive SLE patients. This finding further strengthens the hypothesis that IFN type I and Th17 cells by co-acting contribute to the pathogenesis of SLE. Further research to understand the link between these two pathways is warranted.

A possible mechanism explaining the co-occurrence of IFN type I and IL-17/Th17 immune pathway in SLE could be that both IFN type I and production of IL-6 and IL-23 by DCs are regulated through IRF-5 [34, 35]. Activation of TLR signalling on DCs will then lead to simultaneous enhancement of both pathways. Indeed there is evidence that TLR7 activation of plasmacytoid DCs, the main producers of IFN type I, promotes and modifies Th17 cell differentiation and function [36]. IFN type I itself is also able to promote Th17 differentiation and IL-17 production through induction of STAT-3 in T cells and IL-6 in DCs [37, 38]. In addition, IFN type I conditioned monocytes differentiate into DCs driving the development of Th17 cells from autologous naive CD4+ T cells [39].

In addition to the direct effect of IFN type I on Th17 cells, IFN type I may also act indirectly through the production of BAFF. BAFF is reported to be involved in DC maturation and DC driven Th17 cell differentiation *in vitro* [40]. BAFF gene silencing ameliorated joint pathology and inhibited the generation of Th17 cells in the joints of a collagen induced arthritis (CIA) mouse model [40]. In turn IL-17A can induce the formation of neutrophil extracellular traps (NETs) [41], which could potentially provide new auto-antigens to active TLRs on DCs, thereby forming a pro-inflammatory loop.

We find a correlation between BAFF, an IFN type I inducible factor, and the Th17 produced cytokine IL-21. Ettinger *et al.* showed that IL-21 together with BAFF promotes B cell responses by bypassing the need for T cell help or TLR signalling [23]. As these downstream factors are

both involved in activation and selection of B cells, these findings again support the concept that IFN type I and the Th17 pathway act together in driving the disease process of SLE.

In contrast to others we did not find a correlation between SLEDAI and Th17 cytokines [10]. This might be due to the relatively low patient number, which is a limitation of our study.

Although we don't show a functional link between IFN type I and the Th17 pathway, our findings provide the first support for co-occurrence of increased IFN type activity and increased IL-17/Th17 system in SLE. The Th17-IFN type I interaction found in this study might have implications for future treatment of SLE and other systemic autoimmune diseases where IFN type I plays a role. Preliminary results from a phase IIa trial with human IgG1κ anti-IFNα antibody in 87 SLE patients, showed so far a 40% reduction in IFN type I induced gene expression but no clinical effect compared to placebo (abstract Merrill J *et al.**). Our data indicate that IFN type I might act in concert with Th17 cytokines, paving the way for combination therapies possibly resulting in more significant clinical effects in the future.

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CHAPTER 8

GENERAL DISCUSSION

SUMMARY OF DATA IN GENERALIZED AUTOIMMUNE DISEASES

Increased IFN type I activity, reflected by the IFN type I signature in monocytes, was observed in about half of the pSS patients, one third of SSc patients and two thirds of SLE patients compared with HCs. pSS patients with the IFN type I signature (IFN+) showed higher disease activity and IFN+ SSc patients displayed signs of more progressive disease. IFN type I signature showed stability over time in pSS patients. MxA protein expression by Enzyme immunoassay (EIA) in whole blood lysates and by FACS analysis in monocytes were practical and rapid approaches for assessment of IFN type I bioactivity in pSS and SLE.

Next to increased disease activity, signs of increased BAFF system and IL-17/Th17 system were found in IFN+ patients. IFN type I signature strongly correlated to BAFF mRNA expression in monocytes from pSS, SSc and SLE patients. Moreover, BAFF expression could be induced in cultured monocytes by serum of IFN type I signature positive pSS patients.

Chemokine receptor defined Th17 cells and IL-17 producing CCR6+ T memory cells were significantly increased in IFN+ pSS and SLE patients, respectively. Chemokine receptor defined Th17 cell percentage was significantly higher in pSS patients with anti-SSA antibodies, hypergammagloblulinemia and elevated monocyte BAFF mRNA expression, all previously found in IFN type I signature positive patients.

Finally collagen synthesis was increased in IFN+ SSc patients, as determined by N-terminal propeptide of type III collagen (PIIINP) levels.

In summary, IFN+ patients with a systemic autoimmune disease form a separate group of patients with a more severe disease and various signs of activation of other immune systems, such as the BAFF system, IL-17/Th17 system and fibrosis.

This chapter will discuss the implications of these findings in relation to the pathogenesis and treatment of IFN type I positive generalized autoimmune diseases. Finally, the limitations of the studies and future perspectives will be given.

INCREASED IFN TYPE I ACTIVITY IN GENERALIZED AUTOIMMUNE DISEASES

Development of pSS, SLE and SSc has been described upon treatment with IFN type I in patients with malignancies and hepatitis C [1-4] supporting the role of IFN type I in the pathogenesis of these diseases. About half of the SLE patients exhibit upregulation of IFN type I induced genes which has been found to correlate with disease activity and severity as well as with the presence of autoantibodies against RNA binding proteins [5-8]. Increased IFN type I activity was detected in salivary glands, whole blood, PBMCs and monocytes from pSS patients [9-12]. In SSc increased IFN type I activity was reported both in the affected skin and in immune cells isolated from the circulation [13-15]. However the prevalence of the increased IFN type I activity in pSS and SSc was not established yet. Given that there are 17

different IFN type I subtypes, it is difficult to measure serum protein levels using an ELISA. Therefore we used the monocyte mRNA expression of a set of IFIGs previously detected in a microarray study [10], as our operational definition for the increased IFN type I activation – the so-called IFN type I signature. By performing RQ-PCR, we found in 55% of pSS patients the IFN type I signature present compared with 4.5% of HCs **(Chapter 2)**. In SSc, 29.3% of the patients displayed the IFN type I signature versus none of the HCs **(Chapter 4)**. In line with previously reported data, the IFN type I signature was detected in 64% of SLE patients **(Chapter 7)**.

IFN type I activity has been associated with more active and severe disease in SLE and SSc [5-8, 16]. By stratifying pSS patients according to their IFN type I signature status (IFN+ and IFN-), significantly higher EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores were observed in the IFN+ pSS patients **(Chapter 2)**. In SSc patients a correlation between increased IFN type I activity and the mean Rodnan Skin Score (mRSS) could not be replicated **(Chapter 4)**. A possible explanation for this might be the fact that this study comprised patients who had been included in two different centers in which the mRSS was performed by different clinicians. We did observe a markedly shorter time between the onset of Raynaud's phenomenon and development of the first non-Raynaud symptom in IFN type I positive patients, suggesting a more progressive disease in the IFN+ positive SSc patients.

A relationship between the presence of IFN type I activity and autoantibodies has been described in autoimmune diseases [17-22]. IFN type I induced gene expression was higher in pSS patients positive for anti-SSA and/or anti-SSB antibodies **(Chapter 2)**. In SSc the IFN+ patients were characterized by the presence of anti-RNP autoantibodies and absence of anti-centromere antibodies **(Chapter 4)**. The autoantibodies present can form ICs with the autoantigens released upon apoptosis. Interestingly, IFN type I is able to upregulate the expression of Fas, Fas-L and Ro52 autoantigen [23, 24]. ICs containing both RNA and DNA are found to be potent IFN type I inducers in autoimmune diseases [20, 25-29]. This occurs through FcγR-dependent internalization of the ICs by pDCs, followed by TLR7 and 9 activation. In line with these findings, in IFN+ pSS patients decreased C3 levels and increased serum IgG levels were observed **(Chapter 2)**.

Why one IFN type I driven autoimmune disease exhibits manifestations mainly in the salivary and lacrimal glands, while other IFN type I driven autoimmune diseases primarily manifest in the skin and kidneys, remains to be elucidated. Although not limited to this, a possible explanation could be genetic or acquired aberrancies in the target tissues.

Implications for therapy

The data in this thesis and in the literature indicate a relationship between activated IFN type I system and generalized autoimmune diseases, suggesting that downregulation of this

system could be a therapeutic approach. Interestingly, anti-IFN-α autoantibodies have been detected in 27% of serum samples from SLE patients. These patients had lower IFN type I inducible gene expression and were clinically less active [30]. Blockade of the IFN type I system with biologic agents can be exerted by interfering with different upstream and downstream targets of the IFN type I system.

One of the therapeutic targets might be IFNα and currently 2 anti-IFNα monoclonal antibodies, rontalizumab and sifalimumab, are studied in clinical trials for treatment of SLE. Results from phase I trial of sifalimumab revealed that there was a significant dose-dependent inhibition of the IFN type I signature and corresponding changes in related proteins in affected skin (ESI-1 and USP18) after treatment compared with placebo [31]. No serious adverse events were reported. Preliminary results from a phase IIa trial with sifalimumab in 87 SLE patients presented at the EULAR 2011, showed a 40% reduction in IFN type I induced gene expression but no clinical effects compared to placebo (abstract Merrill J *et al.**). Treatment duration from this study may have not been long enough to detect clinical effects.

Rontalizumab was successful in turning off IFIGs (IFI27, MX1, IFI44, IFIT1, OAS1, OAS2, and OAS3) in mildly active SLE patients in a phase I trial [32]. Remarkably, the decline in IFIG expression following treatment was similar in all patients regardless of their IFIG expression at baseline. None of the patients who were designated as being IFN type I high reached the levels of IFIG expression seen in the IFN low patients, nor did they reached the basal levels detected in the HCs. Also no apparent decline in the levels of IFN type I inducible proteins (BLC, I-TAC, MCP-1, BAFF and IP-10) was detectible. An explanation for these results could be attributed to the residual activity of other type I interferons, such as IFNβ, IFNω, IFNλ. Also other stimuli that are present in the serum of the patients and have the capacity to induce expression of the same genes are possible. It is currently not known whether complete or partial normalization of IFIGs is required in order to achieve clinical efficacy. Levels of autoantibodies (anti dsDNA, anti-SSA, anti-RNP) also did not decline following treatment with rontalizumab. For these changes possibly longer treatment periods may be required, since these autoantibodies are derived from long-lived plasma cells. An acceptable safety profile was demonstrated for rontalizumab in this study.

A limitation of the above discussed monoclonal antibodies is that each antibody targets only a subset of the 13 subtypes of IFNα. Induction of anti-IFNα antibodies by active immunisation with kinoids (conjugates of cytokines to keyhole limpet haemocyanin), involves a polyclonal reaction to all the 13 subtypes of IFNα [33]. A first trial of 28 SLE patients treated with IFNα-kinoid showed dose-related reduction in IFIGs in all immunised patients who were IFN+ at baseline [34].

Another possible therapeutic target within the IFN type I pathway might be the IFNAR. A more profound inhibition of IFN type I effects can be expected when the IFNAR is blocked.

Also the pDCs could be directly targeted using human monoclonal antibodies directed against BDCA-2 or BDCA-4 [35, 36]. Furthermore, an oligonucleotide that blocks TLR7/9 has been tested as experimental therapy for skin inflammation in SLE mice [37]. Finally, the use of RNAse to block the stimulation of TLR7 by RNA containing ICs could be a treatment possibility and recently showed to be effective in an SLE mouse model [38].

Table 1. Monoclonal antibodies for treatment of pSS, SSc and SLE discussed in this chapter

Name	Target
Rontalizumab	IFNα
Sifalimumab	IFNα
Belimumab	BAFF
Rituximab	CD20
Ustekinumab	Il-12/IL-23
Ixekizumab	IL-17
Brodalumab	IL-17 receptor
Tocilizumab	IL-6

A CLINICALLY APPLICABLE BIOMARKER FOR IDENTIFYING SYSTEMIC IFN TYPE I ACTIVITY

Since the IFN type I activity is assessed via laborious expression profiles of multiple IFN type I inducible genes, we explored in **Chapter 3** the possibilities for an easy and practical assay for identifying systemic IFN type I bioactivity. This study was the first to simultaneously assess protein levels of MxA, CD64, CD169 and BAFF as biomarkers for IFN type I detection. Herein MxA was distinguished as the best functional biomarker for systemic IFN type I activity in pSS. Intracellular MxA protein was assessed using an EIA method on whole blood and flow cytometric analysis on circulating $CD14^+$ monocytes. Flow cytometric detection of MxA in monocytes showed a slightly better distinction between IFN+ and IFN- patients than the EIA in whole blood. Nevertheless, the MxA-EIA was a more practical assay as it requires only 25µl of whole blood, without necessity of laborious PBMC- and subsequent monocyte-isolation.

Implications for therapy

Whole blood MxA levels correlated with ESSDAI scores and patients using HCQ showed reduced MxA levels **(Chapter 3)**. HCQ has been shown to impair systemic IFNα production [39]. Our data suggests that identifying IFN type I signature by performing MxA-assays might prove useful in predicting HCQ treatment responsiveness. Also in the anti-IFN trials, MxA might be useful predictor for responsiveness of therapy.

IFN TYPE I AND BAFF

BAFF has been implicated in the pathogenesis of generalized autoimmune diseases [40-48]. Furthermore, a link between IFN type I and BAFF has been reported [49-51]. In this thesis we find a strong correlation between BAFF and IFN type I signature in all 3 autoimmune disease **(Chapter 2, 4 and 7)**. BAFF mRNA expression could be induced in cultured monocytes by serum from IFN type I signature positive pSS patients **(Chapter 2)**. Moreover a significant correlation between BAFF and chemokine defined Th17 cells **(Chapter 6)** as well as between BAFF and IL-21 producing Th17 cells **(Chapter 7)** was observed. Interestingly, BAFF is reported to be involved in DC maturation and DC driven Th17 cell differentiation *in vitro* [52]. BAFF gene silencing ameliorated joint pathology and inhibited the generation of Th17 cells in the joints of a collagen induced arthritis (CIA) mouse model. IL-17 was furthermore shown to be involved in BAFF-mediated exacerbation of CIA. These findings shed light on IFN type I as a link between the innate and adaptive immune system through upregulation of BAFF and point to IFN type I as well as BAFF as good candidates for therapy.

Implications for therapy

As shortly discussed in chapter 1, an antibody to BAFF, belimumab (Table 1), has been shown to be beneficial in SLE patients, especially in patients with high disease activity, positive anti-dsDNA antibodies, low complement or who are on corticosteroids [53]. Without significant safety concerns belimumab was well-tolerated [54, 55]. Although the additional benefits of belimumab over placebo were modest in patients receiving standard therapy, the main benefits are to maintain remission, slow disease progression and reduce steroid use [56]. Given these results, belimumab might also be a good therapeutic option for pSS and SSc. Interestingly, increased BAFF levels were reported following rituximab therapy in pSS [57] and recently also increased IFN type I activity [58]. IFN type I activity negatively predicted the therapeutic outcome for rituximab (Table 1) in rheumatoid arthritis [59]. Taken this into account, anti-IFN therapy or the addition of belimumab might be more efficacious than rituximab solely.

IFN TYPE I AND FIBROSIS

BAFF mRNA was increased in the IFN type I signature positive SSc patients **(Chapter 4)**. BAFF serum levels were found to correlate with the extent of skin fibrosis in SSc [43]. We therefore investigated whether an indicator of *de novo* collagen type III synthesis, namely PIIINP, is increased in SSc patients positive for the IFN type I signature. We found increased PIIINP levels in SSc compared to HC and by far the highest levels in IFN+ patients **(Chapter 4 and 5)**. A possible factor linking the IFN type I to fibrosis could be TLR3. IFNα2 has been reported to induce TLR3 expression on human dermal fibroblasts, which resulted in increased TLR3-induced production of the profibrotic cytokine IL-6 [60]. SSc fibroblasts showed an

augmented TLR3 response to IFNα2 compared with HC fibroblasts. In addition, TLR3 appears to be increased in the skin of SSc individuals [61]. Transcutaneous administration of several TLR ligands to mice, especially of TLR3 ligand induced inflammation and progression of fibrosis [62].

Implications for therapy

SSc is a difficult to treat severely debilitating disease. Imatinib mesylate is used as a treatment for therapy-refractory SSc with high variability in therapeutic outcomes ranging from ineffective/toxic responses to extremely encouraging clinical improvement [63]. We performed an open label study with Imatinib in 10 therapy resistant SSc patients **(Chapter 5)**. Similar to previous studies, a small percentage of patients responded [63]. Interestingly only the responding patients showed a high IFN type I activation, high BAFF mRNA levels and high PIIINP serum levels, indicating that IFN type I signature might be a potential biomarker for the selection of SSc patients for Imatinib treatment. The use of such a biomarker could avoid high costs and potential side effects in patients who are unlikely to respond to Imatinib. IFN type I might also be a suited target for treatment of SSc patients. Blocking the IFN type I activity could result in decrease of fibrosis both directly by diminished IFN type I activity, as well as indirectly through inhibition of BAFF.

IFN TYPE I AND TH17 PATHWAY

The role of IFN type I on the T cell activation and differentiation is complex and consists of contrasting data. The results of the performed studies depend on whether the influence of IFN type I was studied on naive or activated T cells, and whether in vitro or in vivo experiments were conducted [64]. Early on, IFN type I has been shown to play a role in the differentiation of human Th1 cells through induction of STAT4 [65]. In contrast to these data, in this thesis decreased Th1 cell percentages were observed for pSS patients with the IFN type I signature **(Chapter 6)**. A role of IFN type I on the development of CD4+ helper memory T cells upon cognate Ag exposure has been described [66]. Furthermore, IFN type I was observed to stimulate cytotoxic T cells and long lived central memory CD8+ T cells [64, 67]. Recently, a co-activity between IFN type and Th17 subset has been suggested. TLR7 activation promotes and modifies Th17 cell differentiation and function mainly related to increased IL-1β and IL-23p19 production of pDCs [68]. IFN type I itself is also able to promote Th17 differentiation and IL-17 production through induction of STAT3 in T cells and IL-6 induction in DCs [69, 70]. Finally, IFN type I conditioned monocytes differentiate into DCs driving the development of autologous naive CD4 T cells into Th17 cells through IL-23 production [71].

Next to these data on an association between IFN type I and Th17 pathway, 2 mouse models have led us to hypothesize that Th17 cells are increased in patients with the IFN

type I signature. The first mouse model is the EAE model which showed improvement upon IFN type I treatment if the disease was Th1 driven, and exacerbation if the disease was Th17 driven [72], suggesting IFN type I as a driving force behind Th17. The second model is the Ro52-/- mouse model [73]. Ro52 is involved in the ubiquitination of IRFs, a mechanism reported to limit the IFN type I response. The Ro52-/- mice appeared phenotypically normal if left unmanipulated. However, these mice developed severe dermatitis with neutrophil infiltrates induced by metal ear tags. These affected mice also developed signs of systemic lupus with hypergammaglobulinemia, autoantibodies to dsDNA, proteinuria, and kidney pathology. Next to the inflamed skin, Th17 cells were detected in the draining lymph nodes in these mice. Interestingly, when these mice were crossed on an IL-23p19-/- mouse line, they did not develop the systemic autoimmune symptoms upon ear tagging, indicating that the development of an SLE phenotype through enhanced IFN type I production in these mice is dependent on the IL-23/Th17 pathway.

To test our hypothesis that Th17 cells are increased in patients with the IFN type I signature, we used flowcytometry to phenotypically characterize the PBMCs from pSS and SLE patients and study the differences between IFN+ and IFN- patients. We detected increased IL-17/Th17 proportions in the peripheral blood of both pSS and SLE patients positive for the IFN type I signature **(Chapter 6 and 7)**. In pSS we observed a significant increase in chemokine receptor defined Th17 cells (CD4+CD45RO+CCR6+CCR4+CXCR3-CCR10- cells), while in SLE we found an increased percentages of IL-17A and IL-17F producing CCR6+ T memory cells. Furthermore, in SLE we detected a significant correlation between the downstream factors of IFN type I and Th17 pathway, namely BAFF and IL-21 production by CCR6+ T memory cells, respectively **(Chapter 7)**. In pSS patients, BAFF correlated significantly with chemokine receptor defined Th17 cell percentages and negatively with Th1 cell proportions **(Chapter 6)**. These data provide the first *ex vivo* support for the co-occurrence of IFN type I and Th17 pathway in patients with generalized autoimmune diseases, underscoring the role of IFN type I in linking the innate and adaptive immunity.

Interestingly, IL-21 is the main effector cytokine produced by follicular Th (Tfh) cells. Tfh are important for germinal center formation and B cell differentiation into plasma cells and memory B cells [74, 75]. Increased frequencies of circulating Tfh-like cells are found in SLE and pSS patients [76]. Moreover increased frequency of Tfh-like cells in SLE correlates with autoantibody titers and tissue damage. Although not studied in this thesis, it can be envisaged that next to Th17 cells also Tfh cells play an important role in IFN type I signature positive patients.

Implications for therapy

Regarding the above mentioned data, various biological drugs directed against Th17 cytokines might be effective as therapeutic options. Since preliminary results from a phase IIa trial with sifalimumab showed so far disappointing results, combination therapies

blocking both IFN type I and Th17 pathway might result in more significant therapeutic effects, provided that safety concerns can be addressed.

Monoclonal antibodies directed against Th17 cytokines are being studied in autoimmune diseases such as psoriasis and rheumatoid arthritis. Ustekinumab, a fully human IgG κ monoclonal antibody that binds to the common p40 subunit shared by IL-12 and IL-23, has shown promising results in patients with active psoriatic arthritis in a phase III trial [77]. The benefits of ustekinumab in psoriatic arthritis might be related to the dual effects of inhibiting IL-23 and IL-12, with downstream effects on Th1 cells, or could be because of inhibition of the IL-23–Th17 axis alone. No deaths, opportunistic infections, tuberculosis infection or malignancies were reported, although 3 patients had major adverse cardiovascular events**.** Use of a humanized anti-IL-17 monoclonal antibody, ixekizumab, improved the clinical symptoms of psoriasis in a phase II trial [78]. No serious adverse events or major cardiovascular events were observed. In another phase II study, the use of a human anti-interleukin-17-receptor monoclonal antibody, brodalumab, significantly improved plaque psoriasis without major adverse side effects [79]. Finally, tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody is now in clinical use in RA. In a 5-year extension study, tocilizumab demonstrated sustained long-term efficacy and a good safety profile [80]. These agents might be useful in future therapy for pSS, SLE and SSc. As with other biological agents, the safety of these anti-Th17 biologic therapies merits close long-term follow up, especially to fully assess major adverse events such as cardiovascular risks.

THE HYPOTHETICAL MECHANISM FOR THE PATHOPHYSIOLOGY OF IFN TYPE I MEDIATED AUTOIMMUNE DISEASES

Figure 1 shows a hypothetical mechanism for the pathophysiology of IFN type I mediated autoimmune diseases, based on the data given in this thesis. Increased apoptosis and impaired clearance of apoptotic material have been proposed to play a role in the pathogenesis of systemic autoimmune diseases [81, 82]. Whether a viral infection, target organ specific abnormalities such as the increased expression of Fas, Fas-L, Perforin and granzyme B in pSS salivary glands, damage due to environmental factors like sunlight, decrease in estrogens, several medications or a combination of aforementioned factors initiate apoptosis, remains to be elucidated. Due to insufficient clearance of apoptotic cells, these cells undergo secondary necrosis and disintegrate into apoptotic blebs. Under normal conditions, chromatin does not induce an immune response. However during apoptosis, many chromatin modifications take place and chromatin containing apoptosis-associated modifications present in the apoptotic blebs are a target for autoantibodies [83]. After DCs take this chromatin up, these cells have been reported to mature, stimulate autoreactive T cells and finally induce an antibody production by B cells [84-86].

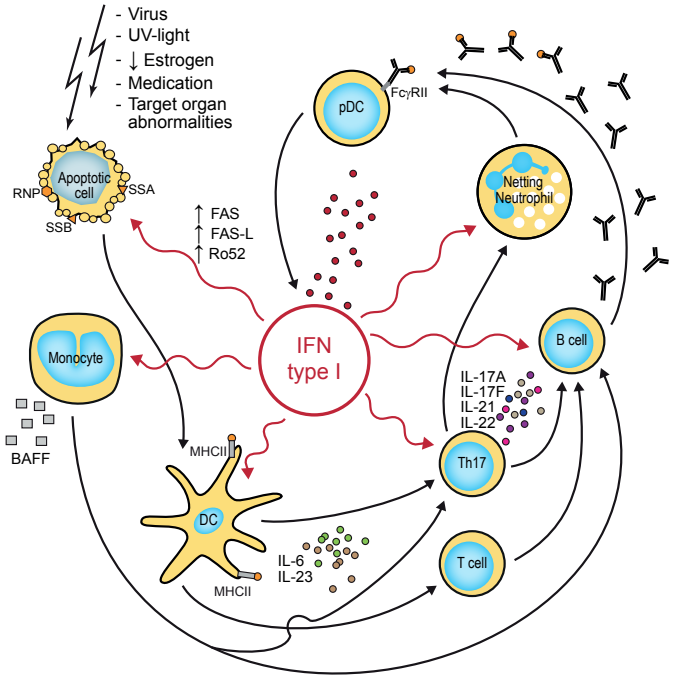

Figure 1. Simplified hypothetical mechanism for the pathophysiology of IFN type I mediated autoimmune diseases

Subsequently, the abundantly produced autoantibodies can form ICs with autoantigens present in the apoptotic remains. These ICs induce IFN type I production, IFIG expression and production of IFN type I induced proteins such as MxA via FcγRIIa-mediated endocytosis and subsequent TLR-7/9 activation. Produced IFN type I stimulates the monocyte to produce BAFF, which stimulates the differentiation/maturation and survival of B cells. IFN type I also induces Th17 differentiation through production of IL-6 and IL-23 by conventional DCs for example. Th17 cells produce subsequently IL-17A, IL-17F, IL-22 and IL-21. IL-17A was recently reported to stimulate the neutrophils to release extracellularly chromatin structures termed as neutrophil extracellular traps (NETs) [87]. Chromatin structures loaded with antimicrobial peptides such as LL37 or HNP (typically present in neutrophil granules) are externalized by neutrophils during NETosis [88]. Neutrophils are able to cause tissue damage and promote autoimmunity by aberrant formation of NETs [89]. Importantly, netting neutrophils are great inducers of IFN type I [90]. In turn, IFN type I has been shown to prime neutrophils to produce NETs more easily [91]. Autoantibodies induce NETosis in IFN type I primed neutrophils. Thus upon apoptosis, B cells are stimulated to produce autoantibodies that form on the one hand ICs with the autoantigens, resulting in pDC activation and IFN type I production. On the other hand, the produced autoantibodies induce NETosis in IFN type I primed neutrophils,

amplifying the IFN type I response. Hereby a vicious circle is initiated. Our observation that IFN type I activation is correlated with decreased neutrophil count in pSS patients **(Chapter 2)** is in line with these data. Next to induced NETosis by apoptotic material, impaired NET degradation is described in SLE patients [92, 93]. Lastly IFN type I is able to upregulate the expression of Fas, Fas-L and Ro52 autoantigen and prime for even more enhanced IFN type I production by pDCs.

LIMITATIONS

Small study samples

Mainly the studies in chapter 6 and 7 as well as the clinical trial for imatinib mesylate in chapter 5 involved relative low numbers of patients. These studies nevertheless turned out to have enough power to detect differences between IFN+ patients, IFN- patients and HC. Furthermore, in all studies patients and healthy controls were matched for age and gender. Nevertheless, our studies should be repeated and confirmed in larger study samples, making correction for all possible confounders possible.

Peripheral blood samples

All our studies were carried out on peripheral blood samples and information is lacking on the immune cells in the actual affected organs such as the salivary glands, skin and kidneys. We studied the IFN type I inducible gene expression in CD14+CD16- monocytes instead of PBMCs, as monocytes were found to be involved in the pathogenesis of pSS [94]. Moreover, CD14CD16- monocytes are thought to be precursors for the DCs and macrophages in the peripheral tissue, offering possibilities to study the mononuclear phagocyte system in these diseases. Comparing the three autoimmune diseases studied in this thesis, pSS target organs are the most accessible to perform research on. However, at our immunology outpatient clinic relatively few salivary gland biopsies are performed due to low diagnostic sensitivity of the biopsies and the possible complications for the patient.

Influence of medication

The majority of the patients in our studies used medication, with possible effects on the immune system. Lower MxA levels were detected in pSS patients using HCQ **(Chapter 3)**. To draw clear conclusion on the effect of medication on IFN type I activity, longitudinal follow-up studies are required in which IFN type I activity is compared between medication naive patients before the start of treatment and the same patients after taking medication.

Technical limitations

Due to limitations in current antibody-fluorochrome availability, we were not able to analyze cytokine expression on chemokine receptor defined Th17 cells (CD4+CD45RO+CCR6+CXCR3-

CCR4+CCR10-cells) in chapter 6. Future studies where both chemokine receptor expression and cytokine expression can simultaneously be assessed, are warranted.

PERSPECTIVES

Generalized autoimmune diseases are evidently heterogeneous diseases with a multi-factorial pathophysiology. Currently, therapy is mainly symptomatic without clear treatment guidelines. Variation in chosen therapy exists between doctors and there is also variation in therapy outcome between patients. Stratification of patients in subgroups according to the pathophysiological mechanisms (e.g. increased IFN type I activity versus low IFN type I activity) and appropriate evidence-based personalized therapies (e.g. anti-IFN therapy) will lead to improved management of these diseases and therefore increased quality of life for the patients. Taking into account the before described pathophysiological mechanism and the results from clinical trials with biologicals, it is questionable whether inhibition of one pathogenic mechanism such as solely IFN type I, BAFF or Th17 will be sufficient for significant clinical responses. Perhaps that combination therapies such as anti-IFN type I together with anti-CD20 or anti-Th17 will lead to more significant therapeutic effects. Determining the IFN type I signature is the first step in the right direction regarding personalized therapies and monitoring responsiveness of therapy. Longitudinal studies have to clarify whether the in this thesis used cut-off values for the definition of a positive IFN type I signature (i.e. increased IFN type I activity) are sufficient for stratifying patients into therapy subgroups.

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ADDENDUM

SUMMARY

SAMENVATTING

LIST OF ABBREVIATIONS

ACKNOWLEDGEMENTS

CURRICULUM VITAE

PORTFOLIO

LIST OF PUBLICATIONS

SUMMARY

When the immune system fails to recognize the body's own constituent parts as 'self' and responds against its components, it is called autoimmunity and an autoimmune disease can develop. Primary Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) are examples of autoimmune diseases where multiple organs and tissues are affected by an autoimmune process. These diseases are called 'generalized autoimmune diseases', because of the multisystem involvement. Interferon (IFN) type I has been implicated in the pathogenesis of generalized autoimmune diseases. IFN type I in general is produced in humans upon viral infection and originally defined by its capacity to interfere with viruses; this is reflected in its name. In patients with generalized autoimmune diseases IFN type I seems to be produced without evidence for a viral infection.

The aim of this thesis was to determine the prevalence of the increased IFN type I activity in pSS and to elucidate the relation between the increased IFN type I activity and:

a) disease manifestations
b) other immune abnormalities, typical for generalized autoimmune diseases

In humans IFN type I includes more than 17 different subtypes. Therefore assessing IFN type I levels in blood of patients is not practical to determine the total IFN type I activity. The IFN type I inducible gene expression levels in circulating blood cells (the so-called 'IFN type I signature') are used as a tool for the assessment of systemic IFN type I activity. This has been addressed in **Chapter 2**. By assessing the IFN type I signature in pSS patients, we observed in about half of the pSS patients increased IFN type I activity. This increased IFN type I activity seems to be stable over time and correlates with increased EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores and various disease manifestations such as higher autoantibody levels, higher serum IgG, lower complement and lymphocyte and neutrophil counts. Also a strong correlation with B cell activating factor (BAFF) is described in chapter 2.

Since the IFN type I activity is assessed via laborious expression profiles of multiple IFN type I inducible genes, we explored in **Chapter 3** the possibilities for an easy and more practical assay to measure systemic IFN type I bioactivity. The levels of MxA protein (also an IFN inducible gene and protein) were distinguished as the best functional biomarker for systemic IFN type I activity in pSS.

The prevalence of the increased IFN type I activity in SSc patients has not been established yet. In **Chapter 4** we describe in about one third of SSc patients the presence of the IFN type I signature. Also the association between IFN type I and disease manifestations, BAFF expression and collagen metabolism was established. In addition, the possible role of the IFN type I signature as a biomarker for progressive disease is investigated.

Imatinib mesylate is used as a treatment for therapy-refractory SSc with high variability in therapeutic outcomes ranging from ineffective/toxic responses to extremely encouraging clinical improvement. This high variability in treatment outcomes stresses the need for

a biomarker identifying SSc patients likely to respond to Imatinib treatment. **Chapter 5** describes the IFN type I signature, together with BAFF and N-terminal propeptide of type III collagen (PIIINP, which is an indicator of activated collagen metabolism) as possible predictors for SSc patients responding to Imatinib treatment.

Recently, a T helper 17 subset (Th17 cells), which produces IL-17A, IL-17F, IL-21 and IL-22, has been implicated in generalized autoimmune diseases. A possible co-activity between the pathogenic IFN type I and the IL-17/Th17 pathway has been suggested. Data about co-occurrence of these two pathways in patients has not been shown yet. In **Chapter 6** chemokine receptor defined Th17 cells have been assessed and found increased in IFN positive pSS patients as compared to IFN negative pSS patients. In **Chapter 7** we compare the actual percentages of IL-17A and IL-17F producing CCR6+ T memory cells in IFN positive and IFN negative SLE patients. We find these cells significantly increased in IFN positive SLE patients.

Finally, the results of the different chapters are integrated in **Chapter 8**. Generalized autoimmune diseases are evidently heterogeneous diseases with a multi-factorial pathophysiology with a prominent role for IFN type I in at least a large subgroup of patients. Why one IFN type I driven autoimmune disease exhibits manifestations mainly in the salivary and lacrimal glands and another primarily manifests signs and symptoms in the skin and kidneys, remains to be elucidated. Stratification of patients in subgroups according to pathophysiological mechanisms described here (e.g. in increased IFN type I activity versus low IFN type I activity) and appropriate evidence-based individualized therapies (e.g. anti-IFN therapies) will most likely lead to improved management of these diseases and therefore increased quality of life for the patients.

SAMENVATTING

Wanneer het immuunsysteem zich richt tegen lichaamseigen cellen en deze gaat aanvallen, spreekt men van auto-immuniteit. Het primaire syndroom van Sjögren (pSS), systemische lupus erythematodes (SLE) en systemische sclerose (SSc) zijn voorbeelden van auto-immuunziekten waarbij meerdere organen en weefsels aangedaan kunnen zijn. Daarom worden deze ziekten ook wel 'gegeneraliseerde auto-immuunziekten' genoemd. Er zijn aanwijzingen voor een rol van Interferon (IFN) type I in de pathogenese van gegeneraliseerde auto-immuunziekten. IFN type I wordt over het algemeen in het lichaam van mensen met een virale infectie geproduceerd en wordt 'interferon' genoemd omdat het interfereert met de infecterende virussen. Echter, in patiënten met een gegeneraliseerde auto-immuunziekte lijkt IFN geproduceerd te worden zonder dat er aanwijzingen zijn voor een virale infectie. Het doel van dit proefschrift was om de prevalentie van verhoogde IFN type I activiteit in pSS te bepalen en de relatie te bestuderen tussen verhoogde IFN type I activiteit en:

a) ziekte verschijnselen

b) andere immuun afwijkingen kenmerkend voor gegeneraliseerde auto-immuunziekten.

In mensen bestaat IFN type I uit een familie van meer dan 17 verschillende soorten, die een iets andere structuur hebben, maar allen IFN type I genoemd worden. Daarom is het meten van IFN type I waarden in bloed van patiënten niet de juiste maat voor de daadwerkelijk aanwezige IFN type I activiteit. IFN type I geïnduceerde gen expressie of de zogenaamde 'IFN type I handtekening', wordt als een maat voor de IFN type I activiteit gebruikt. Dit wordt uiteengezet in **Hoofdstuk 2**. Door het bepalen van de IFN type I handtekening in pSS patiënten, hebben wij in ongeveer de helft van de pSS patiënten verhoogde IFN type I activiteit gevonden. Bezien over een langere tijdsduur lijkt deze IFN type I activiteit stabiel en is deze gecorreleerd aan verhoogde pSS ziekte activiteit welke bepaald werd door middel van de ESSDAI score. Ook verscheidene andere ziekte manifestaties zoals autoantistoffen, hoger serum IgG, lager complement en lymfocyt en neutrofiel aantallen correleren met de IFN type I activiteit. Verder is er een sterke correlatie met B cel activerende factor (BAFF) beschreven in Hoofdstuk 2.

Aangezien IFN type I activiteit bepaald wordt middels arbeidsintensieve gen expressie profielen van meerdere IFN type I induceerbare genen, hebben wij in **Hoofdstuk 3** een snelle en gemakkelijke assay voor het bepalen van IFN type I activiteit onderzocht en vergeleken met de IFN type I handtekening. Het MxA eiwit bleek een goed alternatief voor de IFN geïnduceerde genexpressie profielen en is dus bruikbaar als biomarker voor systemische IFN type I activiteit in pSS.

De prevalentie van verhoogde IFN type I activiteit in monocyten van SSc patiënten is nog niet aangetoond. In **Hoofdstuk 4** beschrijven wij in ongeveer één derde van SSc patiënten de aanwezigheid van de IFN type I handtekening. Ook is er een associatie tussen IFN type I en ziekte verschijnselen, BAFF expressie en collageen metabolisme gevonden. Daarbij is tevens

de mogelijke rol van IFN type I als een biomarker voor progressieve ziekte onderzocht.

Imatinib mesilaat wordt als een behandeling voor therapieresistente SSc patiënten gebruikt en heeft een hoge variabiliteit in therapeutische effecten variërend van geen respons of toxisch respons tot aanmoedigende klinische verbetering. Deze hoge variabiliteit benadrukt de noodzaak voor een biomarker die SSc patiënten kan identificeren die positief op Imatinib zullen reageren. **Hoofdstuk 5** beschrijft dat de IFN type I handtekening, samen met BAFF en N-terminaal propeptide van type III collageen (PIIINP, welke een maat voor geactiveerde collageen metabolisme is) mogelijk voorspellen welke SSc patiënten op behandeling met Imatinib zullen reageren.

Recent is er een nieuwe T helper set namelijk de zogenaamde Th17 cellen gevonden. Deze Th17 cellen, die IL-17A, IL-17F, IL-21 en IL-22 produceren, lijken een rol te spelen in de pathogenese van gegeneraliseerde auto-immuunziekten. Mogelijk bevorderen IFN type I en IL-17/Th17 samen het ziekteproces in deze auto-immuunziekten. Data over de gelijktijdige aanwezigheid van deze twee ziekte-bevorderende mechanismen in patiënten ontbreken nog. In **Hoofdstuk 6** zijn in pSS patiënten de chemokine receptor gedefinieerde Th17 cellen bepaald en verhoogd gevonden in IFN positieve patiënten in vergelijking met IFN negatieve patiënten. **Hoofdstuk 7** vergelijkt de IL-17A en IL-17F producerende CCR6+ T cel percentages tussen IFN positieve en IFN negatieve SLE patiënten. Deze IL-17 producerende cellen zijn verhoogd gevonden in IFN positieve SLE patiënten.

Ten slotte worden de resultaten van de verschillende hoofdstukken geïntegreerd in **Hoofdstuk 8**. Gegeneraliseerde auto-immuunziekten zijn heterogene ziekten met een multifactoriële pathofysiologie waar IFN type I deel van uitmaakt. Waarom de ene IFN gemedieerde ziekte manifestaties in vooral speeksel- en traanklieren heeft, terwijl in andere IFN type I gemedieerde ziekten vooral de huid en nieren aangedaan zijn, moet nog onderzocht worden. Mogelijk kan het indelen van patiënten in subgroepen gebaseerd op het pathofysiologisch mechanisme (bijvoorbeeld met behulp van de IFN type I handtekening) en hierop gebaseerde behandeling (bijvoorbeeld met anti-IFN type I) leiden tot een betere behandeling en kwaliteit van leven voor deze groep patiënten.

LIST OF ABBREVIATIONS

ACA	Anti-centromere antibodies
AIM2	Absent in melanoma 2
ANA	Anti-nuclear antibodies
ANOVA	Analysis of variance
anti-CCP	Antibodies against cyclic citrullinated peptides
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ASMA	Anti-smooth muscle antibodies
BAFF	B cell activating factor of the tumor necrosis factor family
BAFF-R	BAFF receptor
BCMA	B cell maturation antigen receptor
BCR	B cell receptor
BDCA	Blood dendritic cell antigen
BSA	Bovine serum albumin
C3	Complement component 3
C4	Complement component 4
CCR4	Chemokine (C-C motif) receptor 4
CCR6	Chemokine (C-C motif) receptor 6
CCR10	Chemokine (C-C motif) receptor 10
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD19	Cluster of differentiation 19
CD20	Cluster of differentiation 20
CD22	Cluster of differentiation 22
CD28	Cluster of differentiation 28
CD64	Cluster of differentiation 64
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CD169	Cluster of differentiation 169
CD45RO	Protein tyrosine phosphatase, receptor type, C
cDNA	Copy DNA
CIA	Collagen induced arthritis
CNS	Central nervous system
CRP	C-reactive protein
CXCL9	Chemokine (C-C motif) ligand 9
CXCL10	Chemokine (C-C motif) ligand 10

CXCL11	Chemokine (C-C motif) ligand 11
CXCL12	Chemokine (C-C motif) ligand 12
CXCL13	Chemokine (C-C motif) ligand 13
CXCL19	Chemokine (C-C motif) ligand 19
CXCL21	Chemokine (C-C motif) ligand 21
CXCR3	Chemokine (C-X-C motif) receptor 3
CXCR5	Chemokine (C-X-C motif) receptor 5
DAI	DNA-dependent activator of IRF
DC	Dendritic cell
dcSSc	Diffuse cutaneous systemic sclerosis
ΔΔCT	Delta delta Cycle treshold
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DLCO	Diffusing capacity for carbon monoxide
DN	Double negative
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EIA	Enzyme immuno assay
ELISA	Enzyme Linked Immuno Sorbent Assay
ENA	Extractable Nuclear Antigens
ER	Endoplasmatic reiculum
ESR	Erythrocyte sedimentation rate
ESSDAI	EULAR Sjögren's Syndrome Disease Activity Index
FACS	Flow cytometric analysis
Fas-L	Fas Ligand
FcγRII	Fc fragment of IgG, low affinity III, receptor
GC	Germinal center
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hb	Haemoglobin
HC	Healthy control
HCQ	Hydroxychloroquine
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV1	Human T-lymphotropic virus Type I

ICAM	Intercellular adhesion molecule
ICs	Immunecomplexes
IFI27	Interferon, alpha-inducible protein 27
IFI44	Interferon-induced protein 44
IFI44L	Interferon-induced protein 44-like
IFIGs	Interferon type I induced genes
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
IFITM1	Interferon induced transmembrane protein 1
IFN	Interferon
IFN-	Interferon negative
IFN+	Interferon positive
IFNAR	Interferon type I receptor
IFNAR1	Interferon type I receptor 1
IFNAR2	Interferon type I receptor 2
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgM	Immunoglobulin M
IKKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells kinase epsilon
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-17A	Interleukin 17A
IL-17F	Interleukin 17F
IL-17R	Interleukin 17 receptor
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-23	Interleukin 23
IM	Imatinib mesylate
IP-10	Interferon gamma-induced protein 10

IQRs	Interquartile ranges
IRAK 1	Interleukin receptor associated kinase 1
IRAK 4	Interleukin receptor associated kinase 4
IRF	Interferon regulatory factor
IRF3	Interferon regulatory factor 3
IRF5	Interferon regulatory factor 5
IRF7	Interferon regulatory factor 7
ISRE	Interferon stimulated response elements
Jak1	Janus family kinase 1
lcSSc	Limited cutaneous systemic sclerosis
LGP2	Laboratory of Genetic and Physiology 2
LPS	Lipopolysaccharides
LY6E	Lymphocyte antigen 6 complex, locus E
M3R	Muscarinic 3 receptor
Mabs	Monoclonal antibodies
MAVS	Mitochondrial antiviral-signaling
MCP-1	Monocyte chemotactic protein-1
MDA5	Melanoma differentiation antigen 5
MHC	Major histocompatibility complex
mRSS	Mean Rodnan Skin Score
MS	Multiple sclerosis
MX1	Myxovirusresistance protein 1
MxA	Myxovirusresistance protein 1
MyD88	Myeloid differentiation factor 88
ND	Not determined
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NLR	NOD-like receptors
NS	Nonsignificant
NSAIDs	Non-steroidal anti-inflammatory drugs
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
PIIINP	N-terminal propeptide of type III procollagen
PNS	Peripheral nervous system
poly (I:C)	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
pSS	Primary Sjögren's syndrome

RA	Rheumatoid arthritis
RF	Rheumatoid factor
RIG-1	Retinoic acid-inducible gene 1
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RQ-PCR	Real time quantitative polymerase chain reaction
SD	Standard deviation
SERPING1	Serpin peptidase inhibitor, clade G, member 1
SGEC	Salivary gland epithelial cells
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SS	Sjögren's syndrome
SSc	Systemic sclerosis
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STAT4	Signal transducer and activator of transcription 4
TACI	Transmembrane activator and calcium modulator ligand interactor
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
Th2	T helper 2
Th17	T helper 17
Th22	T helper 22
TLC	Total lung capacity
TLR	Toll like receptor
TLR 3	Toll like receptor 3
TLR 4	Toll like receptor 4
TLR 7	Toll like receptor 7
TLR 9	Toll like receptor 9
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor α
TRAF3	Tumor necrosis factor receptor-associated factor 3
TRAF6	Tumor necrosis factor receptor-associated factor 6
Treg	Regulatory T cell
TRIF	TIR containing adaptor molecule

TRIM21	Tripartite motif-containing protein 21
TWEAK	TNF-like weak inducer of apoptosis
Tyk2	Tyrosine kinase 2
USP18	Ubiquitin specific peptidase 18
VCAM	Vascular cell adhesion protein
XAF1	XIAP associated factor 1
XIAP	X-chromosome-linked inhibitor of apoptosis

DANKWOORD

Yes, eindelijk aangekomen bij het schrijven van het meest gelezen deel van dit boekje. Ik zal hierin proberen om iedereen te bedanken die mij de afgelopen 4 jaar geholpen, geïnspireerd en gesteund heeft bij het tot stand brengen van dit proefschrift.

Prof. Benner en Prof. Hooijkaas, ik wil u beiden bedanken voor de mogelijkheid om mijn promotieonderzoek op de afdeling Immunologie te kunnen uitvoeren. Ik heb de afgelopen jaren met veel plezier op de afdeling gewerkt.

Prof. Drexhage, beste Hemmo, ik kan het me als de dag van gisteren herinneren dat ik bij jou als geneeskunde student kwam solliciteren voor mijn keuzeonderzoek. Jij vroeg me meteen of ik zou willen promoveren waarop ik volmondig 'nee' antwoorde met als toevoeging 'dat ik liever 4 jaar klinische ervaring zou willen opdoen dan 4 jaar lang op een lab zitten'. Jouw woorden destijds 'dus jij wilt ergens in Gouda internistje worden' en 'ik zie het al jij bent geen onderzoekster' waren het begin van vele enerverende discussies en dialogen. Het duurde niet lang totdat ik het enthousiasme voor het onderzoek te pakken had en hier ben ik dan 4 jaar later het dankwoord van mijn proefschrift aan het schrijven. Ik wil je bedanken voor alle wetenschappelijke prikkelingen, jouw helpende 'helicopter view' bij het schrijven van mijn manuscripten en je altijd open en uitnodigende houding hoe druk je ook was.

Prof. Van Hagen, beste Martin, ondanks dat wij later in mijn promotie traject met elkaar zijn gaan samenwerken, wil ik je bedanken voor al je hulp, wijze raad en klinische inzichten. Ik kijk met veel plezier uit naar de opleiding interne geneeskunde.

Dr. Versnel, beste Marjan, als copromotor ben jij zeer nauw betrokken geweest bij dit onderzoek. Ik wil je bedanken voor de manier waarop jij dit gedaan hebt. Ondanks dat ik als arts geen ervaring op het gebied van onderzoek had en jij geen eerdere ervaring met het superviseren van klinische OIO's, heb jij mij vanaf het begin de vrijheid geboden om vorm aan mijn eigen ideeën te geven. Dit met het volle vertrouwen dat het wel goed zou komen, zelfs toen dat vertrouwen op momenten bij mij ontbrak. Bovendien was je een enorm toegankelijke supervisor; jouw deur stond altijd open om stukken na te kijken, over ideeën te brainstormen of een luisterend oor te bieden. En als je niet op het lab aanwezig was, stond je wel stand-by per e-mail, telefoon of whatsapp. Bedankt hiervoor!

I would like to thank the members of the reading committee, prof.dr. Jacques-Eric Gottenberg, prof.dr. Timothy Radstake and dr. Bart Jacobs for your review and academic input. Dr. Elke Theander, thank you for coming to the Netherlands to participate in my dissertation committee.

Lieve Corine, mijn paranimf en ook één van de grootste bikkels die ik ken. Jij hebt mij de fijne kneepjes van het vak geleerd. Daar waar ik me nog verwonderde over de ijsmachine op het lab, probeerde jij mij histologie coupes te leren kleuren, RNA te isoleren en andere voor mij wereldvreemde technieken op het lab. Ik kon op mijn beurt ook af en toe wat

voor jou betekenen zoals bloedende duimen verbinden of hechtingen verwijderen. Ik heb in ieder geval op meerdere vlakken veel van je geleerd en ben heel blij dat jij als paranimf naast mij zult staan. Lieve Naomi, my sweet choco! Thanks for being a great partner in crime during this journey! Een betere reisgenoot naar Athene, Washington en New York had ik niet kunnen wensen! And the 'chocolate-vanilla chronicle' will be continued soon in Kyoto! Lieve Wouter, er waren vast momenten dat je het niet makkelijk had als enige man in de groep en op de OIO-kamer, maar wat hebben wij vrouwen met jou een lol gehad! Bedankt voor al je hulp met figuren, statistiek, computer problemen, styling adviezen, je heerlijke espressootjes, je Haagse humor en bovenal je gezelligheid! Lieve Karin W, de afgelopen vier jaar heb jij veel ingrediënten aan mijn PhD traject toegevoegd; van de grootste lol op en buiten de werkvloer, tot de beste filosofische gesprekken onder het genot van een falafelburger en schnaps in het Westerpaviljoen. En natuurlijk niet te vergeten, de door jou zelf samengestelde cd's afgestemd op de verschillende fasen van dit 4-jarig traject en uiteraard Rosie en Jumbo. Vielen Dank für all den Spaß und deine Freundschaft! Angelique, lieve Angie, jij zorgde voor het 'thuis' gevoel op het lab. De Turkse deuntjes die uit jouw telefoon menig keer geklonken hebben in combinatie met jouw open en warme persoonlijkheid, waren vaak een bron van energie in de afgelopen 4 jaar. Beste Harm, voordat ik jou ontmoet had, geloofde ik niet dat er mensen bestonden die nooit slecht gehumeurd waren. Jij hebt me doen inzien dat dit onjuist was! Beste Annemarie, jij hebt mij als student de allereerste techniek op het lab geleerd, namelijk Ficollen. Bedankt voor je geduld en je hulp. Thomas, jij was het muzikale talent op het lab en voor mij ook een vraagbaak voor allerlei gekke weetjes. Ik zal in ieder geval nooit vergeten wie Tapio Wirkkala was. Karin B, door jou heb ik in mijn 4de promotiejaar de wondere wereld van de psychiatrie tijdens de diensten van de GGZ Bavo ontdekt. Ook vond ik in jou een gelijke betreffende de dagelijkse worstelingen van een klinische OIO. Bedankt voor alle gezellige momenten onder het genot van een theetje op het lab. Lieve Jo, door jou had ik het stukje Brabant terug op een Rotterdams lab. Beste Wim, bedankt voor je samenwerking en je kritische blik betreffende de systemische sclerose manuscripten. Alle andere collega's van de Unit Auto-Immuunziekten − Roos, Lucy, A3, Pieter, Joey, Serena, Bas, Anjali, Jeroen, Prayer en Connie − graag wil ik jullie ook allen bedanken voor jullie samenwerking en gezelligheid.

Ook buiten de unit hebben de nodige mensen bijgedragen aan mijn onderzoek. Beste Menno, ik wil jou bedanken voor je hulp met mijn Mozaïek project en de B cellen. Ondanks dat jij gelooft dat ik een trauma aan FACSen heb overgehouden, ik beloof je mijn best te doen om het B cel project na mijn promotie af te maken. Jorn en Diana, ook jullie bedankt voor jullie hulp met FACSen. Erik, Sandra en Odilia, zonder jullie zouden de Th17 manuscripten niet tot stand zijn gekomen. Enorme dank voor de fijne en vlotte samenwerking. Dames van het secretariaat, dank voor al jullie hulp en ondersteuning gedurende de afgelopen 4 jaar. Sandra de Bruin, ik weet dat ik op het laatste moment bij je aankwam voor de figuren, desondanks heb je er iets moois van weten te maken waar ik je voor wil bedanken.

Ook heb ik in de kliniek hulp gehad bij het verzamelen van patiënt materiaal. Beste Paul, Virgil en Jan, bedankt voor jullie samenwerking en de gezelligheid op de poli ondanks jullie drukke poli schema's. Dr. Dolhain bedankt voor uw hulp bij het verzamelen van SLE materiaal. Ook de dames van de poli immunologie en de prikkamer, jullie zijn een enorme steun geweest. Door jullie opgewektheid en vriendelijkheid vonden patiënten het nooit een punt om extra bloed voor wetenschappelijk onderzoek af te staan. Beste Joop, zoals ik ook op je afscheidsdiner verteld heb, jij bent een enorme voorbeeld en inspirator voor mij geweest. Voor Sjögren patiënten is jouw vertrek echt een verlies geweest!

Alle studenten, Ashley, Kim, Samara en Eline, bedankt voor jullie hulp en inzet! Alle andere mede OIO's die ik nog niet genoemd heb, Mati, Prisca, Armanda, Lizenka, Ewout, Hessel, Kim, Leendert, Magda R, Magda B, Hanna, Alice, Anna, Nicole, Wendy, Christina, Britt, dank voor jullie steun, gezelligheid en de leuke AIO weekenden. Veel plezier en succes met jullie verdere carrières! Lieve Chris, not only my colleague, but also my friend, neighbour, running mate, cat sitter and culinary inspiration, danke für alles!

Ook buiten het Erasmus MC hebben de nodige mensen bijgedragen aan dit onderzoek. Ik wil de samenwerkingspartners Tim en Lenny uit Nijmgegen en Utrecht hartelijk danken voor de prettige samenwerking and also dr. Matti Waris thank you for your collaboration.

NWO en in het bijzonder Mirjam Rigterink, bedankt voor de mooie beurs en ook voor de ongelooflijke ervaring gedurende het hele proces. Dear Liz, your presentation trainings were brilliant! Thank you so much for your help and your inspiration!

Zonder de steun van vrienden en familie was dit boekje niet afgekomen. Lieve Myrthe en Stana, bedankt voor jullie onvoorwaardelijke vriendschap en al jullie steun en aanmoedigingen de afgelopen 4 jaar. Lieve Shilpi, Güler en Miljana, bedankt voor de SATC achtige afspraakjes, precies datgene dat ik na een onderzoeksdag vaak nodig had! Het prachtige omslag is geheel aan jouw talent te danken Shilpi, bedankt voor je ontwerp! Lieve Habib Jan, ik heb lang niet met iemand zo veel gelachen als met jou, dank voor al je grappen en verhalen! Lieve Samir, my cousin en mijn raadgever, bedankt voor al de handsfree gespreken en de vakantie in 2011.

Lieve Zina, mijn aller liefste twinny! Ondanks dat jij enkel 10 minuten ouder bent, heb jij mij altijd beschermd en over mij gewaakt als 'the big sister'. Ik zou dan ook niemand liever als paranimf naast me willen hebben! De afgelopen jaren heb ik het jou als ziekenhuisapotheker in opleiding in het Erasmus MC niet makkelijker gemaakt met alle verwarringen in de wandelgangen en over de mail. Ik ben enorm trots dat z.brkic.1@erasmusmc.nl 19 september aan mijn zijde zal staan. Zina en Thomas, bedankt dat jullie onvoorwaardelijk en altijd voor mij klaar stonden!

Draga mama i dragi tata, jullie bijdrage aan dit boekje kan ik moeilijk in woorden vatten. Ondanks de situatie die jullie noodzaakte om een nieuw leven in een heel ander land te beginnen, hebben jullie ervoor gezorgd dat mij nooit aan iets ontbrak en dat ik altijd datgene

kon doen dat ik wilde. Als ik ergens het zelfvertrouwen vandaan kan halen dat ik alles in het leven kan bereiken wat ik wil, dan is dat wel uit jullie goede voorbeelden. Jullie hebben altijd achter mijn keuzes gestaan, al hebben jullie je vast bij sommige keuzes even achter het oor moeten krabben. Bedankt hiervoor en bedankt voor het onvoorwaardelijke vangnet en de liefde! Jullie zijn mijn helden en vandaar dat ik dit boekje aan jullie opdraag.

And last but not least, dragi Mirza, wat heb jij het laatste 1.5 jaar van mijn promotie tot een ware vreugde gemaakt! Hvala ti na strpljenju, podršci i ljubavi. Volim te puno!

Zana

CURRICULUM VITAE

Zana Brkić was born in Livno in Bosnia and Herzegovina on the 17th of May in 1985. There she finished the first class of elementary school at the Ivan Goran Kovačić school and moved in October of 1993 to the Netherlands with her parents and twin sister. In 2003 she graduated from secondary school at the Dongemond College in Raamsdonksveer and attended the medical school at the Erasmus University in Rotterdam. During her medical studies she did a research internship entitled 'Interferon type I signature in Sjögren's syndrome' at the department of Immunology, Erasmus Medical Center, which formed the basis for her PhD project later on. In 2009 she graduated *cum laude* from medical school and started her PhD project at the department of Immunology at the Erasmus Medical Center with a NWO mosaic grant that she successfully obtained. In January of 2014 she will start her residency of internal medicine.

PHD PORTFOLIO

Name PhD student	Zana Brkić
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PhD period	September 2009 – September 2013
Promotores	Prof.dr. H.A. Drexhage, Prof.dr. P.M. van Hagen
Copromotor	Dr. M.A. Versnel

PhD Training	Year
Courses and workshops	
- Workshop on basic data analysis on gene expression arrays	2009
- Scientific writing course	2010
- Biostatistics for clinicians	2010
- Regression analysis for clinicians	2010
- Molecular immunology	2010
- Flow cytometer training by BD	2010
- NIBI time management for PhD students and postdocs	2011
- Photoshop and Illustrator CS5 Workshop	2011
Teaching activities	
- Supervision of 'casus onderwijs' 2nd year medical students	2010-2013
- Lecture 2nd year Master 'Immunity and Infection'	2010-2013
- Lecture 2nd year 'keuzeonderwijs immunologie' medical students	2010-2013
- Supervision 'proefstuderen'	2010-2013
- Supervision of medical students during their internships	2010-2013
Presentations	
- 'Type I Interferon signature in primary Sjögren's syndrome patients correlates with disease activity and BAFF mRNA level' *10th ISSS, Brest, France – Oct 2009*	poster
- 'Prevalence of Interferon Type I signature in Sjögren's syndrome and association with disease activity and BAFF expression' *11th ISSS, Athens, Greece – Oct 2011*	poster
- 'Increased BAFF expression in patients with Interstitial cystitis but in contrast to Sjögren's syndrome patients no activation of the Interferon type I pathway' *11th ISSS, Athens, Greece – Oct 2011*	poster
- 'The Interferon type I signature is increased in monocytes from systemic sclerosis patients' *ACR, Washington DC, USA – Oct 2012*	poster
Grants and awards	
- Netherlands Organization for Scientific Research (NWO) Mosaic grant	Jul 2010
- Young talent travel award for 12th ISSS, Kyoto, Japan	Jul 2013
Extracurricular Activities	
- Active committee member of the PhD student committee of the Immunology department, Erasmus MC	2011

LIST OF PUBLICATIONS

Brkic Z*, Corneth OB*, van Helden-Meeuwsen CG, Dolhain RJ, Maria NI, Paulissen SM, Davleaar N, van Hamburg JP, van Daele PL, Dalm VA, van Hagen PM, Hazes JM, Versnel MA**, Lubberts E**. Th17 cytokines and Interferon type I: partners is crime in SLE? *Submitted for publication*

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