

The Toll of too much Interferon:

The systemic interferon signature in the
pathogenesis of Sjögren's syndrome

Naomi I. Maria - 2015



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The Toll of too much Interferon:
The systemic interferon signature in the pathogenesis
of Sjögren's syndrome

De Toll van te veel Interferon:
De systemische interferon handtekening in de pathogenese
van het syndroom van Sjögren

Thesis

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by

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born in Groningen

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*Un Kurason, yená ku...
Speransa i pashon,
Forsa i union.
Un Kurason...*

- Naomi I. Maria

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Chapter

General Introduction

1



PREFACE

This thesis focuses on the role of interferons in the immunopathogenesis of primary Sjögren's Syndrome (pSS). pSS is a systemic autoimmune disease with a heterogeneous pathogenesis. Over the past decade, vast evidence for a role of interferon (IFN) type I in the pathogenesis of pSS is emerging.

Here, we first give a short introduction on the immune system, with regards to immunity and autoimmunity. Autoimmune diseases are a family of often chronic inflammatory disorders characterized by the dysregulation of the immune system, which finally results in the loss of tolerance to self. Activation of IFN type I has been identified in subgroups of patients with systemic autoimmune diseases, such as pSS, systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and rheumatoid arthritis (RA). Among these diseases, SLE is considered to be the most prototypic IFN-driven autoimmune disease, and most advances in unravelling IFN-related pathology have been made in this disease.

We therefore use SLE as a basis in this introduction with a focus on IFN signalling, followed by a more in depth overview of pSS and its immunopathogenesis. As IFN type I is a suggested key pathogenic factor in pSS, we set out to better understand its role and contribution to the disease. A particular interest is on the main IFN producing cell, the plasmacytoid dendritic cell, its receptors and signalling pathways, leading to IFN and inflammatory cytokine production in immunity and autoimmunity. We elaborate on the difficult diagnosis of pSS, knowledge on genetics and epigenetics. One of the most debilitating symptoms of pSS patients is fatigue. Here we discuss the possible associations between IFNs and fatigue in pSS.

We end this introduction with current and potential therapeutic strategies and shortly give our perspective on the potential of mouse models for pSS-like disease. Finally, the scope of the different chapters in this thesis will be outlined.

IMMUNITY

Immunology is the study of the physiological mechanisms that humans and other animals use as defence against invasion by other organisms such as bacteria and viruses. *Immunity* is the ability to resist these infections, and to be *immune* is to be resistant to infection. The body's defense mechanism against these invaders comprises an organization of cells and molecules with specialized roles, collectively called the immune system [1, 2].

PREFACE

This thesis focuses on the role of interferons in the immunopathogenesis of primary Sjögren's Syndrome (pSS). pSS is a systemic autoimmune disease with a heterogeneous pathogenesis. Over the past decade, vast evidence for a role of interferon (IFN) type I in the pathogenesis of pSS is emerging.

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THE IMMUNE SYSTEM

The immune system is a remarkable complex of mechanisms and biochemical processes, protecting host viability by enabling efficient detection and elimination of host-threatening invaders. An essential feature of the immune system is its ability to distinguish innocuous from dangerous, self from non-self. It has evolved over time to protect the host from a world of microbes, both pathogenic and commensal. The immune system has traditionally been divided into two broad but interconnected subsystems, the innate and adaptive arms of the immune system [3-5]. This traditional dichotomy is however much more complex as previously thought [6].

Innate and adaptive immunity

The host uses both innate and adaptive immune mechanisms in order to identify and eliminate invading pathogens [3].

The *innate* arm of immunity is an evolutionary conserved system providing the first line of defense mediating immediate immune responses, whilst traditionally thought to lack the formation of immunological memory [6]. The cellular composition of innate immunity mainly comprises phagocytic cells (neutrophils, monocytes, and macrophages), cells that largely release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer (NK) cells. Neutrophils are mononuclear cells that form the front-line defenders of innate immunity [1, 5, 7]. The molecular components of innate immune responses include components of the complement system such as C3 and C4, acute-phase proteins such as C-reactive protein (CRP), and cytokines such as the interferons (IFNs) [1, 8].

The *adaptive* arm of immunity is highly specific, using acquired immunological memory for its responses. Adaptive responses improve upon repeated exposure to a given microbe or antigen [1, 5]. Specialized cells that form the basis of this acquired memory are the B and T lymphocytes. These B and T cells display an extremely diverse repertoire of antigen-specific recognition receptors, generated as a consequence of gene rearrangements that hereby provide long-lasting protective immunity. Adaptive immune responses are generated in lymph nodes, spleen and mucosa-associated lymphoid tissues (MALT) [2, 5].

A necessary first step for induction of adaptive immunity is the activation of specialized antigen-presenting cells (APCs), such as dendritic cells (DCs). DCs are unique cells, often called accessory cells, that bridge innate and adaptive immune responses [2]. APCs display antigen to B and T cells, and all these cells collaborate in the response to antigen. B cells are able to secrete immunoglobulins (Ig), the antigen-specific antibodies responsible for microbe-elimination [1].

Monocytes, DCs and macrophages are all derived from the same bone marrow (BM) precursors, arising from a common myeloid progenitor. Furthermore, recent evidence indicates an additional contribution of local tissue precursors to the myeloid lineage [7, 9-12]. Certain DCs

are actually of lymphoid origin, arising from a common lymphoid precursor [13, 14]. DCs themselves can be divided into two main subtypes, the conventional DC (cDC) and the plasmacytoid DC (pDC).

The immune system is able to distinguish between innocuous and dangerous, between self and non-self, by means of a large repertoire of receptors. DCs recognize invading pathogens through specialized germ-line encoded, non-clonal pattern recognition receptors (PRR) [2, 5, 8, 15]. The pattern recognition theory proposed 20 years ago by the late Charles Janeway Jr. provided a conceptual framework for our current understanding of innate immune recognition and its role in triggering the adaptive arm of immunity [2, 16].

Toll-like receptors (TLR) and retinoic acid-inducible gene I (RIG)-like receptors (RLR) serve as PRRs able to recognize evolutionarily conserved motifs associated to microbes, collectively called pathogen-associated molecular patterns (PAMP). These molecular patterns are often essential for the survival of the microorganism, and thus not easily altered. Furthermore, PRRs are constitutively expressed in the host, detecting pathogens regardless of their life-cycle stage [5]. They are expressed in different cellular compartments such as the cell surface or endosomes, on all cells of a given type, and are independent of immunological memory. Upon activation, they can trigger specific cellular immune responses that lead to distinct downstream signalling cascades resulting in expression of antimicrobial genes and inflammatory cytokines [2, 3, 5, 8, 15, 17].

Toll-like receptors

TLRs are a highly conserved transmembrane receptor family of PRRs, playing a crucial role in host-cell defence against microbial pathogens [18, 19]. Triggering of the TLR-pathway initiates various downstream inflammatory signalling pathways which result in expression of many inflammatory genes, including IFNs [20]. Additionally, TLR triggering initiates DC maturation and results in costimulatory molecule induction, increasing the antigen-presenting capacity of these cells [17]. TLRs are particularly found on DCs and macrophages, but are also expressed on neutrophils, B cells, and even on epithelial cells [3].

To date, the mammalian TLR family comprises 13 members [5, 8]. TLR1 through TLR9 are conserved between humans and mice and recognize similar ligands in both species, except for TLR8. TLR10 is non-functional in mice because of a retroviral insertion, and TLR11 through TLR13 are absent in the human genome [8]. Recently TLR8 was speculated to be non-functional in mice, raising numerous questions on the data thus far acquired in TLR8-related mouse models.

The family of TLRs can be divided into two subpopulations based on their cellular location. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are exclusively expressed on the cell surface, recognizing microbial membrane components such as lipids and proteins. Nucleic acid-sensing TLRs such as TLR3, TLR7, TLR8 and TLR9 can localize in intracellular compartments such as endosomes, lysosomes and the endoplasmic reticulum (ER), predominantly recognizing microbial nucleic acids [8]. Their localization consequently minimizes possible exposure to self-antigens [21].

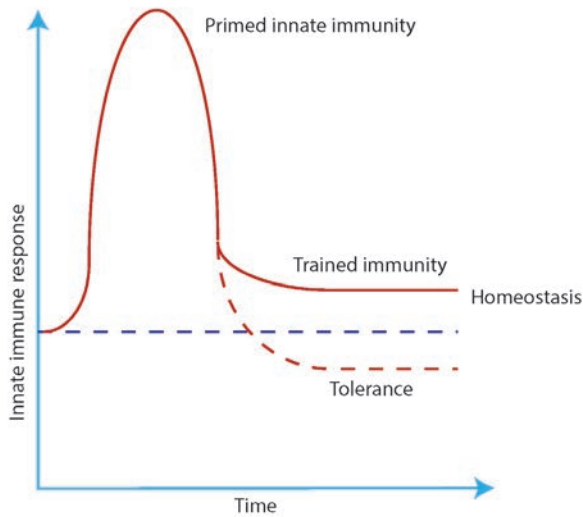


Figure 1. Innate immune responses. *Adapted from Netea M.G 2013, Eur J Clin Invest [23].* Innate immunity can be primed to elicit an enhanced response upon being challenged with a second stimulus (primed innate immunity), which rapidly declines after infection is resolved. The level of innate immune responses often remains in an enhanced state, not immediately returning to basal levels. This long-term enhanced state is termed trained immunity, which can be considered the opposite of immune tolerance.

RIG-I like receptors

RLRs comprise a cytosolic detection system for intracellular PAMPs, belonging to the RNA helicases family that specifically detects viral RNA in the cytoplasm [22]. RLRs consist of retinoic acid inducible gene-I (RIG-I, also DDX58), melanoma differentiation associated gene-5 (MDA-5, also IFIH1), and 'laboratory of genetics and physiology' (LGP2, also DHX58). In resting cells RIG-I is inactive as a monomer. RNA binding triggers a conformational change to facilitate self-association, and activation, which subsequently promotes downstream signalling [22]. LGP2 was suggested to be a negative regulator of RIG-I, as its repressor domain (RD) can bind to that of RIG-I, inhibiting self-association of RIG-I and thereby preventing activation. TLRs and the cytoplasmic RLRs both elicit proinflammatory- as well as IFN-responses upon activation. RLRs are, however, thought to be TLR-independent [8, 19, 22].

Trained immunity: innate immunological memory

It was long thought that innate immunity, in contrast to adaptive immunity, lacked the formation of immunological memory. Recent evidence indicates that innate immunity exhibits enhanced innate immune responses after exposure to secondary infections, which gave rise to the concept of "trained immunity". Trained immunity appears to be a form of innate immunological memory mediated by conceptually innate immune cells such as monocytes, macrophages and NK cells, protecting the host against reinfection in a B and T cell-independent manner [6, 23]. Trained immunity protects organisms that lack adaptive immunity, such as plants, where innate immune memory was previously called systemic acquired resistance (SAR) [24]. Interestingly, these protective mechanisms during reinfection have been attributed

to Toll-dependent mechanisms, with phagocytes as the critical effectors of this priming-induced protection [25]. Long-lasting upregulation of the Toll pathway amongst others was suggested to increase innate immune responses during reinfection, hereby “training” the immune system [6, 26]. According to Netea M.G *et al*, long-term trained immunity is fundamentally different from short-lived priming. It was additionally proposed that trained immunity may be considered as the opposite of immune tolerance (see Figure 1) [23].

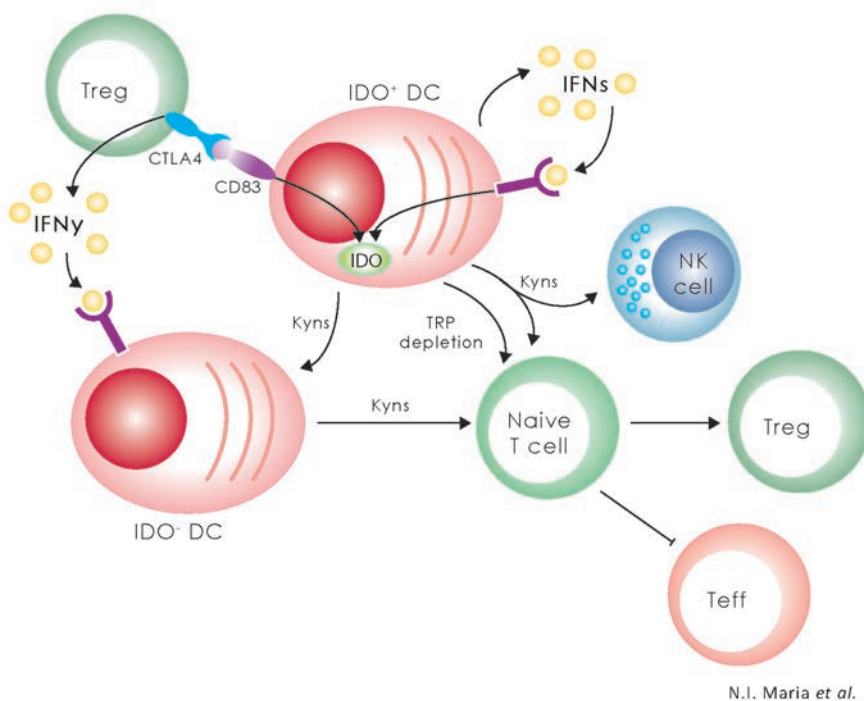


Figure 2. A model for crosstalk between dendritic cells and T cells. Adapted from Puccetti *et al.*, 2008 [36]. The activity of indoleamine 2,3-dioxygenase (IDO)-expressing dendritic cells (DC) is driven by both type I and type II interferons (IFNs), and results in the sustained IDO induced production of immunomodulatory tryptophan metabolites collectively termed kynurenines (Kyns). CD4⁺ naïve T cells are converted into FoxP3⁺ regulatory T cells (Tregs) upon exposure to IDO⁺ DCs, and in response to Tryptophan (TRP) depletion. Furthermore this highly immunosuppressive milieu induces suppression of T-cell responses by inhibition of T effector (eff) cell proliferation, inducing T-cell apoptosis, and expanding and stabilizing Tregs. The CD83-CTLA4 interaction between DCs and Tregs induces IFN γ (IFN type II) production, hereby further inducing production of Kyns by IDO⁻ DCs amongst others. The presence of Kyns can additionally recruit other cell types to the regulatory response, such as Natural killer (NK) cells. The combined effects of TRP depletion, caused by IDO⁺ DCs, and the high downstream Kyn production resulting from actions of both IDO⁺ and IDO⁻ DCs, is expected to have various effects on target T cells and other cell types.



TOLERANCE

The ability of the immune system to avoid damaging and recognizing self-tissues is referred to as self-tolerance [3]. Recognition of endogenous molecules by PRRs allows the immune system to distinguish infectious non-self from non-infectious self [17]. This specific recognition is, however, strongly associated with the pathogenesis of autoimmune diseases [8, 27], and will be discussed in more detail in the next part of this chapter.

Dendritic cells (DCs) form a crucial link between innate and adaptive immunity by translating innate immune responses into antigen specific T- and B-cell responses [28]. Notably, these adaptive immune responses can be silenced by tolerogenic DCs [29]. There is significant heterogeneity within tolerogenic DCs, though DCs expressing the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) are well characterized [30-32].

Indoleamine 2,3-dioxygenase in immunity and tolerance

IDO is a critical participant in the maintenance of peripheral immune tolerance. Enhanced IDO activity results in depletion of tryptophan (TRP), an essential amino acid required for T cell proliferation, thus limiting T-cell responses. Additionally, IDO is able to induce the development of T regulatory (Treg) cells, necessary to preserve tolerance [33-35]. IDO is known to suppress T-cell responses and promote immune tolerance in mammalian pregnancy, tumour resistance, chronic infection, allergy and autoimmunity [36].

The kynurenine (KYN) pathway is the major metabolic pathway in mammals to catabolize TRP and is tightly regulated by the immune system. IDO is the rate limiting enzyme in the catabolism of the essential amino acid TRP, degrading TRP to KYN [37]. Tregs are essential cells for maintaining peripheral tolerance, hereby preventing autoimmunity and limiting chronic inflammation [38]. The transcription factor forkhead box P3 (FoxP3) is required for Treg development, maintenance and function [39]. Cluster of differentiation (CD)4⁺ naïve T cells are converted into FoxP3⁺ Tregs upon exposure to either KYN or IDO⁺ DCs [32, 40-42]. Surface expression of the IL-2 receptor α -chain (CD25) has been used to discriminate CD4⁺CD25^{hi}FoxP3⁺ Tregs [43, 44]. These CD25^{hi}FoxP3⁺ Tregs represent a distinct subset of T cells that play a crucial role in maintenance of self-tolerance by repressing immune responses to self [38, 45-47]. Consequently IDO functions at the interface between immunity and tolerance, regulating and fine-tuning immune homeostasis whilst establishing tolerance to self.

KYN can be converted into immunomodulatory metabolites collectively termed “kynurenines” (Kyns) [37, 42, 48, 49]. Both TRP depletion and KYN accumulation are said to create a highly immunosuppressive milieu, suppressing T-cell responses by inhibition of T-cell proliferation, inducing T-cell apoptosis, and expanding and stabilizing Tregs (Figure 2) [50].

AUTOIMMUNITY: LOSS OF TOLERANCE

The sensitivity and potential destructiveness of our immune system, which is essential in recognizing and battling infections, can under certain circumstances be misdirected towards harmless components. These components, either self-derived or non-self, can trigger numerous kinds of chronic, non-infectious diseases such as autoimmunity. Autoimmunity is thus adaptive immunity directed to self-antigens.

It was thought that PAMPs were broadly expressed by pathogens, and not host cells, denoting that PRRs would specifically discriminate between danger and non-danger, self and non-self. These same PRRs have since been widely implicated in the pathogenesis of autoimmune diseases [8, 51]. How and where exactly it can potentially go wrong in these pathways is not entirely clear. Differentiation of self from non-self is thought to be evolutionarily achieved by restricting localization of host nucleic acids and by localization of PRRs in specific subcellular compartments [52]. Once pathogen-derived antigens activate the immune system, cross-reactivity with self can occur. Also, negative feedback mechanisms of the TLR-signalling pathways have been described as crucial in preventing overactivation and chronic inflammation. Loss of these tight regulatory mechanisms can have autoimmunity as an end result [20].

Autoimmune diseases are a family of often chronic inflammatory disorders characterized by the dysregulation of the immune system, which finally results in the loss of tolerance to self [51]. The onset of autoimmunity appears to be a multifactorial phenomenon, with contributions from gene defects conferring autoimmune susceptibility, to environmental factors. For one, infectious agents or tissue damage may activate the immune system, hereby altering expression of self-antigens or leading to expression of these self-antigens. Often, inadequate control or clearance of stressed or damaged cells contributes to the accumulation of endogenous ligands, danger-associated molecular patterns (DAMPs), which subsequently lead to the activation of PRRs. Thus a combined genetic predisposition with changes in self-antigen can potentially result in autoimmunity. [53]. Some well-known examples of *organ-specific* autoimmune diseases are type I diabetes (T1D) and multiple sclerosis (MS), mainly affecting a specific target organ such as the pancreas and the brain respectively. There are also *systemic* autoimmune diseases, meaning fully throughout the body, such as SLE, SSc, RA, and pSS.

Of course the above portrayed concept is only one of the many possible concepts of how autoimmunity can arise. In this thesis we turn our focus onto PRRs and their potential dysregulation in systemic autoimmunity.

Systemic autoimmunity

As mentioned, B and T cells display a diverse repertoire of antigen-specific recognition receptors [2, 5]. Since this diversity gives rise to numerous antigen specificities, some cells will develop that are specific to self-antigen. In early B-cell development, these self-recognizing B



cells also called autoreactive B cells, will be negatively selected to undergo programmed cell death – apoptosis. Moreover, autoreactive cells that escape this selection process can later undergo additional receptor editing in order to reduce autoreactivity [54, 55]. Lastly, immature circulating B cells that bind antigen in absence of a costimulatory signal, induces a state of non-responsiveness in these cells – anergy [56]. In systemic autoimmune diseases, the number of autoreactive cells is significantly increased [54]. As with autoreactive B cells, self-reactive T cells are present in healthy individuals, however, remaining silent. Inappropriate DC activation contributes to the activation of autoreactive T cells in autoimmunity, possibly by presentation of neo-self antigens [53].

In autoimmunity, self-antigen cannot be easily eliminated due to its excessive presence or ubiquitous nature, as with the SLE-related autoantigen chromatin, creating a chronic eventually incurable state [57, 58]. Autoantigens in SLE are often associated with macromolecular complexes that incorporate DNA (deoxyribonucleic acid) or RNA (ribonucleic acid). Production of autoantibodies recognizing nuclear self-antigens, such as antinuclear autoantibodies (ANAs), are frequently present in systemic autoimmune diseases and function as biomarker for diagnosis. In SLE, circulating immune complexes (ICs) are formed by these ANAs, eliciting damage and inflammation upon deposition in multiple organs including the kidneys, lungs, joints, skin and even the central nervous system (CNS) [59-61].

THE INTERFERONOPATHOLOGY IN SYSTEMIC AUTOIMMUNE DISEASES

Activation of the IFN type I signalling pathway has been identified in subgroups of patients with systemic autoimmune diseases, such as SLE, SSc and RA [62-66]. Interestingly these diseases frequently coincide with SS. Among these disease, SLE is considered to be the most prototypic IFN-driven autoimmune disease, and most advances in IFN-related pathology have been made in this disease. Many systemic autoimmune models used for SLE, extensively direct their attention to IFNs and their role in autoimmune development [67-70]. These models amongst others, gave rise to the currently available knowledge on the role of IFN in the pathogenesis – ‘interferonopathology’ – in systemic autoimmunity.

SLE is a debilitating systemic autoimmune disease predominantly affecting females of child-bearing age. SLE is characterized by the production of autoreactive antibodies, and flares and remissions affecting multiple organs. Symptoms include amongst others Raynaud’s phenomenon, butterfly rash, photosensitivity, arthritis, pleuritis and nephritis [71, 72]. The serological hallmark of SLE is elevated autoantibodies, including ANAs, double-stranded DNA (ds-DNA), nucleosomes and various small nuclear ribonucleoproteins (snRNPs). Autoreactive B and T cells have long been the main focus with regard to SLE pathogenesis. Shlomchik *et al.* previously proposed a model to explain how self-tolerance is initially lost and how the loss of tolerance

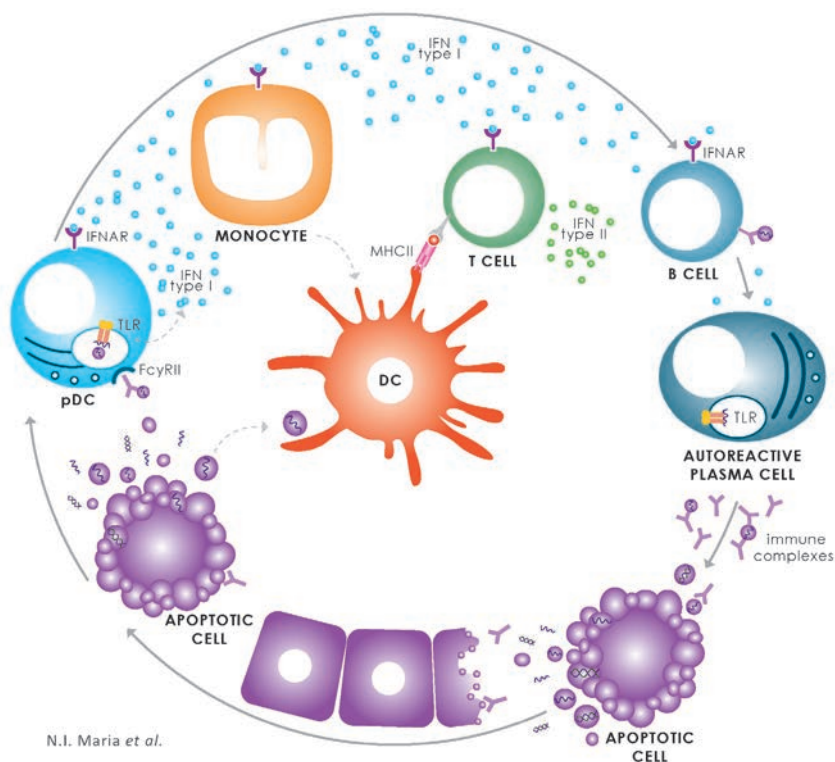


Figure 3. The interferonopathology in systemic autoimmunity: a simplified hypothetical scheme. IFN type I induces a self-amplifying loop in the pathogenesis of systemic autoimmune diseases, such as SLE. The aberrant clearance of nucleic-acid-containing debris and immune complexes activate pDCs to produce large amounts of IFN type I in a TLR-dependent manner. IFN can further activate adjacent monocytes, B and T cells. IFN-primed DCs activate T cells to produce vast amounts of IFN type II (IFN γ). Abnormally activated B cells can produce large amounts of autoantibodies that can further trigger pDCs, coming full circle in autoimmune interferonopathy. DC, Dendritic cell; Fc γ RII, Fc gamma Receptor II; IFNAR, IFN- α/β receptor; MHC, major histocompatibility complex; pDC, plasmacytoid DC; TLR, Toll-like receptor.

is then amplified and maintained. Herein the self-reinforcing interactions of B and T cells were suggested to lead to perpetuation and maintenance of autoimmunity [73]. Recent focus has however shifted towards DCs, with specific interest in pDCs. As pDCs are the major source of IFN type I during antiviral immunity, they are thought to be the most likely producer of IFN type I overexpression in autoimmune diseases [63, 74-78].

Over half of SLE patients display a marked over expression of IFN-inducible genes (IFIGs), defined as the IFN type I signature. The presence of this IFN type I signature was previously shown to correlate with disease activity in SLE, however, the exact relationship between IFNs and



disease activity in SLE remains controversial [62, 79-81]. More specific, the IFN type I signature identified a subgroup of SLE patients characterized by increased disease activity and severity, which included renal disease, complement activation, the presence of anti-RNA binding protein autoantibodies [79] and/or involvement of the central nervous system (CNS) [62]. These initial studies provided insights into the genetic pathways underlying not only SLE, but also the other systemic autoimmune diseases, and opened up a path of research which formed a basis for this thesis. Genetic studies have identified numerous functional pathways in SLE pathogenesis, of which the main pathways are the aberrant clearance of nucleic-acid-containing debris and ICs, excessive innate immune activation involving TLRs and IFN type I, and abnormal B and T cell activation [82, 83]. Nucleic-acid-containing debris and ICs can activate pDCs to produce large amounts of IFN type I in a TLR-dependent manner. IFN can further activate B and T cells, and

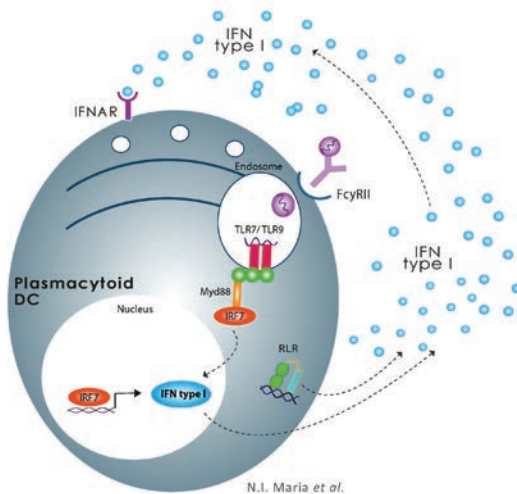


Figure 4. Simplified scheme of IFN type I production by plasmacytoid DCs. The pDC produces large amounts of IFN type I, after internalizing ICs through the FcγRII. In particular, endosomal triggering of either TLR7 (RNA-containing ICs) or TLR9 (DNA-containing ICs) signals in a MyD88-IRF7 dependent fashion to produce IFN type I. A second IFN type I producing route is through cytosolic RIG-I like receptors (RLRs). TLRs and RLRs both elicit proinflammatory- as well as IFN-responses upon activation.

abnormally activated B cells can produce autoantibodies that can further trigger pDCs, coming full circle in SLE interferonopathy (Figure 3).

Plasmacytoid dendritic cells

Plasmacytoid DCs are bone marrow-derived cells released into the blood, recirculating in secondary lymphoid organs such as the lymph nodes, spleen, MALT, thymus and liver. Resting or alternatively activated pDCs may promote tolerance, while activated pDCs can promote immunity or even autoimmunity [84-87]. pDCs were first described as plasmacytoid T cells or plasmacytoid monocytes, based on their plasma cell-like morphology and expression of T cell and myeloid-specific markers [88, 89]. Although their function was initially unknown, the large endoplasmic reticulum (ER) present in pDCs, indicated their potential role in cytokine secretion

[86]. The pDC was later described as the natural interferon-producing cell, owing to its capacity to produce large amounts of IFN type I following viral or bacterial exposure *in vitro* [90-92].

pDCs represent a small but distinct DC-population, representing approximately 0.2 to 0.8% of the peripheral blood mononuclear cells (PBMCs). pDCs play a key role in linking the innate and adaptive immunity [85, 93-96]. Human pDCs lack the lineage markers CD3, CD14, CD16, CD19 and the DCs marker CD11c [86], and can be identified by the expression of blood dendritic cell antigen (BDCA)2 (also CD303) and BDCA4 (also CD304/ Neuropilin1) as well as high expression of CD45RA and CD123. Interestingly, BDCA2 is known to mediate antigen-capture and has inhibitory effects on IFN type I production in pDCs [97-99]. The specific expression of FcγRII on pDCs surface membrane contributes to the internalization of ICs. These ICs are transported into TLR-containing endosomes, causing the continuous activation of pDCs to produce large amounts of IFN type I (Figure 3&4) [100-102].

pDCs can secrete high amounts of IFN type I. They execute their antiviral capacity by inducing the expression of numerous antiviral IFIGs, making cells resistant to infections while promoting apoptosis of virally infected cells [86]. Their IFN type I-producing capacity has also been described in response to self-derived nucleic acids originating from damaged tissues – recognized by TLR7 and TLR9 through MyD88-IRF7 dependent signalling – further implicating these “IFN type I producing cells” in systemic autoimmunity [103]. Interestingly, a marked increase in pDCs is evident in target organs of numerous diseases, such as in the skin of SLE patients [104]. pDCs are considered to produce IFN type I via 2 main signalling pathways, namely in a TLR-dependent way through endosomal TLR7 and TLR9, and in a TLR-independent way through cytosolic RLRs (see Figure 4) [105, 106]. To date, circulating pDCs have not been fully characterized, as present research is mostly on murine pDCs or on expanded human CD34⁺ bone marrow-derived stem cells [107]. The low frequency of these cells in the peripheral blood has clearly hampered attempts to understand their biology [108]. Deciphering the mechanisms by which pDCs are culprit in SLE pathogenesis has revealed interactions with neutrophils and autoreactive B cells amongst others [109, 110], which are most probably IFN-driven. Recent focus is shifting towards TLRs and RLRs, in trying to better understand their mechanisms of action and possible dysfunctions in autoimmunity. This will aid in the development of targeted therapies to inhibit these IFN-producing pathways. Here we first briefly mention the family of IFNs and subsequently continue into the known IFN-producing PRRs and their roles in autoimmunity.

Interferons

Over the last century, the discovery and molecular understanding of IFNs have been a major advance in biomedicine [111]. IFNs are a large family of multifunctional cytokines involved in anti-viral defense mechanisms, cell growth regulation and immune activation, enhancing both innate and adaptive immunity [111, 112]. After being discovered in the 1950s, it quickly became apparent that this family of cytokines could ‘interfere’ with both DNA- and RNA-viruses, hence

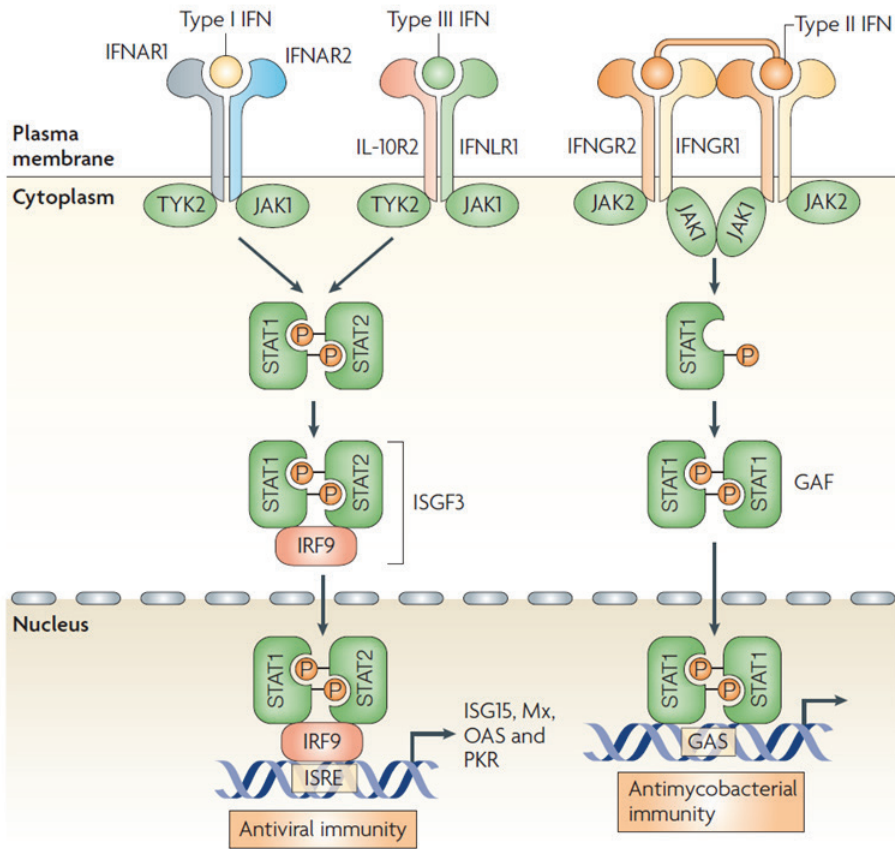


Figure 5. Receptor activation by Type I, Type II and Type III IFNs. Adapted from Sadler A.J. et al., 2008 [125]. The activation of the interferons (IFNs) is mediated through three receptor complexes: Type I (13 IFN α subtypes, IFN β , IFN ϵ , IFN κ and IFN ω) interact with a heterodimer of IFN receptor 1 (IFNAR1) and IFNAR2. Type III (IFN λ subtypes, IFN λ 1, IFN λ 2 and IFN λ 3; also IL28A, IL28B and IL29 respectively) interact with IFNLR1 (also IL28RA) and the interleukin-10 receptor 2 (IL-10R2; also IL10RB). IFN type I and III have similar downstream signalling pathways through phosphorylation of STAT1 and STAT2. Following binding by type I IFNs to the IFNAR-IFNAR2, or type III IFNs to the IL10R2-IFNLR1 heterodimers, signal transduction is initiated by pre-associated tyrosine kinases (JAK1 and TYK2), which lead to the recruitment and phosphorylation of the signal transducers and activators of transcription (STATs). STAT heterodimers associate with IRF9 to ISGF3. These complexes translocate to the nucleus to induce IFN-stimulated genes from IFN-stimulated response elements (ISREs), such as ISG15. Type II (IFN γ) interacts with IFNGR1 and IFNGR2, which form a tetramer consisting of two IFNGR2 chains and two IFNGR1 chains that bind dimers of the type II IFN. Signal transduction is initiated by JAK1 and JAK2, recruiting and phosphorylating STAT homodimers, translocate to the nucleus to induce GAS promoter elements. GAS, gamma IFN-activated site; IRF9, IFN regulatory factor 9; ISGF3, IFN-stimulated gene factor 3, refers to the STAT1–STAT2–IRF9 complex; ISG15, IFN-stimulated protein of 15 kDa; Mx, myxovirus resistance; OAS, 2',5'-oligoadenylate synthetase; P, phosphate; PKR, protein kinase R.

Table 1. Members of the IFN family of cytokines. Adapted from Hall J et al., 2010 *Nat Rev Rheumatol* [150].

Feature	Type I IFNs	Type II IFN	Type III IFNs	References
Subtypes	IFN- α (13 subtypes)		IFN- λ -1(also known as IL-29)	
	IFN- β		IFN- λ -2 (also known as IL-28A)	116,118,
	IFN- ω	IFN- γ	IFN- λ -3 (also known as IL-28B)	119, 129
	IFN- ϵ			
	IFN- κ			
Chromosome localization	9p22	12q14	19q13	108,110, 118
Produced by	All nucleated cells	NK cells	All nucleated cells	127, 130
		T cells		
Receptor subunits	IFNAR1	IFNGR1	IFNLR1	116, 118,
	IFNAR2	IFNGR2	IL-10R2	119
Receptor expression	All nucleated cells	All nucleated cells	Epithelial cells	126, 131
			DCs	
Signalling molecules	JAK1 and TYK2	JAK1 and JAK2	JAK1 and TYK2	116, 118,
				119, 129
Transcription factors*	STAT1/STAT2/IRF9	STAT1/STAT1	STAT1/STAT2/IRF9	116, 118,
	STAT1/STAT1		STAT1/STAT1	119, 129

*Dimerization partners.

Abbreviations: IFN, interferon; IFNAR, interferon- α receptor; IFNGR, interferon- γ receptor; IFNLR, interferon- λ receptor; IL, interleukin; IL-10R, interleukin-10 receptor; IRF, interferon regulatory factor; JAK, Janus kinase; NK, natural killer; NKT, natural killer T; pDCs, plasmacytoid dendritic cells; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase.

the name ‘interferon’, and had potential as treatment against viral infections. Later IFNs were also found effective in treating certain forms of cancer as well as a subset of patients with MS [113-115].

The IFN *type I* family of cytokines comprises 17 subtypes, all binding to a shared cell-surface IFN- α/β receptor (IFNAR) (see Figure 5) [76, 116]. The IFNAR is composed of two ubiquitously expressed transmembrane polypeptide chains, IFNAR1 and IFNAR2 [117]. In humans, the genes encoding the IFN type I subtypes are clustered on chromosome 9. The 17 mammalian subtypes constitute 13 IFN α subtypes, IFN β , IFN ϵ , IFN κ and IFN ω [118-120]. Although the IFN type I subtypes differ in their antiviral properties and other biological effects, the reason for origin and maintenance through evolution of all these related subtypes remains unknown [121-125].



IFN type II consists of IFN γ and signals through its own IFN γ receptor (IFNGR), consisting of the two subunits IFNGR1 and IFNGR2. IFNGR1 has a janus kinase (JAK)1 binding domain and a signal transducer and activator of transcription (STAT)1 docking site. IFNGR2 contains a JAK2 binding domain. IFN γ is mainly produced by activated T cells or NK cells [126, 127].

Recently, the novel IFN type III family of cytokines consisting of three subtypes IFN λ 1, IFN λ 2 and IFN λ 3, also known as IL28A, IL28B and IL29 respectively, was identified [128-131]. IFN type III activates the same main downstream signalling pathway as IFN type I, but has evolved with a completely different receptor structure (Figure 5) [132]. IFN type I and III signal downstream through JAK1 and tyrosine kinase (TYK)2 activation. These activated kinases recruit and phosphorylate the transcription factors STAT1 and STAT2, which translocate to the nucleus where the STAT1-STAT2 heterodimer associates with IFN regulatory factor (IRF9) to form an IFN-stimulated gene factor 3 (ISG3) complex. ISG3 refers to the STAT1-STAT2-IRF9 complex, that binds to IFN-stimulated response elements (ISRE) in the DNA, to activate transcription of over 300 IFIGs (Figure 5) [132-135]. Both IFN type I and II also induce the formation of STAT1-STAT1 homodimers that translocate to the nucleus and bind gamma-IFN-activation site (GAS) elements that are present in the promoter of certain IFN stimulated genes (ISGs), thereby initiating gene transcription [116, 136].

Type I, type II and type III IFNs (also see Table 1) signal via different receptors and display different levels of functional diversity, but share overlapping patterns of activated genes downstream [116, 134]. Especially IFN type I and III share numerous IFIGs, which can be expected due to their similar downstream signalling pathways. A key mediator in antiviral responses which is transcriptionally upregulated by both IFN type I and III is MxA, encoded by the myxovirus-resistance protein 1 (Mx1) gene [137]. MxA is a guanosine triphosphate hydrolysing enzyme (GTPase), tightly regulated by IFN type I [125, 138-140]. MxA, in contrast to other IFIGs, is not directly inducible by viruses and depends strictly on IFN signalling [140]. In SSC, MxA gene expression has been proposed as a biomarker for IFN type I bioactivity, and found to correlate to disease activity [141].

The IFN type I family of cytokines signal in an autocrine and paracrine manner for the induction of hundreds of IFIGs, also known as ISGs, which have antiviral, antiproliferative and immunomodulatory properties [135, 142]. A crucial role for IFN type I signalling in host protection is supported by the observation that individuals with genetic abnormalities in this pathway (in STAT1 or TYK2 for example) die of viral infections. Herein, defects in IFNAR signalling are more significant than that of IFNGR [143-145]. IFN type I has vast effects on other cells of the immune system. It has been shown to increase major histocompatibility complex (MHC) class I and II-expression, enhance co-stimulatory molecule expression on DCs, modulate Ig production, synergize with IL-12 to enhance IFN γ production, and augment NK and cytotoxic T cell responses [108]. Many of these effects are even more enhanced when IFN type I signalling is overactive, which can be the case in SLE. Overactive or sustained IFN type I signalling can have vast effects

on B cell survival and class switching, possibly perpetuating the pathogenic loop. IFNs induce BAFF- and APRIL- (A proliferation inducing ligand) expression in monocytes, hereby contributing to antibody-producing plasma cell survival resulting in prolonged pathogenic autoantibody production. This further triggers IFN-signalling, as well as increased IC formation and deposition in target tissues leading to chronic inflammation, damage, and ultimately loss of function [146].

IFNs are currently used therapeutically in cancer, MS and hepatitis C virus (HCV) infection amongst others. Due to their immunomodulatory properties, however, treatment with IFNs has shown upon occasion to induce autoimmune phenomena, such as lupus-like autoimmunity, showing elevated ANA-titers and clinical manifestations [147-149]. These observations, already in the early 1990s, support an important role for IFNs in autoimmunity. Interestingly however, the autoimmune symptoms that manifest can resolve after termination of treatment, suggesting additional factors such as genetic predisposition to be involved in self-propagation and sustenance of autoimmunity [150].

Intracellular PRRs and interferon type I

As mentioned, DCs are specialized accessory cells that bridge innate and adaptive immune responses, and have been implicated in the pathogenesis of most autoimmune diseases. DCs recognize invading pathogens using specialized PRRs, including TLRs and RLRs [15]. PRRs either recognize microbe-derived PAMPs or stress/damaged-induced host-derived DAMPs, subsequently leading to the activation of PRRs [53].

In contrast to infections by pathogens that replicate autonomously from the host, viral infections can blur the boundaries of self and non-self. Differentiating host from virus is achieved by restricting the localization of host nucleic acids and by placing PRRs that recognize these nucleic acids, in specific subcellular compartments [52]. PRRs detect the most intrinsic part of a virus, namely its nucleic acid genome, either single or double stranded RNA and/or DNA [52]. Nucleic acids are however not unique to viruses, since they are essential to all cellular life, and distinction between virus and host therefore becomes complex.

PRRs use specific mechanisms in order to distinguish self and non-self. PRRs are able to recognize unusual biochemical features present in viral but not host nucleic acids, such as the recognition of tri- (or di-)phosphorylated RNAs also lacking a 7-methylguanosine cap by RIG-I. Although host mRNAs are also tri-phosphorylated, the presence of the cap minimized recognition [151-154]. Additionally, the host is able to restrict its own nucleic acids to specific locations within the cell—host DNA should not be present in the cytoplasm of non-infected cells—enabling PRRs such as cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) to detect cytoplasmic DNA which is presumably of viral origin [155-157]. However, if host DNA for some reason presents itself in the cytoplasm, or host RNA in absence of their protective ‘cap’, signalling through these PRR-mediated pathways might be inevitable.



The 3 main PRR-pathways known to produce IFN type I signal through the TLRs, the RLRs and cGAS, are described in the next paragraphs.

Toll-like receptor signalling

The nucleic acid-sensing TLR3, TLR7, TLR8 and TLR9 localize in intracellular endolysosomal compartments, consequently minimizing possible exposure to self [8, 21]. TLR3 recognizes double stranded (ds)RNA such as a synthetic analogue of dsRNA polyinosinic-polycytidylic acid (poly I:C). TLR7 and TLR8 recognize single stranded (ss)RNA such as the imidazoquinoline derivatives imiquimod and resiquimod respectively, and TLR9 recognizes DNA containing CpG motifs present in numerous viral and non-viral pathogens [158-162]. TLR7 and TLR9 are predominantly expressed in pDCs, while TLR3 and TLR8 predominantly in monocytes and cDCs [8, 158, 159]. Binding of nucleic acids promotes the interaction of 2 TLRs, consequently dimerizing their toll-IL-1 receptor (TIR) domains, recruiting the adaptor protein MyD88, a cytoplasmic protein encoded by the myeloid differentiation primary response gene 88 [163]. Downstream signal transduction through most TLRs is dependent on MyD88 [3]. A second adaptor is TIRAP, encoded by the toll-TIR domain containing adaptor protein gene, was previously thought to signal in a MyD88-independent manner but was recently shown to be required for anti-viral signalling through TLR7 and TLR9 (Figure 6) [164]. TLR activation causes TIRAP-oligomerization with MyD88 and IL-1 receptor associated kinase (IRAK) kinases, initiating the formation of a large helical oligomer called the myddosome – functionally considered a so called supramolecular organization center (SMOC) [165-167]. This SMOC mediates the activation of IRF7 (IFN-regulatory factor 7) and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factors, after which IRF7 and NFκB translocate to the nucleus to promote the downstream expression of IFNs and other inflammatory cytokines respectively [167].

To minimize detection of host-encoded nucleic acids, TLRs are restricted to endolysosomal compartments by a UNC93B1-mediated process. UNC93B1 is a polytopic membrane protein, essential for signalling of TLR3, TLR7 and TLR9, that traffics the TLRs from the ER to endolysosomes. Interestingly, a mutation in UNC93B1 completely abrogates IFN expression elicited by these TLRs [168-170]. Furthermore, the cleavage of TLRs by proteases such as cathepsins is essential for effective downstream signalling to occur [171, 172]. This proteolytic regulation is of great importance to prevent autoimmunity, as having nucleic acid sensing TLRs mislocalized to the plasma membrane increases the change of autoimmune reactivity [173]. However, IFN-induced signal transduction is thought to solely occur from endosomal compartments.

Specifically the endosomal TLRs have been implicated to play a role in recognition of self-derived nucleic acids originating from damaged tissues [103, 174]. Negative feedback mechanisms of the TLR-signalling pathways have been described as crucial in preventing overactivation and chronic inflammation. Loss of these tight regulatory mechanisms can result in autoimmunity [20]. For the generation of autoantibodies by B cells and IC-mediated IFN production by

pDCs, endosomal Toll-like receptors TLR7 and TLR9 are crucial. Recently, opposing effects were described for TLR7 and TLR9 in murine lupus models, where TLR7-deletion limited autoimmunity, while TLR9 deletion paradoxically exacerbated disease. TLR7 deletion prevented RNA-associated antibody formation, whereas TLR9 deletion resulted in increased systemic inflammation and IC-induced glomerulonephritis [70]. That controlling TLR7 expression is essential in restricting autoimmunity already became clear when TLR7 gene duplication was demonstrated to be the sole requirement for accelerated autoimmunity in lupus-prone mice. A substantial TLR7 increase even caused fatal acute inflammatory pathology and extensive DC dysregulation [68]. The imbalance in endosomal TLRs, which results in much more prominent lupus-like disease compared to wild type (WT)-mice, was also recently shown using mice deficient in TLR8 and/or 9. B6.TLR8^{-/-} and particularly double TLR8/9 deficient mice (B6.TLR8/9^{-/-}) displayed marked induction of TLR7, which was associated with more severe disease. This study concludes that both TLR8 and TLR9 act together in controlling TLR7 function, TLR8 particularly controlling TLR7 function in DCs and TLR9 restraining TLR7 response in B cells [69]. Other TLR-related models elaborate on possibilities for exacerbated renal disease in TLR9^{-/-}, versus reduced disease in their TLR7^{-/-} counterpart. TLR9-deficient autoreactive B cells no longer undergo class switching to pathogenic Ig isotypes (IgG2a and IgG2b). These TLR9^{-/-} mice have significantly smaller IgG deposits in the glomeruli and a prolonged survival compared to their TLR9 sufficient littermates. In contrast, TLR7 deficient mice no longer produce RNA-specific autoantibodies, developing less severe clinical disease than their TLR7 sufficient littermates [67]. Further investigation of this imbalanced endosomal TLR-signalling in IFN-driven autoimmunity is warranted.

RIG-I like receptor signalling

In contrast to TLRs, RLRs are a family of DExD/H box RNA helicases that function as cytoplasmic sensors, expressed in virtually all cell types. RIG-I (DDX58) binds 5'tri- and di-phosphates present on short double dsRNA, whereas MDA5 (IFIH1) binds longer dsRNA [5, 151, 154]. RIG-I and MDA5 activate IRF3 and NFκB through the adapter termed mitochondrial antiviral signalling protein (MAVS), forming filaments upon RNA binding which subsequently oligomerizes the RLR caspase recruitment domain (CARD), and enhances interactions with the MAVS CARD (Figure 6) [22, 175, 176]. RIG-I contains tandem CARD-like regions at its N-terminus that function as an interaction domain with other CARD-containing proteins, the central DExD/H helicase domain. RIG-I also has a C-terminal repressor domain (RD), which binds to RNA [177]. MDA5 also contains tandem CARD-like regions and a DExD/H helicase domain, however the functionality of its C-terminal repressor domain is unknown. LGP2 (DHX58) contains a repressor domain (RD) and a DExD/H helicase domain, but lacks the CARD-region. LGP2 is the suggested negative regulator of RIG-I and uses its RD to bind to that of RIG-I, thereby inhibiting self-association of RIG-I and preventing further activation [8, 22]. The adaptor protein IFN-β promotor stimulator 1 (IPS-1), contains an N-terminal CARD-like structure that mediates interaction with the CARD of RIG-I

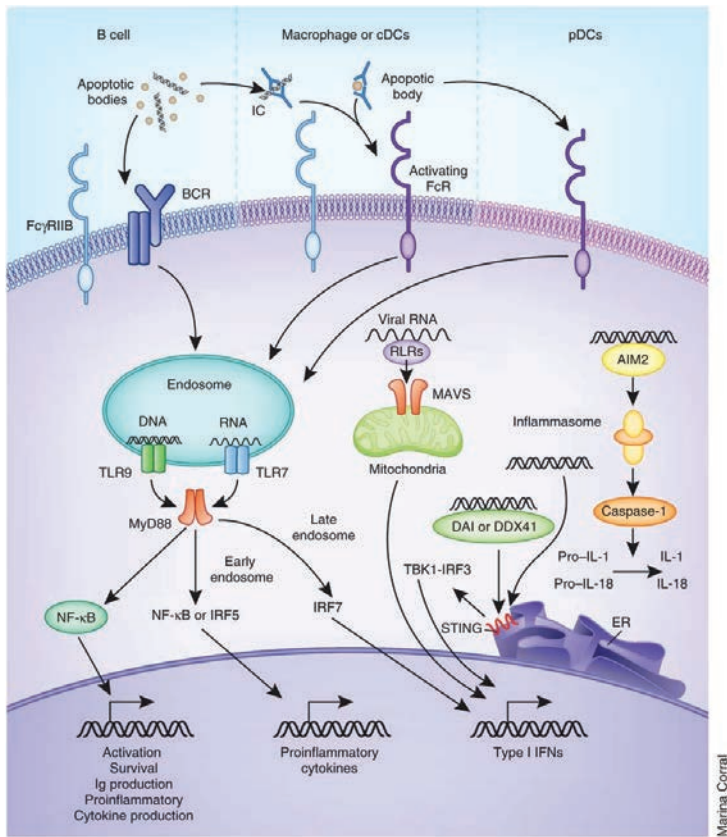


Figure 6. Recognition of nucleic acids by intracellular TLRs, RLRs and the cytosolic DNA sensor STING. Adapted from Liu Z. *et al.*, 2012 [83]. Nucleic acids or apoptotic particles can be taken up by B cells through the B cell receptor (BCR), and immune complexes containing these antigens are taken up by monocytes, myeloid dendritic cells and pDCs through FcR-mediated recognition and internalization. Within endosomes, DNA and RNA then interact with TLR9 and TLR7, respectively. Viral RNA can also be delivered to endosomes by autophagosome formation in pDCs. The ligation of TLRs recruits the adaptor protein MyD88, which activates the NF- κ B signalling cascade in B cells and leads to B cell activation and survival, as well as various effector functions. In pDCs, the recruitment of MyD88 preferentially triggers an IRF7-mediated signalling pathway, which initiates type I IFN production. In other cell types, MyD88 recruitment in late endosomes leads to inflammatory cytokine production. Cytosolic DNA and RNA can be recognized by sensors that, through adaptors, lead to type I IFN production. Cytosolic DNA can also be recognized by AIM2, which activates caspase-1, leading to proteolytic cleavage of pro-IL-1 and pro-IL-18 into active forms. Immune complexes may signal directly through both activating and inhibitory FcRs whose relative expression on the cell surface varies with cell activation status. How these positive and negative signals are integrated with each other and with TLR-mediated signals has not yet been fully elucidated. cDC, conventional dendritic cell; RLR, RIG-I like receptors; DDX41, a member of the DEXDc family of helicases; TBK1, TANK-binding kinase 1; ER, endoplasmic reticulum; Ig, immunoglobulin

and MDAs. Tank-binding kinase (TBK)1 and inducible-I κ B kinase (IKKi) protein kinases are crucial for IPS-1-mediated IFN induction [176]. Activation of IRF3 or IRF7 leads to the production of IFN type I, especially IFN- β . A second pathway involves the recruitment of TNF receptor-associated factor 6 (TRAF6) or receptor interacting protein 1 (RIP1), with subsequent activation of the transcription factors NF- κ B and AP-1. Activation of this pathway triggers the production of inflammatory cytokines and chemokines such as TNF- α , IL-6 and CXCL10 [178]. Interestingly in addition to TLR3, poly(I:C) also engages the nucleic acid sensors RIG-I and MDA5 [179], although 5'ppp-dsRNA is a more specific ligand for RIG-I [151].

RLRs have long been thought to be TLR-independent [180]. Recent findings are however pointing towards a collaborative effort between TLRs and RLRs, thereby enhancing and sustaining IFN-overactivation [19, 181]. In pDCs, this collaboration even seems to take place independent of IFN type I signalling [182].

RLRs are however also able to recognize processed self-RNA, and aberrant RLR-signalling or dysregulation has now been implicated in the development of autoimmunity [183]. RIG-I recognizes tri- (or di-) phosphorylated RNAs, lacking a 7-methylguanosine cap, and can under certain circumstances also recognize (capped) tri-phosphorylated host mRNAs [151-154]. Genetic screening studies have led to the identification of a number of polymorphisms in IFIH1 (de MDA5 gene) associated with autoimmunity. Very recent evidence shows a crucial role for IFIH1 in SLE where a gain-of-function mutation was described in the IFIH1-gene, pointing towards IFIH1 as the culprit of sustained IFN-signalling [184, 185].

cGAS and the STING pathway

While TLRs and RLRs detect intracellular RNA, the parallel STING pathway detects intracellular DNA. The stimulator of IFN genes (STING) is an ER- or mitochondria-localized protein that initiates IFN type I production, predominantly activated by the DNA sensor cGAS (Figure 6) [186-188]. In mitochondria, STING interacts with IPS-1 and RIG-I and activates NF- κ B and IRF3. In the ER, STING plays an essential role in the responses to dsDNA. STING activation normally requires dimerization, which is induced by cGAMP, produced by cGAS in response to cytosolic dsDNA. DsDNA activates NF κ B and IRF3 via the IKK complex and TBK1–IKKi, respectively [155-157, 189].

The host is able to restrict its own nucleic acids to specific locations within the cell. Host DNA should not be present in the cytoplasm of non-infected cells, enabling cGAS to detect cytoplasmic DNA that is presumably of viral origin [155-157]. However, if host DNA finds itself in the cytoplasm, signalling through the STING pathway might be inevitable and could induce self-activated IFN induction.

A germline dominant gain-of-function mutation in the gene encoding STING, TMEM173, was recently described in patients affected by systemic inflammatory and autoimmune conditions, including SLE patients. Here they suggest STING to represent a possible new therapeutic target in these disorders, where IFN type I production is prominently dysregulated [189]. Interestingly,



a recent study on STING in a lupus-prone murine model actually revealed STING to suppress inflammation in these mice, pointing towards a possible unexpected negative regulatory role for STING [190]. Clearly this pathway warrants further studies to better understand its role in immunity and autoimmunity.

INTRODUCTION INTO SJÖGREN'S SYNDROME

Many similarities exist between the pathogenesis of SLE and pSS. The main pathogenetic difference between the diseases is the association of mucosal tissue in pSS [191]. This thesis focuses on the role of IFNs in the pathogenesis of pSS.

It was already in 1933 that the Swedish ophthalmologist Henrik Sjögren described 19 women with symptoms of dry eyes and dry mouth in his doctoral thesis entitled “On knowledge of keratoconjunctivitis sicca” [192], an autoimmune disease ever since termed as Sjögren’s Syndrome (SS) [193]. SS is a systemic autoimmune disease characterized by lymphocytic infiltrates in salivary and lacrimal glands, sialoadenitis and dacryoadenitis respectively. The disease can occur alone – primary SS (pSS) – or together with other systemic autoimmune diseases such as SLE, SSc or RA – secondary SS (sSS). pSS is estimated to be the second most common systemic autoimmune disease, with RA being the most prevalent. The estimated prevalence of pSS ranges from 0.09-2% with a nine fold predominance in females, primarily in premenopausal women [194-197]. Characteristic symptoms are dry eyes (keratoconjunctivitis sicca) and mouth (xerostomia), with frequent presence of multiple extraglandular manifestations, such as vasculitis, arthritis, polyneuropathy, severe fatigue and multi-organ (often renal or pulmonary) involvement [195, 196, 198-200]. Disabling fatigue and reduced well-being are often experienced, with 40% of pSS patients experiencing fatigue as their most severe symptom [201-204]. Fatigue and factors causing fatigue should therefore be considered as important treatment targets. At present, no common evidence-based intervention therapy is available and treatment is mainly symptomatic. Further unravelling the pathophysiology of pSS is essential for finding novel biomarkers and the identification of new treatment targets.

DIAGNOSIS

As pSS is a heterogeneous disease, its diagnosis commonly requires a multidisciplinary approach and may be difficult to establish [205]. It can take up to 10 years, after onset of first symptoms, until a correct diagnosis is made and often patients are misdiagnosed. Symptoms do not always present concurrently and the diversity of symptomatic expressions adds to the difficulty of initial diagnosis [206]. Regrettably, as the disease frequently occurs in women in early-menopause, symptoms are frequently attributed to hormonal changes instead of the disease, increasing the chance of under- or misdiagnosis. A study in 2004 on early SS diagnosis stated

Box 1. Revised international classification criteria for Sjögren's syndrome [AECG-criteria by Vitali et al., 2002]

- I. Ocular symptoms: a positive response to at least one of the following questions:
 1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
 2. Do you have a recurrent sensation of sand or gravel in the eyes?
 3. Do you use tear substitutes more than 3 times a day?
- II. Oral symptoms: a positive response to at least one of the following questions:
 1. Have you had a daily feeling of dry mouth for more than 3 months?
 2. Have you had recurrently or persistently swollen salivary glands as an adult?
 3. Do you frequently drink liquids to aid in swallowing dry food?
- III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests:
 1. Schirmer's I test, performed without anaesthesia (≤ 5 mm in 5 minutes)
 2. Rose bengal score or other ocular dye score (≥ 4 according to van Bijsterveld's scoring system)
- IV. Histopathology: In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score ≥ 1 , defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm² of glandular tissue
- V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:
 1. Unstimulated whole salivary flow (≤ 1.5 ml in 15 minutes)
 2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
 3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer
- VI. Autoantibodies: presence in the serum of the following autoantibodies:
 1. Antibodies to Ro (SSA) or La (SSB) antigens, or both

Revised rules of classification:

Primary Sjögren's syndrome

In patients without any potentially associated disease, primary SS may be defined as follows:

- a) The presence of any 4 of the 6 items is indicative of primary SS, as long as either item IV (Histopathology) or VI (Serology) is positive
- b) The presence of any 3 of the 4 objective criteria items (that is, items III, IV, V, VI)
- c) The classification tree procedure represents a valid alternative method for classification, although it should be more properly used in clinical-epidemiological survey

Secondary Sjögren's syndrome

In patients with a potentially associated disease (for instance, another well-defined connective tissue disease), the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS

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



that SS remains undiagnosed in more than half of the patients. Timely diagnosis and appropriate treatment are therefore essential for optimal management of SS, and could aid in preventing or limiting the many clinical manifestations associated with the disease [206].

From a clinical perspective patients often mention constant straining flu-like symptoms with extreme muscle (myalgia) and joint pain (arthralgia). Patients describe a ‘sandy feeling’ in their eyes that gives a painful burning or itching sensation, grittiness, soreness and dryness, due to the diminished secretion of tears. Oral dryness can have a profound effect on quality of life, affecting daily functioning such as eating, speaking and even sleeping. Patients also complain not being able to eat without drinking a glass of water as a result of hyposalivation (cracker sign). Tears and saliva exhibit antibacterial properties, serving to clean and lubricate the eyes and mouth respectively. Lack of lubrication and thus loss of protection can result in many opportunistic

infections, such as candidiasis. Diminished tear-secretion may result in chronic irritation and destruction of corneal and bulbar conjunctival epithelium, keratoconjunctivitis sicca. Loss of saliva in pSS patients may accelerate infection, tooth decay and periodontitis, often resulting in loss of some or even all teeth. Also salivary or parotid gland swelling can occur, either uni- or bilateral, which can be a risk factor for lymphoma-development [206-209].

In order to develop universal classification criteria for pSS, the American-European consensus group (AECG) was formed, consisting of experts in the field. To date, pSS patients are positively diagnosed according to the revised 2002 AECG criteria for Sjögren’s syndrome (see box 1), as is the case in this thesis, which consists of 6 elements (both subjective and objective) [210]. Still a number of patients do not completely fulfil the criteria, and remain classified as incomplete SS (iSS). New criteria will be developed in the near future, possibly by combining the existing AECG criteria with the recently described American college of Rheumatology (ACR) criteria [211]. Presently, multiple groups are comparing the two sets of criteria [205, 212, 213]. Interestingly, due to the current presence of multiple criteria for diagnosis as well as classification, the incidence and prevalence of pSS can vary markedly [197]. The development of universally accepted criteria is warranted.

EXTRAGLANDULAR MANIFESTATIONS AND DISEASE ACTIVITY

To develop a disease activity index for pSS patients, the European League Against Rheumatism (EULAR) developed a consensus systemic disease activity index, the EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) [214]. Experts identified 12 organ-specific domains contributing to disease activity in pSS, hereby representing practically all possible systemic complications in the disease. For each domain the features of disease activity were classified into 3 or 4 levels, according to domain-severity [214]. In box 2 a brief overview and description of the 12 components of the ESSDAI is given.

Next to these extraglandular manifestations, as assessed by the ESSDAI, pSS patients often describe fatigue to be the most debilitating of all symptoms [204, 215]. pSS patients often

Box 2. Brief description of the domains of the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI)

[See Seror *et al.*, 2010 for full weighted domain description]

I. Constitutional:	Mild or intermittent, to severe fever/night sweats and/or involuntary weight loss.
II. Lymphadenopathy:	<i>Low to Moderate</i> , as determined by size in cm of (palpable) nodal or inguinal region. <i>High</i> : current malignant B cell proliferative disorder, such as lymphoma
III. Glandular:	Small to major glandular swelling with enlarged parotid, or limited submandibular or lachrymal swelling.
IV. Articular:	<i>Low, moderate or high</i> arthralgias in hands, wrists, ankles and feet accompanied by morning stiffness (moderate and high associated to total count of synovitis).
V. Cutaneous:	<i>Low</i> : erythema multiforma, <i>Moderate</i> : Limited cutaneous/ urticarial vasculitis limited to feet and ankle or subacute cutaneous lupus, <i>High</i> : Diffuse cutaneous / urticarial vasculitis or diffuse purpura, or ulcers related to vasculitis.
VI. Pulmonary:	From persistent cough or bronchial involvement, interstitial lung disease, to moderately and highly active pulmonary involvement, such as interstitial lung disease with shortness of breath or abnormal lung function.
VII. Renal:	<i>Mild</i> (tubular acidosis without renal failure), <i>moderate</i> (such as tubular acidosis with renal failure or glomerular involvement with proteinuria and without hematuria or renal failure, or histological evidence of extra-membranous glomerulonephritis) or <i>highly</i> active renal involvement.
VIII. Muscular:	<i>Mild, Moderate or Highly</i> active myositis, shown by abnormal electromyogram or biopsy (with assessment of muscle weakness and levels of creatine kinase).
IX. Peripheral nervous system:	<i>Mild</i> (such as pure sensory axonal polyneuropathy or trigeminal neuralgia), <i>Moderate</i> (such as axonal sensory-motor neuropathy or pure sensory neuropathy with presence of cryoglobulinemic vasculitis) or <i>Highly</i> active PNS involvement.
X. Central nervous system:	<i>Moderate</i> (such as cranial nerve involvement of central origin or optic neuritis) or <i>Highly</i> (cerebral vasculitis with cerebrovascular accident or transient ischemic attack, seizures, transverse myelitis or lymphocytic meningitis) active CNS features.
XI. Hematological:	<i>Low or Moderate</i> Cytopenia of autoimmune origin with neutropenia, and/or anemia, and/ or thrombocytopenia or lymphopenia.
XII. Biological:	<i>Low</i> : Clonal component and/or hypocomplementemia (Low C3 or C4) and/or hypergammaglobulinemia or high IgG levels >16 g/L. <i>Moderate</i> : presence of cryoglobulinemia and or/ hypergammaglobulinemia or high IgG levels >20 g/L, or recent decrease of IgG levels <5 g/L.

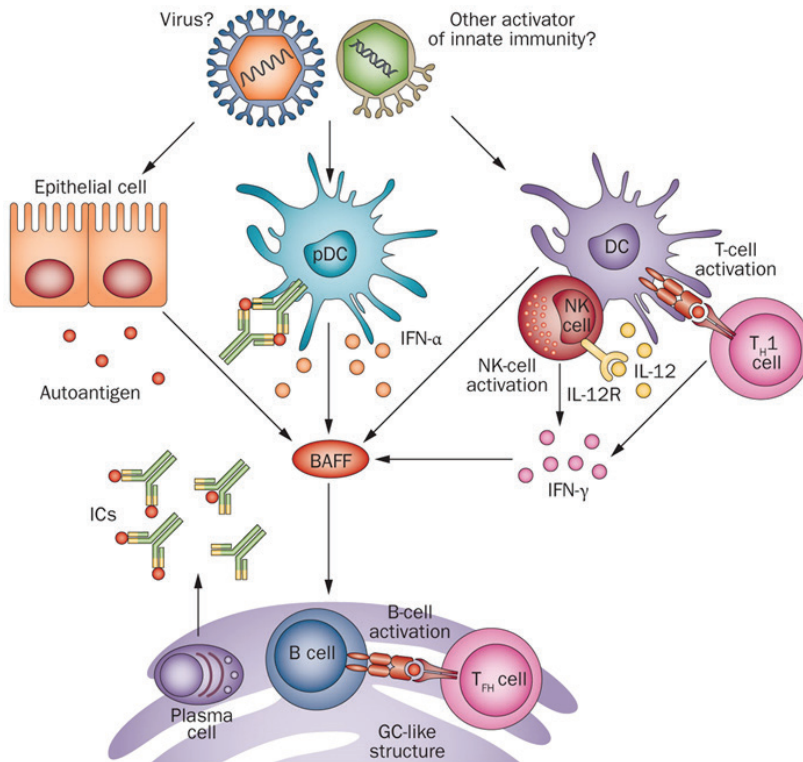


Figure 7. Multifactorial pathogenesis of primary Sjögren's syndrome. Adapted from Nocturne G. et al 2013 [191]. Environmental factors (viruses or other activators of innate immunity) can cause epithelial cell activation/ damage, giving rise to autoantigens in the circulation of pSS patients. These autoantigens can form interferonogenic ICs which can subsequently trigger pDCs to produce large amounts of IFN type I. Stimulation of DCs induces the production of both IFN type I and Type II (IFN γ), which further enhance BAFF secretion and leads to the activation of both B and T cells. IL-12 can further promote IFN γ activation via both NK and T $_H$ 1 cells. In genetically susceptible individuals, activation of B cells is thought to drive autoantibody production within GC-like structures. BAFF, B cell-activating factor; DC, dendritic cell; GC, germinal centre; IFN, interferon; ICs, immune complexes; IL12R, IL-12 receptor; NL, natural killer cell; pDC, plasmacytoid DC; pSS, primary Sjögren's syndrome; T $_{FH}$, follicular helper T cell; T $_H$ 1, T-helper1 cell.

experience disabling fatigue and a depressed mood, reducing overall health-related quality of life. This extensively effects physical, psychological and social functioning, being a source of major disability in these patients [204, 215-217]. To date it is however largely unknown what factors cause these disabling symptoms in pSS.

To assess the main symptoms of pSS – dryness, limb pain, and fatigue-related symptoms – the EULAR consensus group also developed a patient-administered questionnaire, the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) [218]. The ESSPRI consists of

a unidimensional 0-10 global assessment score for the 3 components. For example, scoring fatigue can range from 'no fatigue = 0' to 'maximal imaginable fatigue = 10', which is similar for dryness and joint or muscular pain in arms or legs [218, 219]. Both the ESSDAI and ESSPRI are now widely being used to facilitate clinical research and function as possible outcome measure in clinical trials [220].

IMMUNOPATHOGENESIS

The pathogenesis of pSS – as that of other systemic autoimmune diseases – is multifactorial, with contributions from gene defects conferring autoimmune susceptibility, to environmental factors [198-200, 221]. The innate immune system has an important role in early stages of pSS, through activation of IFN type I signalling [191]. IFN type I itself has vast effects on B cell survival, possibly perpetuating the pathogenic loop (see Figure 7).

Environmental factors and genetics

Pathogenic infections such as viral infection have been speculated to be possible triggers, initiating pSS pathogenesis. Based on the environment within the mouth as well as the presence of the IFN signature in pSS, viral infection of the salivary epithelial cells has long been suspected to be the initial trigger of pSS development [191]. Viruses or other activators of innate immunity can cause epithelial cell activation and damage, giving rise to autoantigens in the circulation. These autoantigens can form ICs, which can subsequently trigger pDCs to produce large amounts of IFN type I (Figure 7). Stimulation of DCs induces the production of both IFN type I and type II. IFNs induce B cell activating factor (BAFF)- and a proliferation inducing ligand (APRIL)-expression in monocytes, hereby contributing to antibody-producing plasma cell survival and resulting in prolonged pathogenic autoantibody production. This further triggers IFN-signalling as well as increased IC deposition in target tissues leading to chronic inflammation, damage, and ultimately loss of function [146]. Several studies have described associations between viral infections and pSS, such as Epstein-Barr virus (EBV), Coxsackie virus and human T lymphotropic virus type I [222-224], although direct evidence between onset of pSS and one single viral infection remains absent.

Also the internal hormonal environment has been proposed at the basis of pSS pathogenesis, such as the imbalanced presence of sex hormones, especially given the large female predominance and the onset of pSS during early-menopause. Some studies have found interesting links to estrogen and the estrogen receptor (ER) in pSS [225], however, evidence for hormonal impact on initiation of pSS pathogenesis has remained elusive. Recent evidence is pointing towards a X-chromosomal link to disease-onset in autoimmunity [226-228]. Murine models comparing females (XX) to males (XY) have concluded the sex chromosome complement in females to confer greater susceptibility to autoimmunity [226]. Various sex-related difference have been described in immunity, where females generally have increased cellular and humoral immune



responses (greater antibody production) compared to males, exerting a more vigorous immune response. Furthermore, changes in clinical features of autoimmune severity have been associated with hormonal changes during pregnancy. For example, disease severity in RA patients is known to improve during pregnancy, whereas in contrast SLE can worsen or remain stable. Interestingly, females have been speculated to produce higher amounts of immunomodulatory cytokines, including IFNs [229]. Differences could arise from genes that are encoded on a particular sex chromosome. These genes are then potentially differentially expressed in male vs. female cells. One extensively studied organ in this respect has been the brain, being sexually dimorphic [230, 231]. A more recent study compared XX (genetic and phenotypic female) with XY(-) (phenotypic female) mice, observing XX sex chromosome complement to be associated with accelerated spontaneous lupus development [228]. Studies by Dillon S. *et al.* have shown that men with an additional X (47,XXY Klinefelter's syndrome) are approximately 11 times more likely to have SLE, even though their circulating sex hormone profile is closer to that of a man. In triple X syndrome (47,XXX), which has a hormone profile comparable to 46,XX females, the prevalence of SLE and SS was respectively 3 and 4 times higher compared to 46,XX women and 25 and 52 times higher compared to 47,XY men. These data indicate the X chromosome number to possibly explain the sex-bias in SLE and pSS, independent of circulating sex hormones [232-234].

Furthermore photosensitivity to ultraviolet (UV) radiation from sunlight has been implicated as a trigger of autoimmunity, mainly in skin-related autoimmune diseases such as subacute cutaneous lupus and dermatomyositis. Current data suggests increased keratinocyte apoptosis can result from genetic polymorphisms in the TNF promoter, or decreased clearance of apoptotic cells due to polymorphisms associated with genes in the complement cascade [235]. If this also holds true for pSS remains to be established.

Genetic predisposition

Various genetic polymorphisms affecting the function of the immune system, in particular genes involved in the production and response of IFN type I are associated to increased risk for pSS [236]. These candidate gene studies in pSS were mainly inspired by findings in SLE [191]. In a large genome-wide association study (GWAS) in pSS patients, strong associations were found within the human leukocyte antigen (HLA) region, IRF5, TNIP1, STAT4, IL12A and (DDX6-)CXCR5 among others, highlighting the importance of genes involved in both innate and adaptive immunity [237]. In particular polymorphisms in IRF5, a pivotal transcription factor of the IFN type I signalling pathway, and polymorphisms in STAT4 encoding signal transducer and activator of transcription 4, a protein involved in the IFN type II signalling pathway, received vast interest in pSS [237-241]. A study aimed at finding single nucleotide polymorphism (SNP) variations in pSS in relation to fatigue found genetic associations with chronic fatigue in pSS, in SLC25A40 (a mitochondrial carrier protein) and PKN1 (of the protein kinase C superfamily involved in signal

transduction) [242]. Such candidate gene studies might be a good step towards better understanding disabling fatigue in pSS patients.

Perhaps out of the scope of this thesis, but intriguingly early after birth and even prenatal abnormalities have been observed, in DC precursors amongst others, in studies performed by our group in murine autoimmune models [9, 243, 244].

Epigenetics

Beyond genetics, epigenetic abnormalities related to DNA hyper- and hypo-methylation, histone modification and non-coding RNAs such as microRNAs (miRNA) also play key roles in pSS pathogenesis [191, 245]. miRNAs are small non-coding RNAs that can modulate expression of multiple protein-encoded genes at a post-transcriptional level, and have recently been recognized as powerful regulators in the pathogenesis of autoimmune diseases [246, 247]. Distinct miRNA patterns have been described in pSS salivary glands [248]. Interesting miRNAs implicated in pSS thus far are miR-146a, miR-155 and miR-181a [249-251]. MiRNA-146a is a negative regulator of NFkB activation through regulation of IRAK1/ TRAF6 in the MyD88-dependent pathway [246, 252], implicating its role in TLR- and possibly downstream IFN-signalling.

INTERFERON TYPE I IN PRIMARY SJÖGREN'S SYNDROME

Insights into the pathogenesis of pSS over the past decade, have indicated an eminent role for the activation of IFN type I. As with SLE, also SS-like symptoms have been described on occasion in cancer and HCV-infected patients treated with IFN [253-255], strongly supporting 'interferonopathology' in pSS. pSS is a systemic autoimmune disease characterized by autoantibodies targeting RNA-associated antigens, anti-SSA (Ro52/Ro60) and -SSB [256]. The abundant presence of autoantibodies leading to circulating ICs is thought to be the main trigger inducing the expression of IFNs and IFN-inducible genes in pSS and SLE [67]. To date, it is however still not clear what causes this marked overexpression of IFN-inducible genes.

Endosomal TLR7 and TLR9 are crucial for both the generation of these autoantibodies by B-cells as well as IC-mediated IFN production by pDCs [257]. Abnormalities in these pDCs have been suggested to play a distinct role in pSS pathogenesis [258]. Infiltrates of pDCs are found in the salivary glands of pSS patients, concurrent with increased IFN type I expression in the glands [75, 95, 96, 259]. The low frequency of these cells in the peripheral blood, with even lower numbers in pSS, has however limited extensive studies on understanding their possibly pathogenic role. Using a genome-wide microarray approach, our group identified a systemic upregulation of IFN type I signalling in peripheral blood monocytes in a small pSS sample study [258], followed by similar observations in pSS PBMCs [260].

As already portrayed above, all roads lead to a crucial role of IFNs in the initiation and immunopathogenesis of pSS. A postulated disease model is that an initial viral trigger induces IFN type I production in the salivary glands with subsequent activation of the adaptive immune



system resulting in the production of autoantibodies against the RNA-binding proteins SSA/SSB. Interferogenic immune complexes are formed, which trigger the pDCs to an ongoing type I IFN production, sustaining the disease process (Figure 7). As a recent study in human salivary gland tissue provided evidence that also IFN type II (IFN γ) contributes to the IFN signature in SS glands [261], the role of IFN γ in pSS needs to be further investigated.

IFNs and Fatigue

Fatigue is defined as extreme subjective feeling of tiredness resulting from mental or physical exertion, or illness. The constant production of pro-inflammatory cytokines due to peripheral immune activation, can act on the brain causing so called sickness behaviour [262]. Development of depressive symptoms in vulnerable individuals suffering from systemic infections, cancer or autoimmune diseases, could in part be caused by a sustained systemic inflammatory state. However at present, associations of clinical or biological markers with fatigue are limited [215, 263-265]. A role for type I IFNs in fatigue and depression is supported by induction of these symptoms in subjects receiving IFN-treatment for infectious diseases [266, 267]. On occasion these IFN-treated patients also reported characteristic pSS-like symptoms, such as dry eyes (keratoconjunctivitis sicca) and mouth (xerostomia) [253-255], supporting a role of IFN type I in pSS pathogenesis as well as various disease hallmarks.

IDO – the rate-limiting enzyme in TRP-catabolism – is driven in part by type I and type II IFNs [36, 37]. As mentioned above IDO is able to induce the development of Tregs, necessary to preserve immune tolerance [33-35]. Interestingly however, IDO has also been implicated in autoimmunity, and enhanced TRP-catabolism has been associated with mood disturbances [36, 37, 49]. There is increasing interest in the role of kynurenines in regulating and maintaining immune homeostasis, and recent studies suggest this pathway to reflect a crucial interface between the immune and nervous system [42]. The exact role of IDO and the regulatory kynurenine pathway in autoimmunity has, however, remained elusive.

TREATMENT

Although pSS is a not a life-threatening disease, prescribing the appropriate treatments is still crucial to improve quality of life and avoid unnecessary complications. Unfortunately, no common evidence-based intervention therapy is available and treatment has been mainly symptomatic [206]. Moisture replacement such as tear and saliva substitutes and moisturizing lotions can help relieve ocular and oral dryness [206, 268].

Evidence from placebo-controlled trials suggests benefits for the stimulation of salivary and tear-flow with the muscarinic agonists pilocarpine and cevimeline for sicca symptoms, and topical cyclosporine for moderate to severe dry eyes [268-271].

The antimalarial agent hydroxychloroquine (HCQ) has long been considered an effective treatment for SLE and is frequently used for pSS. Chloroquines are considered to block

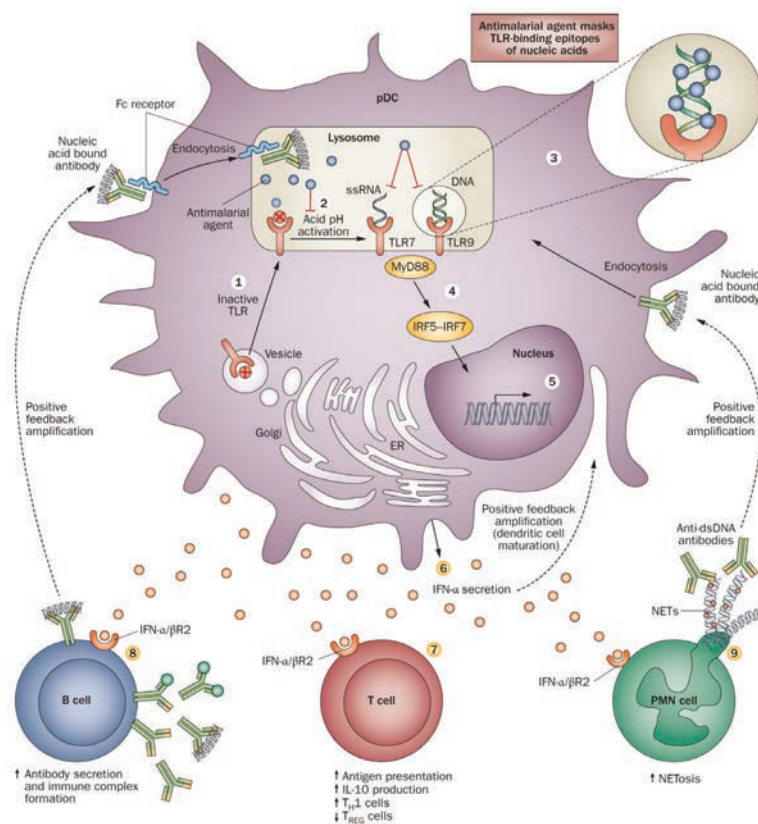


Figure 8. Role of chloroquines in Toll-like receptor-mediated pathways in systemic autoimmunity. Adapted from Wallace *et al.*, 2012 [21]. TLRs are in an inactive state when they exit the Golgi complex (1), are cleaved and activated in endosomes by acid-dependent proteases (2), hereby interacting with nucleic acids presented to endosomal compartments by the Fc γ receptor (3). Putative actions of antimalarial agents involve blocking acid-dependent proteases as shown in (2) or nucleic acid presentation as shown in (3), or both. Upon interaction with their respective ligands, TLRs stimulate the synthesis of IFN type I (4,5). The release of IFN- α (6) has widespread effects on both the innate and adaptive immune systems; most importantly, it stimulates gene expression of TLRs as well as feedback activation of more pDCs, perpetuating a vicious cycle. IFN- α promotes T cell survival, upregulation of the T $_H$ 1 response, proliferation of CD8 $^+$ cells, and suppression of T $_{REG}$ cells (7). IFN- α affects B cells by causing maturation of plasmablasts, immunoglobulin class-switching, and increased antibody secretion (8). IFN- α also stimulates the development of memory B cells and induces BAFF, a B-cell maturation and survival factor. In the presence of immune complexes and interferons, PMNs undergo NETosis and NETs in turn stimulate pDCs—possibly via TLRs (9). Additionally, IFN- α promotes maturation of monocytes into DCs, which are more efficient in antigen processing and presentation. IFN- α further upregulates expression of co-stimulatory molecules and HLA, and stimulates synthesis of IL-10 and IL-12 by DCs. Abbreviations: DCs, dendritic cells; dsDNA, double-stranded DNA; ER, endoplasmic reticulum; IRF, interferon regulatory factor; NET, neutrophil extracellular trap; pDC, plasmacytoid DC; PMN, polymorphonuclear cell; SLE, systemic lupus erythematosus; ssRNA, single-stranded RNA; T $_H$ 1 cell, type 1 T helper cell; TLR, Toll-like receptor; T $_{REG}$ cell, regulatory T cell.



TLR7/9-activation, either by preventing acidification and maturation of the endosomes or by interacting with nucleic acids and thereby preventing TLR-triggering (Figure 8) [21]. The exact mechanisms and effects of chloroquines however remain controversial, and the effect of other TLR-blocking agents needs to be evaluated. In fact, a recent clinical trial evaluating the efficacy of HCQ for main symptoms of pSS concluded that 24 weeks of treatment with HCQ did not improve symptoms compared to placebo. As the authors conclude, indeed further studies are warranted to determine longer-term outcomes of HCQ-use [272]. Interestingly, small-molecule dual TLR7/9-antagonists are currently being tested in SLE patients.

Corticosteroids and certain biologicals can be used to treat systemic disease manifestations [206]. Immunosuppressive drugs such as corticosteroids are effective but limited in use by their well-known, sometimes destructive side effects, and are often used for manifestations such as vasculitis, renal nephritis and neuropathies [268]. Experienced clinicians often state that the use of corticosteroids could possibly do more harm than good, especially in the long run, and advise to always proceed with caution by trying to lower the dose and eventually stopping the treatment as soon as possible. As Robert Fox recently stated “A rheumatologist is a person that can get a patient of steroids”.

The development of targeted therapies specifically addressing disease pathogenesis has been difficult, especially due to the very heterogeneous nature of the disease. Biologicals specifically directed against molecules involved in disease pathogenesis are currently being tested in pSS [273]. Focus has largely been on the B cell compartment, due to high prevalence of auto-antibodies and increased B cell hyperactivity. Observations in lupus-prone mice led to rapid development of biologicals interfering in the BAFF/ APRIL system such as belimumab (anti-BAFF) and atacicept (dual BAFF/APRIL-inhibitor) [274]. Presently, belimumab is tested in human pSS, showing encouraging results. Interestingly, the belimumab in patients with pSS (BELLIS)-trials justifies future studies with BAFF-targeting drugs in the autoantibody positive subset of pSS [275, 276].

Another biological targeting the B cell compartment is rituximab. Rituximab is an anti-CD20 monoclonal antibody, used to treat B cell lymphoma and RA [277]. CD20 is considered to be specific B cell marker, being a highly expressed surface marker of pre B cells and both residing and activating mature B cells [278]. Rituximab is considered relatively well tolerated and effective in a subset of pSS patients [273, 279-283]. Rituximab was effective in reducing subjective and objective symptoms until 24 weeks after treatment, whereas at 48 weeks, symptoms returned to base line [284, 285]. Furthermore, Rituximab was recently shown to predominantly decrease proinflammatory cytokines in pSS [286]. Surprisingly, BAFF levels further increased in pSS after Rituximab treatment, heading caution with the use of this biological [287]. Further studies on combination therapies with anti-BAFF are warranted. The high incidence of ‘serum-sickness’, the variable response, and the return of symptoms, urge for novel evidence-based approaches and illustrate the heterogeneity of the disease. The symptoms of fatigue and reduced well-being

remain largely ignored by clinicians as the tools and experience to treat this component of pSS effectively is lacking. Interestingly, in a randomised, double-blind, placebo-controlled pilot study in pSS with rituximab, reduction of fatigue was observed [288].

Abatacept, a soluble fusion protein consisting of the extracellular domain of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) linked to the modified FC-portion of human IgG1, is a selective costimulation modulator targeting the T cell compartment of the disease [273]. A small pilot study with Abatacept already revealed promising results, showing a significant increase in saliva production and reduced glandular inflammation amongst others [289]. Presently, abatacept is being tested in multiple clinical trials in pSS.

Recently vast interest in development of therapeutic interventions to target the IFN type I signalling pathway are emerging in this field. Thus far however, clinical trials in SLE with anti-IFN α monoclonal antibodies have not been very successful [290-292]. The IFN type I family of cytokines comprise 17 subtypes, all signalling through the common IFNAR. As blocking one single subtype with specific monoclonal Antibodies (mABs) has not shown the promising results as anticipated in SLE, directly blocking the IFNAR might prove more beneficial. Interestingly, mABs against human IFNAR are currently tested for effectivity in SLE and SSc [293, 294], and could potentially be therapeutically beneficial in pSS.

Despite the frequency and clinical relevance of pSS, the development of targeted therapies has lagged behind, which has made the management of the disease relatively difficult. The recent development of more targeted therapies tailored to disease pathogenesis show great promise, however, the heterogeneity of pSS heads caution and calls for more in depth research into specific subgroups of the disease. Overall, use of the readily available treatment modalities as well as novel development of more targeted therapies to target fatigue in pSS amongst others, will aid in increasing the health-related quality of life in pSS patients.

MOUSE MODELS FOR SJÖGREN'S SYNDROME

Mouse models have been widely used to elucidate the pathogenic mechanisms of human diseases. The advantages of using these models include the ability to (I) study different stages of the disease with particular respect to specific target organs, (II) focus on the role of specific pathogenic factors and (III) investigate the effect of possible therapeutic interventions. Murine models are a sophisticated way to model complex pathogenic mechanisms for diseases such as SS, despite discrepancies in the immune system between human and mouse [295]. These models give the opportunity to manipulate disease processes and look at multiple organs in depth during early disease state as well as disease progression, which is particularly difficult to achieve in humans but essential in developing new therapeutic strategies. Ongoing studies in murine models are aimed at developing more effective and targeted therapies in SS. A broader review of interesting mouse models studied in the context of SS and autoimmunity, with particular focus on IFNs, will be given in **Chapter 4** of this thesis.



SCOPE OF THIS THESIS

This thesis describes the toll that pSS patients pay, in the presence of too much IFN. We set out to better understand the role of too much IFN and its contribution to the immunopathogenesis of pSS.

In **chapter 2** the presence of a monocyte IFN type I inducible signature is described, as a tool to sub-divide pSS patients according to high or low IFN type I bioactivity. The monocyte IFN type I signature identifies a subgroup with more active disease. The pSS patients are then stratified according to their IFN signature positivity, into IFN type I positive (IFNpos) and IFN type I negative (IFNneg) pSS subtypes. The assessment of the signature is however a laborious measurement of multiple IFN-inducible gene expression profiles. **Chapter 3** describes the identification of an easy to measure and clinically applicable biomarker for assessing IFN type I bioactivity in pSS – MxA. Multiple assays are compared, revealing a MxA-enzyme immunoassay (EIA) in whole blood lysates as most practical. This MxA-EIA also shows promise for identifying patients eligible for specific therapies, prediction of therapeutic efficacy, as well as potential for monitoring patients longitudinally.

As not only pSS, but also other systemic autoimmune diseases have an IFN-driven component, an addendum of *Chapter 3* shows the broad applicability of MxA as a biomarker for IFN type I activity, not only in pSS, but also in SLE and SSc. A separation of 2 groups is clearly observed for each of the 3 systemic diseases. Here we show that using the MxA-EIA to measure MxA protein levels has high potential to function as a biomarker in a clinical setting. The MxA-EIA could in part be used as a classification tool for the systemic IFN type I signature in autoimmunity, creating more homogeneous subgroups for both basic and clinical research settings. This brings us well along the way towards more individualized, tailor-made therapeutics.

In this thesis we focus our studies mainly on human SS. In **Chapter 4**, however, we review the clinical relevance of the IFN signature in SS-like animal models and provide our perspective on the importance of subdividing pSS patients according to their IFN signature. As the heterogeneity of pSS will most likely benefit from optimizing therapies tailored to specific subgroups of the disease, we provide our perspective on the importance of subdividing SS patients according to their IFN signature, and recommend choosing appropriate mouse models for IFNpos and IFNneg SS-subtypes. Murine models better resembling human-disease phenotypes will be essential in this endeavour.

As pDCs are the main producers of IFN type I, in **Chapter 5** we set out to assess the differences in expression profiles of IFNpos and IFNneg pDCs, compared to that of healthy individuals. Here we find the TLR7 pathway in particular, to be highly upregulated in IFNpos pDCs compared to both IFNneg and HC pDCs. Assessing other cell types such as monocytes we find a full lineage upregulation of the RNA-sensing TLR7 pathway. Furthermore, in depth analysis reveals the RLR family of RNA-sensing receptors to be upregulated in IFNpos pSS, potentially triggered by TLR7.

These RNA-sensing receptors might be collaborating to amplify the pathogenic IFN-driven loop in pSS, and give rise to novel therapeutic targets in pSS.

pSS patients often experience disabling fatigue and depressed mood as their most severe symptom. Part of this thesis aims at better understanding the pathogenic origin of fatigue and depressed mood in pSS. In **Chapter 6** we focus on IDO and the kynurenine pathway, which can rapidly be induced by both type I and type II IFNs. Here we explore IDO enzyme activity and the downstream metabolites, in relation to the IFN type I signature. Also we assess the percentage of Tregs in pSS subgroups, and speculate on possible implications our findings have on symptoms of fatigue and neurotoxicity. We then take it a step further in trying to quantify fatigue in pSS. In **Chapter 7**, levels of fatigue and depressed mood are assessed in relation to the IFN type I signature.

Finally, **Chapter 8** summarizes and discusses the findings of this thesis, with regard to current knowledge in the field, ending with implications for further studies as well as perspectives for the future.

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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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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 \geq 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.

Keywords: Sjögren’s syndrome, Interferon type I, BAFF, ESSDAI

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 mm².

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.

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.

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 \geq 10) and the disease activity scores were compared. Patients with a positive IFN type I signature (IFN score \geq 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).

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).

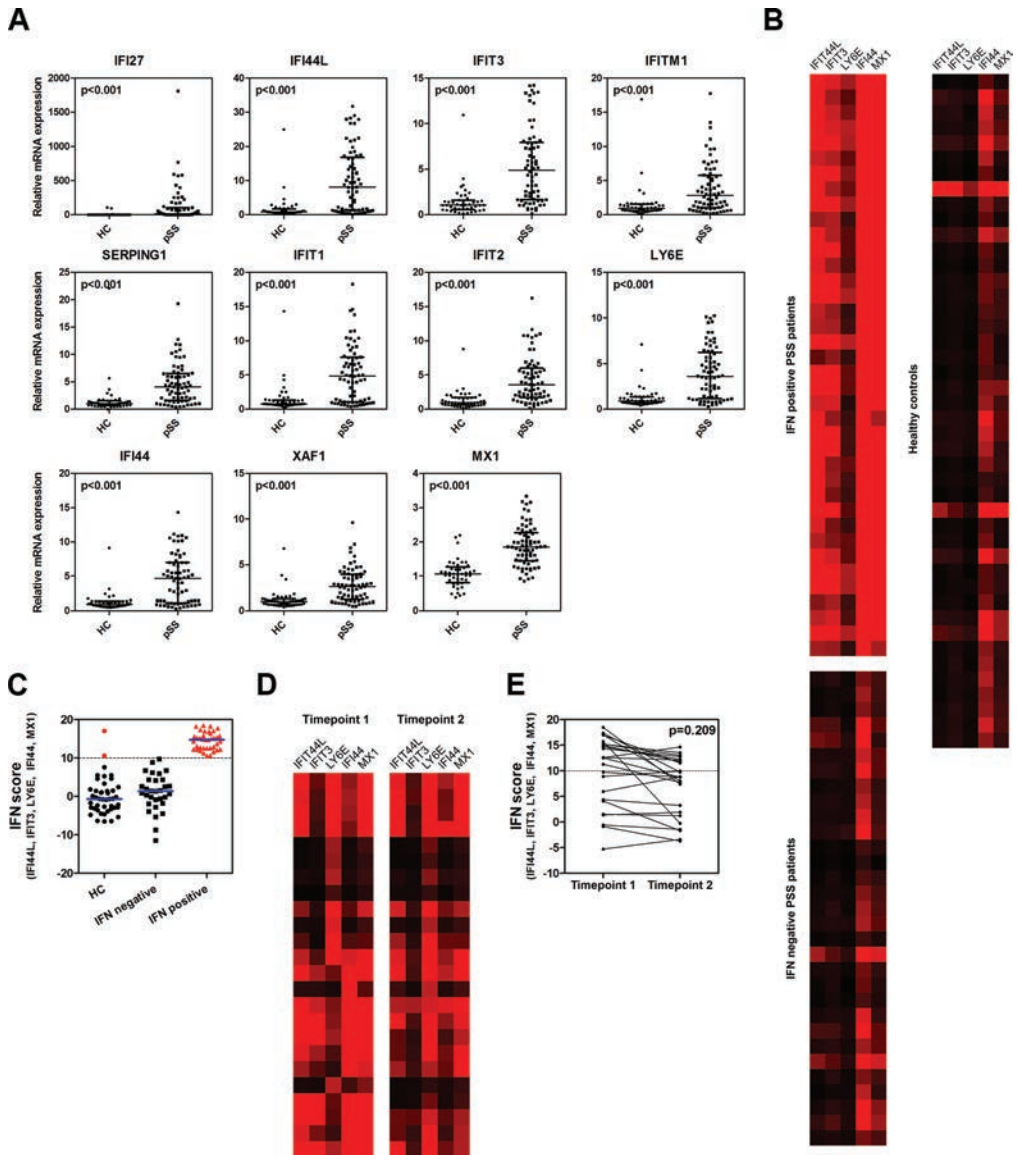


Figure 1. A, Gene expression of 11 IFN type I inducible genes in pSS patients (n=69) and healthy controls (n=44). To compare means the Mann-Whitney U test was applied. B, Heatmap showing gene expression of 5 IFN type I inducible genes in monocytes of pSS patients (n=69) and healthy controls (n=44). On 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.

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

ESSDAI feature	Present		Absent		P
	No. of patients	Median (IQR)	No. of patients	Median (IQR)	
Constitutional	6	6.28 (-1.74,14.92)	32	5.19 (0.10,13.97)	NS
Lymphadenopathy	-		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 nerous system; NS, nonsignificant; ND, not determined.

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).

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
Neutrophils (*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.

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



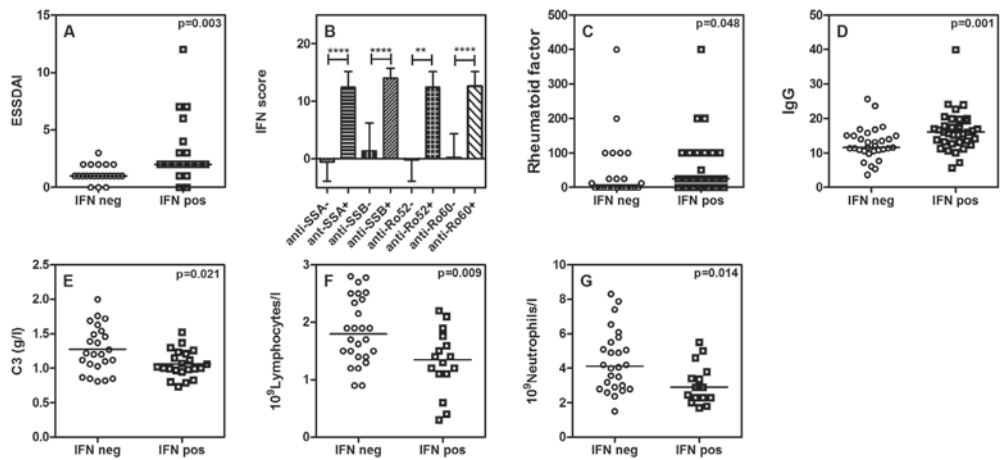


Figure 2. **A**, ESSDAI scores in IFN type I signature positive and negative pSS patients (n=38). **B**, IFN scores in pSS patients positive or negative for anti-SSA (both Ro52 and Ro60) and anti-SSB (n=69). **C**, Rheumatoid factor levels in IFN type I signature positive and negative pSS patients (n=50). **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.

3A). Significant positive correlation between IFN score and BAFF mRNA expression was also thus found (Figure 3B).

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

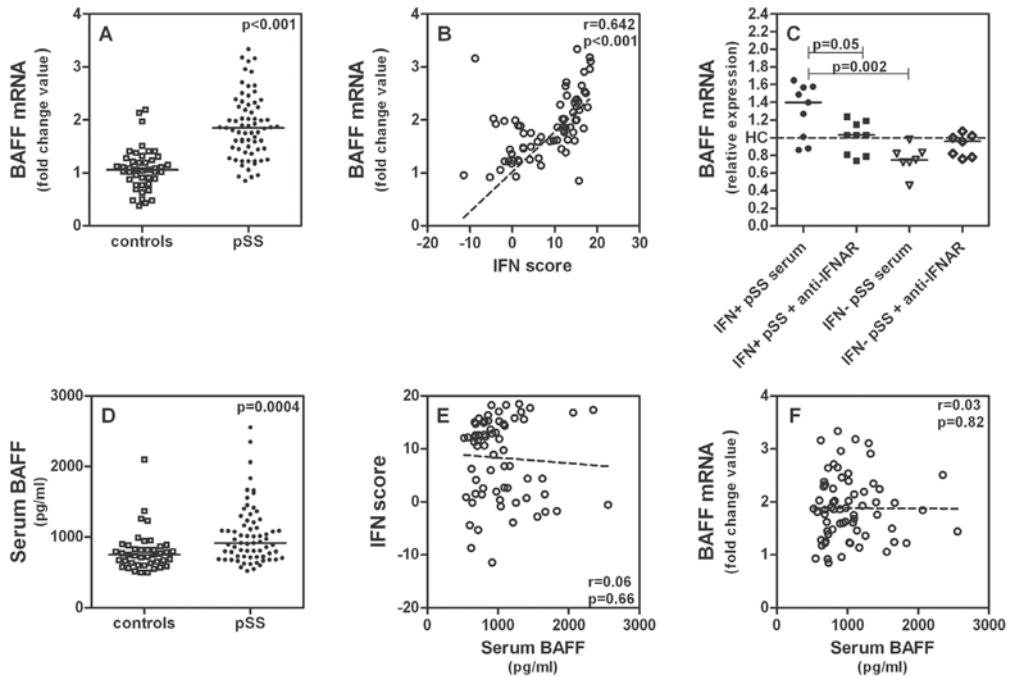


Figure 3. A, BAFF mRNA expression in monocytes in pSS patients (n=69) and healthy controls (n=44). BAFF mRNA expression was determined by RQ-PCR. B, Correlation between IFN type I score and BAFF mRNA expression in monocytes in pSS patients (n=69). C, Induction of BAFF mRNA expression in THP-1 cells by 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.

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.

ACKNOWLEDGMENTS

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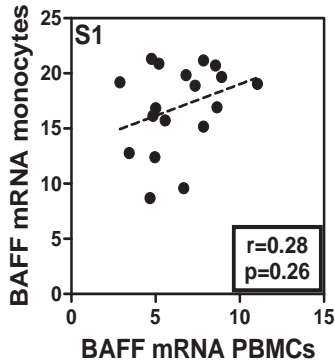
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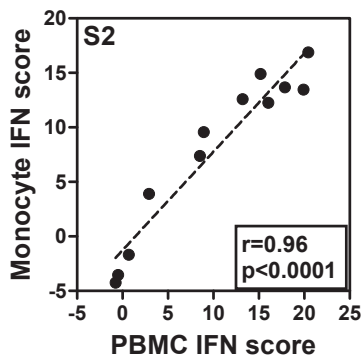


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SUPPLEMENTAL DATA



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.





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Chapter

3

MxA as a clinically applicable biomarker for identifying systemic Interferon type I in primary Sjögren's syndrome

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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.

Keywords: Sjögren's syndrome, Disease Activity, Treatment

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 (Fcγ RI), 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 RNeasy 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

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) ¹	53 ± 11	57(30-85)	55 (37-76)
Disease duration (years) ¹	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)

¹Data are presented as means with corresponding range or SD

pSS, primary Sjögren's syndrome; IFNpos, IFN type I signature positive; IFNneg, IFN type I signature negative; NA, Not applicable

determined from normalized CT values (CT gene – CT housekeeping gene) using the Comparative CT (2- $\Delta\Delta$ CT) method (User Bulletin #2, Applied Biosystems, Foster City, California).

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, absorbance₄₅₀ 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 (rs) or Pearson correlation coefficient (rp) 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.

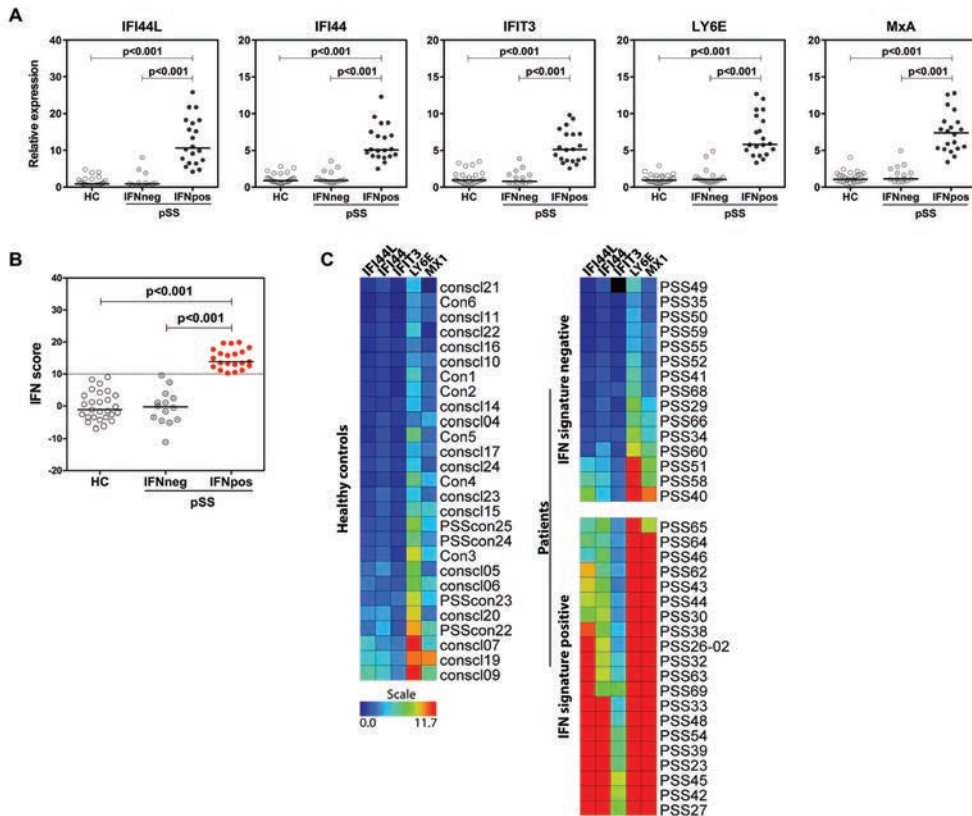


Figure 1. (A) Relative mRNA gene expression is shown for the 5 IFN type I inducible genes of the IFN type I signature in CD14+ monocytes of pSS patients (n=35) and healthy controls (HC) (n=27). Patient group is stratified in patients negative for the IFN type I signature (IFNneg) (n=14) and patients 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).

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).

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

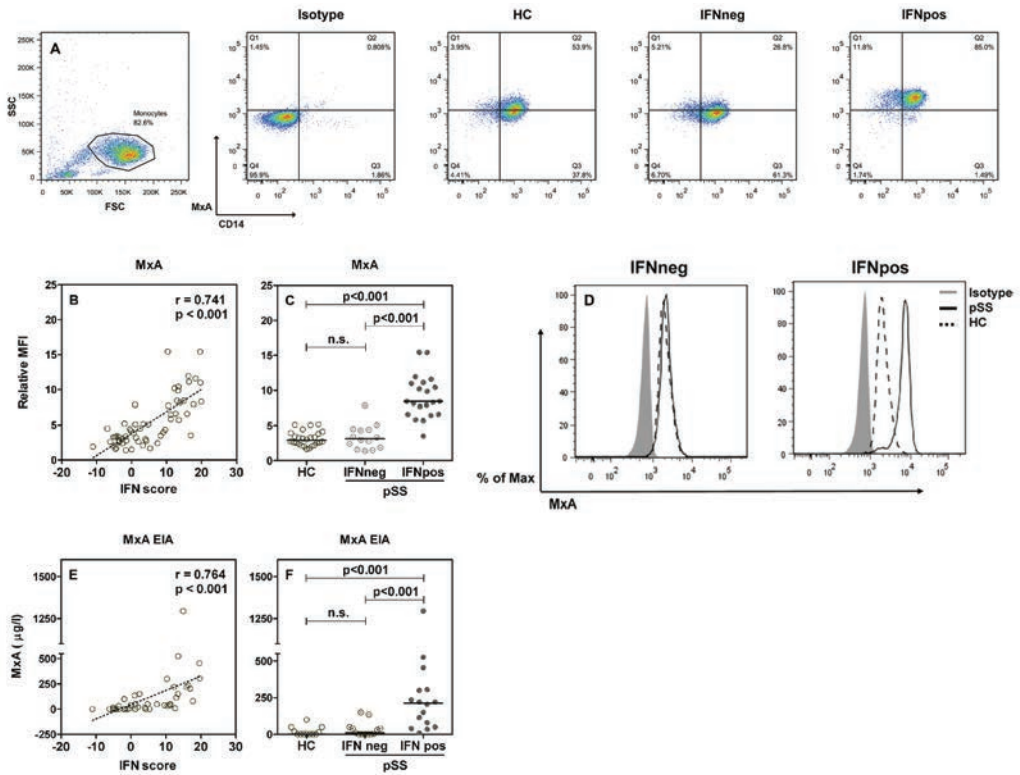


Figure 2. Flow cytometric analysis of MxA protein expression in CD14⁺ monocytes from HC (n=27) and pSS patients stratified in IFNneg (n=14) and IFNpos (n=21) patients. **(A)** Representative dot plots show CD14 and MxA protein expression on monocytes from study subjects. **(B)** Correlation plot shown 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 ($\mu\text{g/l}$) 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 ($\mu\text{g/l}$) levels. **(F)** MxA-EIA ($\mu\text{g/l}$) 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.

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

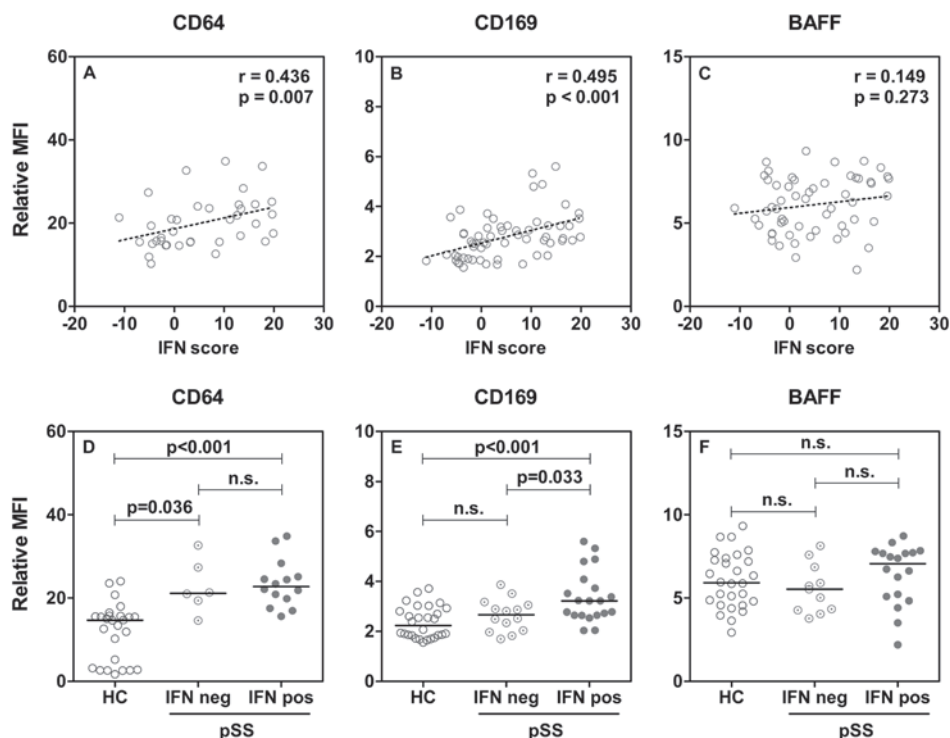


Figure 3. Flow cytometric analysis of CD14⁺ monocytes from HC and pSS patients. Correlation plots shown between IFN scores for all study subjects and (A) CD64, (B) CD169 and (C) BAFF protein levels, respectively. Relative mean fluorescent intensity (MFI) for (D) CD64 (E) CD169 and (F) BAFF protein levels 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.

correlation was strongest when the EIA was used (Table 2, Figure 4B,C). For the other biomarkers no correlation was found (Table S1).

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

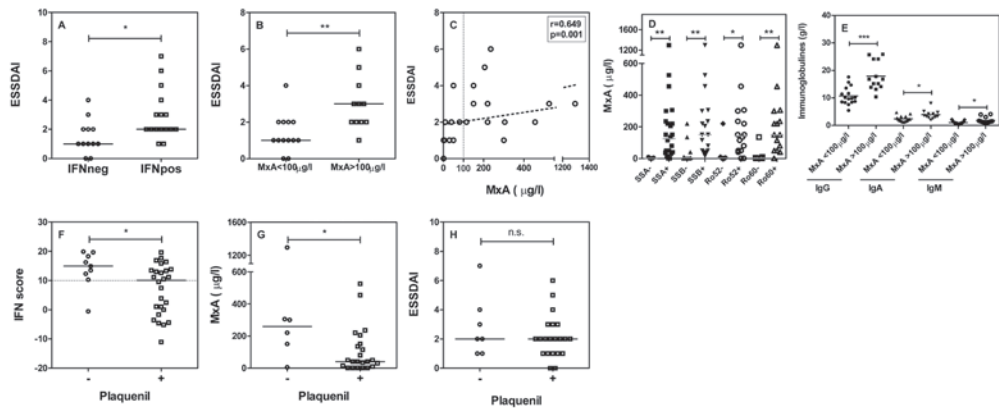


Figure 4. EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores stratified in **(A)** IFNneg and IFNpos pSS patients **(B)** MxA ($\mu\text{g/l}$) levels as measured by the MxA-EIA; MxA<100 $\mu\text{g/l}$ (low) and MxA>100 $\mu\text{g/l}$ (high). Cut-off determined as $\text{mean}_{\text{HC}} + 2 \times \text{SD}_{\text{HC}}$ **(C)** Correlation plot shown between ESSDAI scores and MxA ($\mu\text{g/l}$) levels. **(D)** MxA ($\mu\text{g/l}$) 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<100 $\mu\text{g/l}$ and MxA>100 $\mu\text{g/l}$. 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 ($\mu\text{g/l}$), 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.

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).

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

Variable	MxA-EIA			MxA-Facs		
	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.
Neutrophils (*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 κ^2 test (Z), unless otherwise mentioned

^aData normally distributed are presented as Pearson's rho (r)

Rf, Rheumatoid factor; CRP, C-reactive protein; Hb, Hemoglobin

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 IFNβ, 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 hemoglobin 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 IFN α -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 IFN α -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.

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SUPPLEMENTAL DATA

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.
Neutrophils (*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 κ^2 test (Z), unless otherwise mentioned

^aData normally distributed are presented as Pearson's rho (r)

Rf, Rheumatoid factor; CRP, C-reactive protein; Hb, Hemoglobin

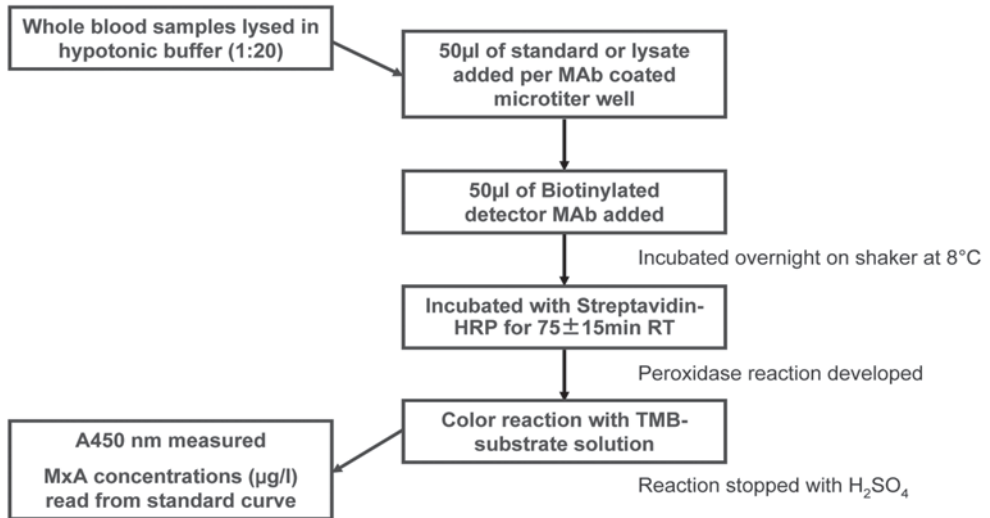


Figure S1. MxA Enzyme immunoassay (EIA). MxA-EIA was executed as previously described with new monoclonal antibodies and recombinant MxA as standard, subsequent to validation for corresponding results [19]. Mouse MABs AFI-7B3 and AFI-10G10 (Institute of Clinical Medicine, University of Eastern Finland) 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-PEO₄-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% NaHCO₃ 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-peroxidase-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) peroxidase substrate solution (Ani Labsystems, Vantaa, Finland) for 10–30 min in the dark. Color development was stopped by adding 100µl of 0.5 N H₂SO₄ and absorbance values were measured at 450 nm using Victor³ 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.

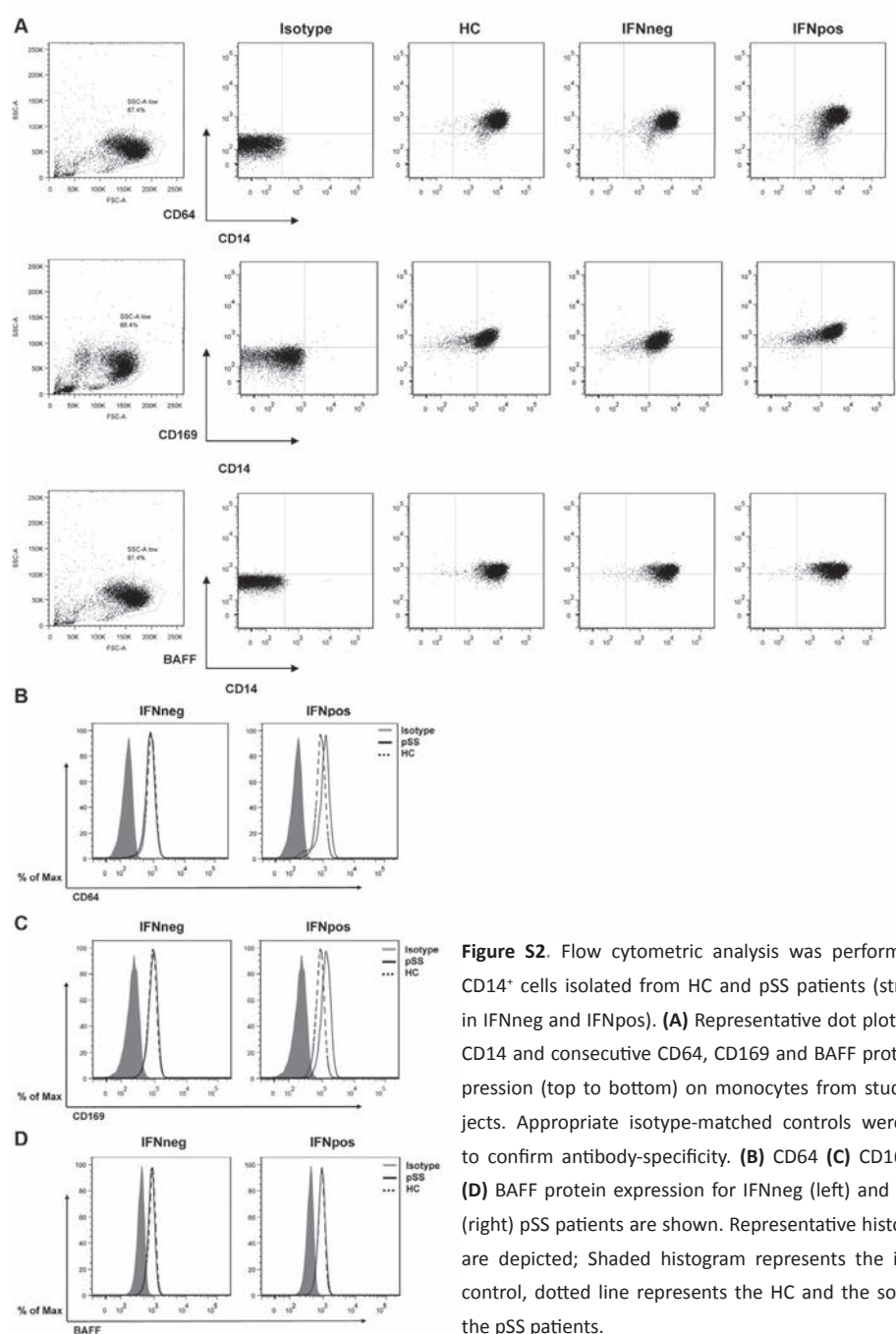


Figure S2. Flow cytometric analysis was performed on CD14⁺ cells isolated from HC and pSS patients (stratified in IFNneg and IFNpos). **(A)** Representative dot plots show CD14 and consecutive CD64, CD169 and BAFF protein expression (top to bottom) on monocytes from study subjects. Appropriate isotype-matched 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.

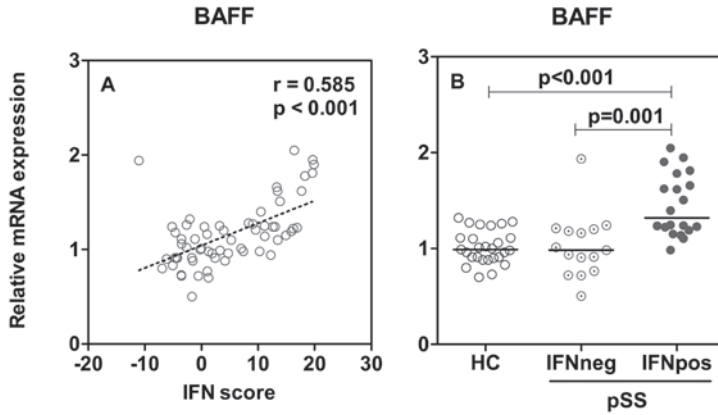


Figure S3. For relative BAFF mRNA gene expression (A) a correlation plot shown with IFNscores for all study subjects and (B) BAFF mRNA gene expression levels stratified in HC (n=27), IFNneg (n=14) and IFNpos (n=21) pSS patients. Each symbol represents an individual sample; horizontal lines represent 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.

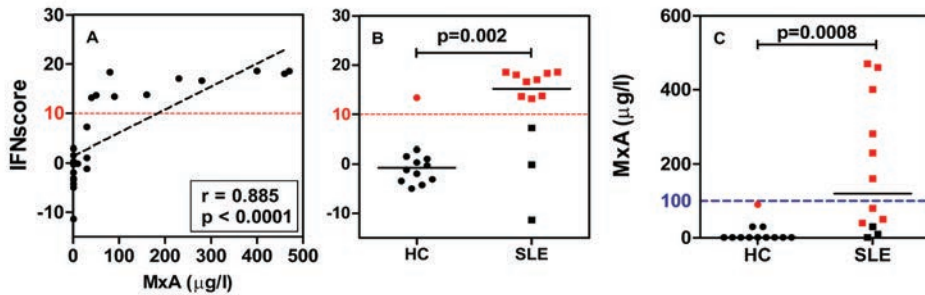


Figure S4. In a small cohort of systemic lupus erythemathosis (SLE) patients (A) a correlation plot is shown between IFNscores (for all age and gender matched study subjects) and intracellular MxA (μg/l) levels, as assessed by the MxA-EIA. (B) IFNscores shown for HC (n=12) and SLE patients (n=12), stratified 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.



2012

Chapter

MxA as a clinically applicable biomarker and classification tool for the systemic Interferon type I signature in systemic autoimmunity: en route to individualized therapy

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Manuscript in preparation: Brief communication

3

Addendum

There is a significant heterogeneity among patients suffering from systemic autoimmune diseases, in clinical phenotype and course as well as in responsiveness to therapy [1]. Researchers in the field have been agreeing, that a better understanding of the components and mechanisms of the IFN signature is essential in systemic autoimmunity. Recent data indicate that the IFN signature can no longer be seen as “the IFN type I signature”, in that it is a broad marker of IFN activity, containing various components or even modules that can be contributed to IFN type I, II and even III, all not mutually exclusive. Hall J. *et al* stated that “Defining whether distinct molecular subgroups exist may facilitate novel disease classification and allow more precise selection of therapy”, which highlights the vast importance of finding disease-related biomarkers for precise subset definition [1, 2].

A recent editorial by Gregersen P. *et al*, on the power of modeling a combination of data from transcriptional blood signatures and distinct clinical profiles, describes that despite early promise, the various approaches to measuring the IFN signature have not yet provided us with a clinically useful biomarker for disease management [3]. In our opinion, we are “en route” to finding these useful biomarkers. We recently described the use of MxA (encoded by Myxovirus-resistance protein gene 1, Mx1) as a promising and clinically applicable biomarker for identifying systemic IFN type I activity in primary Sjögren’s Syndrome (pSS) [4]. In the described study we simultaneously tested four different candidate biomarkers for systemic IFN type I activity in pSS patients (n=35). Several methods for detection of MxA have been previously described [5-8]. We therefore analyzed MxA protein expression using both an enzyme immunoassay (EIA) in whole blood and flow cytometric analyses (FACS) of isolated CD14⁺ monocytes, concluding the MxA-EIA to be the easiest and most reliable of the two assays. Additionally, we showed the potential of MxA in a small cohort of systemic lupus erythematosus (SLE) patients (n=12), where MxA (μg/l), as measured by the MxA-EIA, highly correlated with the SLE-IFN score in these patients (r=0.885, p<0.0001), suggesting the broad applicability of the MxA-EIA in systemic autoimmunity [4].

The MxA-EIA was previously developed by Vallittu *et al*. for monitoring IFN type I bioactivity in multiple sclerosis (MS) patients treated with IFN-β, and considered by the authors to be a faster and more reliable method compared to flow cytometric analysis of MxA [8]. MxA is a key mediator of the IFN-induced antiviral response and is tightly regulated by both type I and type III IFNs [9-12]. A study by Holzinger D. *et al* in 2007, interestingly revealed MxA as a unique marker for the detection of type I and Type III IFN activity during both viral infections and IFN therapy [12]. In systemic sclerosis (SSc), MxA gene expression was previously proposed as biomarker for IFN type I bioactivity and found to correlate with disease activity [7].

The IFN type I signature is present in over half of pSS [13] and SLE [14] patients, and in one third of SSc patients [15], identifying a subgroup with higher disease activity and more progressive disease, respectively. In this concise report, we present the applicability of MxA as a biomarker and classification tool for the IFN type I signature in systemic autoimmunity, using the MxA-EIA in a set of 3 different types of systemic autoimmune diseases: pSS (n=139), SLE

($n=113$), and SSc ($n=30$), compared to healthy controls (HC; $n=78$). We observed MxA levels to be significantly different for all 3 disease groups ($p<0.0001$), compared to HC (see Figure 1). Using a cut-off at $100 \mu\text{g/l}$ (red-dotted line) clearly divides all patients groups into *IFN-low* and *IFN-high*, hereby identifying distinct subgroups.

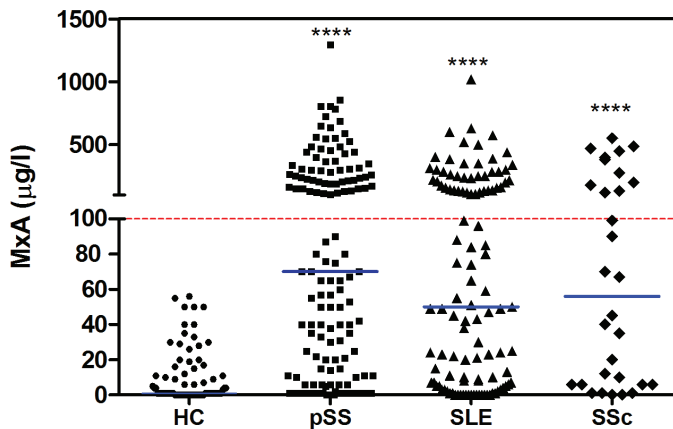


Figure 1. MxA as a biomarker for IFN type I activity in systemic autoimmunity. Here we present an extension of the previously described cohorts of MxA levels in pSS ($n=35$) and SLE patients ($n=12$) [4]. MxA levels ($\mu\text{g/l}$) as determined by the MxA-enzyme immunoassay (EIA) in whole blood lysates of HC ($n=78$), pSS ($n=139$), SLE ($n=113$), and SSc ($n=30$) patients. A cut-off at $100 \mu\text{g/l}$ (red-dotted line) clearly divides all patient groups into *IFN-low* and *IFN-high* subgroups. Disease groups were compared to HC. Each symbol represents an individual sample (median of duplicates of 2-3 separate EIA-measurements per study subject); horizontal blue lines represent the median. To compare medians the Mann-Whitney U test was used (**** $P>0.0001$).

Herein we show that the MxA-EIA is not only an easy and practical tool to assess IFN type I activity in pSS, but also in the systemic autoimmune diseases, pSS, SLE and SSc. Stratifying patients according to their IFN signature positivity has various clinical applications, hereby building the path towards more subgroup-driven, or even individualized therapy in the near future.

Now we need to relate the presence or absence of MxA activity to the clinical status of the patients. We already previously showed the association between MxA protein levels, assessed by the MxA-EIA, and distinct clinical features in pSS [4]. Furthermore, MxA is not only inducible by IFN type I (α , β ...), but also by IFN Type III (λ). This assay does not discriminate between these 2 IFN types, and the contribution of IFN type III to the IFN signature remains to be established for these disease groups. As recently described, there are distinct IFN signatures present in pSS and SLE, some patients displaying only a type I phenotype, others only a type II (γ) and

some displaying a dual phenotype (I/II) [1, 2, 15, 16]. Evidently, further studies are warranted to determine the feasibility of MxA in distinguishing different IFN pheno-types, whereas a feasible marker for IFN type II needs to first be established.

This study shows the broad clinical applicability of MxA as a biomarker for IFN activity, and its feasibility as a classification tool for identifying the IFN type I signature in the systemic autoimmune diseases, pSS, SLE and SSc. Its power lies in the practicability, in solely requiring 25µl of whole blood obtained during routine blood collection and directly stored without necessity of further processing up to time of assessment.

Defining these distinct subgroups will facilitate disease classification, paving the way for more precise and selective therapies. Here we propose MxA as a tool to monitor therapeutic efficacy during IFN-targeted therapies. Clearly further research is warranted to determine the applicability of MxA as a biomarker in disease prognostics, diagnostics and therapeutics, these features being essential for each of the discussed systemic autoimmune diseases with IFN type I involvement.

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Chapter

4

The clinical relevance of animal models in Sjögren's syndrome: the Interferon signature from mouse to man

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ABSTRACT

Mouse models have been widely used to elucidate the pathogenic mechanisms of human diseases. The advantages of using these models include the ability to (I) study different stages of the disease with particular respect to specific target organs, (II) focus on the role of specific pathogenic factors and (III) investigate the effect of possible therapeutic interventions.

Sjögren's syndrome (SS) is a systemic autoimmune disease, characterised by lymphocytic infiltrates in the salivary and lacrimal glands. To date, effective therapy is not available and treatment has been mainly symptomatic. Ongoing studies in murine models are aimed at developing more effective and targeted therapies in SS.

The heterogeneity of SS will most likely benefit from optimizing therapies, tailored to specific subgroups of the disease. In this review, we provide our perspective on the importance of subdividing SS patients according to their interferon (IFN) signature, and recommend choosing appropriate mouse models for IFNpositive and IFNnegative SS-subtypes. Murine models better resembling human-disease phenotypes will be essential in this endeavour.

Keywords: Sjögren's syndrome; autoimmunity; mouse models; Interferon type I; Interferon signature; subgrouping; Non-obese diabetic mouse

INTRODUCTION INTO SJÖGREN'S SYNDROME

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by lymphocytic infiltrates in salivary and lacrimal glands, sialadenitis and dacryoadenitis respectively. The disease can occur alone - primary SS (pSS) - or together with other systemic autoimmune diseases such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc) or rheumatoid arthritis (RA) - secondary SS. The prevalence of pSS is estimated between 0.05-1%, with a nine fold predominance in females. Characteristic symptoms are dry eyes (keratoconjunctivitis sicca) and mouth (xerostomia), with frequent presence of multiple extraglandular manifestations, such as vasculitis, severe fatigue and multi-organ involvement [1-3]. At present, no common evidence-based intervention therapy is available and treatment is mainly symptomatic. Thus, further unravelling the pathophysiology of pSS is essential for finding novel biomarkers and identifying new treatment targets.

Murine models are a sophisticated way to model complex pathogenic mechanisms for diseases such as SS, despite discrepancies in the immune system between human and mouse [4]. These models give the opportunity to manipulate disease processes and look at multiple organs in depth during early disease state as well as disease progression, which is particularly difficult to achieve in humans but essential in developing new therapeutic strategies. Recently, an extensive update on SS-like murine-models was published in this journal [5].

Here we focus on the present knowledge of the pathogenesis of human pSS with special regard to the interferon (IFN) signature. As IFN type I is a suggested key pathogenic factor, we discuss how the currently used mouse models fit with subdividing patients into IFNpositive and IFNnegative subgroups. Taking this subdivision as a starting point we also recapitulate relevant disease features and interventional studies in the non-obese diabetic (NOD) mouse model, which is the most commonly used SS mouse model.

SJÖGREN'S SYNDROME: FROM MAN TO MOUSE

Over the past decade, vast evidence for a role of IFN type I in the pathogenesis of pSS has emerged. We as well as others, have described the presence of an IFN type I signature, assessed as upregulation of a distinct set of IFN type I inducible genes [6-12]. We describe the prevalence of the systemic IFN type I signature in over half of pSS patients (referred to as IFNpositive pSS), identifying a subgroup of patients with higher EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores, presence of anti-SSA (Ro52&60) and/or -SSB (La) autoantibodies, higher immunoglobulin (Ig) G and B-cell activating factor (BAFF)-levels, and lower C3 complement levels [10]. The abundant presence of autoantibodies leading to circulating immune complexes (IC), is thought to be the main trigger inducing the IFN signature in pSS and SLE [13].



In addition to IFN type I, novel evidence indicates IFN activation in SS glands to be partly attributed to IFN type II activity (IFN γ) [14]. Whether this also holds true for systemic IFN activation in pSS, remains to be established. A recent study in SLE however, revealed distinct systemic type I and type II signatures [15]. In figure 1 we depict the multifactorial pathogenesis of pSS, where IFNs are centered as the main culprits in the self-amplifying pathogenic loop. Better understanding these distinct IFN pathways is crucial in selective therapeutic targeting.

Here we provide our perspective on the importance of subdividing pSS patients according to their IFN signature. In order to better resemble the human situation, we will need to find models best fitting our subdivision, where one can be used as an IFNnegative model and the other, possibly accelerated by triggering the IFN-system, can be used as its IFNpositive counterpart.

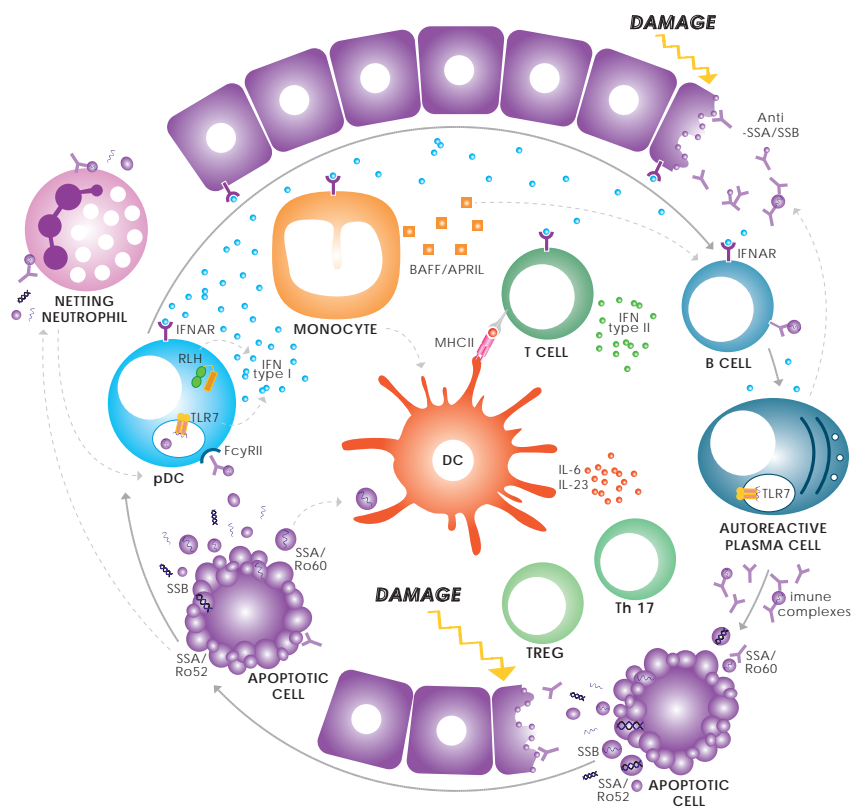
IFN-RELATED MURINE MODELS AS IFNPOSITIVE SS

As IFNs play an eminent role in human pSS pathogenesis, we studied the NOD mouse model for the presence of systemic IFN activation and found no indication for the presence of a systemic IFN signature [McGuinness B, Beumer W *et al.*, unpublished work]. We therefore suggest the NOD mouse to be systemically IFNnegative, and set out to look at some previous studies in murine models for a possible IFNpositive counterpart. Many systemic autoimmune models used for SLE, amongst other diseases, extensively direct their attention to IFNs and their role in autoimmune development [13, 16-18]. Here we highlight existing autoimmune murine models, which are in our opinion models to be revisited for potential SS-like disease.

IFN-inducible models in murine autoimmunity

External triggering of the IFN system in order to mimic systemic IFN overactivation is an approach often used in autoimmune-prone mice. Interestingly, the group of Deshmukh *et al.* applied this strategy to the lupus-prone New Zealand Black/White F1 (NZB/W F1) mouse strain to study SS-like disease. After treatment of the mice with the Toll-like receptor (TLR)3 agonist poly(I:C), chronic systemic activation accelerated sialadenitis [19]. Interestingly in addition to TLR3, poly(I:C) also engages the nucleic acid sensors RIG-I (retinoic acid-inducible gene I; DDX58) and MDA5 (melanoma differentiation associated antigen 5; IFIH1). These RIG-like helicases have recently been suggested to collaborate with endosomal TLRs in amplifying IFN overactivation (see Figure 1) [20-22], and thus might also contribute to overactivation of IFN in the poly(I:C) treated mice. RIG-like helicases might be relevant therapeutic targets.

New Zealand Mixed (NZM) 2758 were additionally demonstrated to develop SS-like disease, after prior triggering of innate immunity by alum and induction of antibodies by immunization with Ro52. This recent study focused on Ro52-induced salivary gland dysfunction and



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hypothesized that autoantibody deposition in the glands might be crucial to induce xerostomia and SS-like disease [23]. However the role of IFNs in disease induction was not assessed.

Imbalanced Toll-like receptor signalling leads to murine autoimmunity

For the generation of autoantibodies by B cells and immune complex (IC)-mediated IFN production by plasmacytoid dendritic cells (pDCs), myeloid differentiation primary-response gene 88 (MyD88)-dependent endosomal TLR7 and TLR9 are crucial. Nucleic acid-sensing TLRs such as TLR3, TLR7, TLR8 and TLR9 are located in intracellular endosomal compartments, consequently minimizing possible exposure to self-antigens [24]. Intriguingly, our group recently found that the TLR7 pathway was upregulated in peripheral blood cells of IFNpositive pSS, whereas TLR9 was not [Maria N.I. et al.; manuscript in preparation]. A similar imbalance in TLRs was observed in murine lupus models. Opposing effects were described for TLR7 and TLR9: deletion of TLR7 limited autoimmunity, whereas TLR9 deletion paradoxically exacerbated disease. TLR7 deletion prevented RNA-associated antibody formation, whereas TLR9 deletion resulted in increased systemic inflammation and IC-induced glomerulonephritis [18]. That controlling TLR7 expression is essential in restricting autoimmunity already became clear when TLR7 gene duplication was demonstrated to be the sole requirement for accelerated autoimmunity in B6.Yaa mice. A substantial TLR7 increase even caused fatal acute inflammatory pathology and extensive DC dysregulation [16]. This mouse model, portraying the Yaa phenotype (TLR7 gene duplication; BXSb/MpJ-Yaa), was recently described to develop autoimmune dacryoadenitis in a study focusing on SS-like features [25], showing ample reason for revisiting the herein mentioned autoimmune models for SS.

The imbalance in endosomal TLRs, which results in much more prominent lupus-like disease compared to wild type (WT)-mice, was also recently shown using mice deficient in TLR8 and/or 9. TLR8 deficient mice (B6.TLR8^{-/-}) and particularly double TLR8/9 deficient mice (B6.TLR8/9^{-/-}) displayed marked induction of TLR7, which was associated with more severe disease. This study concludes that both TLR8 and TLR9 act together in controlling TLR7 function, TLR8 particularly controlling TLR7 function in DCs and TLR9 restraining TLR7 response in B cells [17].

Other potential TLR-related models to be revisited are reviewed elsewhere [13], elaborating on possibilities for exacerbated renal disease in TLR9^{-/-}, versus reduced disease in their TLR7^{-/-} counterpart. TLR9-deficient autoreactive B cells no longer undergo class switching to pathogenic immunoglobulin isotypes (IgG2a and IgG2b), these TLR9^{-/-} mice having significantly smaller IgG deposits in the glomeruli and a prolonged survival compared with their TLR9 sufficient littermates. In contrast, TLR7 deficient mice no longer produce RNA-specific autoantibodies, developing less severe clinical disease than their TLR7 sufficient littermates [13].

Further investigating this imbalanced endosomal TLR-signaling in IFN-driven pSS is warranted as most studies focus on SLE. The above mentioned murine models will be crucial in this endeavor, as the tight regulation of TLR7 is preventing autoreactivity leading to autoimmunity.

TLR inhibitors in IFN related autoimmunity

The antimalarial agent hydroxychloroquine (HCQ) has long been considered an effective treatment for SLE and is frequently used for pSS. Chloroquines are said to block TLR7/9-activation either by preventing acidification and maturation of the endosomes or by interacting with nucleic acids and thereby preventing TLR-triggering. The exact mechanisms and effects of chloroquines remain controversial (reviewed in [24]), and the effect of other TLR-blocking agents needs to be evaluated. In fact, a recent clinical trial evaluating the efficacy of HCQ for main symptoms of pSS concluded that 24 weeks of treatment with HCQ did not improve symptoms compared to placebo. As the authors conclude, indeed further studies are warranted to determine longer-term outcomes of HCQ-use [26].

In a recent study, NZB/W F1 mice were treated with a TLR7/8/9-antagonist. Treated mice had lower serum autoantibody levels, reduction in proteinuria and kidney histopathology compared to their untreated counterparts [27]. Interestingly, small-molecule dual TLR7/9-antagonists are currently being tested in SLE patients, however, selective TLR7 inhibition might be a more promising approach and should, in our opinion, be evaluated in SS-like murine models for therapeutic efficacy.

Targeting IFNs and the IFNAR

The IFN type I family of cytokines comprises 17 subtypes, all signalling through a common receptor, the IFNAR (IFN α , β -receptor). Blocking one single subtype with specific monoclonal Antibodies (mABs) has not shown the promising results anticipated in SLE trials, thus directly blocking the IFNAR might prove more beneficial. This approach is supported by a study showing that deletion of *Ifnar1* prevented severe disease in at least two lupus-prone strains. Interestingly, mABs against human IFNAR are currently tested for effectivity in SLE and SSc [28, 29]. Recently, B6.IFNAR^{-/-}-mice and WT mice triggered with IFN-inducing poly(I:C) were tested for salivary gland hypofunction. Loss of glandular function was evident in WT mice and limited in IFNAR^{-/-}-mice thus indicating a crucial role for IFN type I in xerostomia [30]. This points towards possible therapeutically beneficial effects in blocking the IFNAR in pSS.

Type I and type II IFNs signal via different receptors but share overlapping patterns of activated genes downstream. A recent study in human salivary gland tissue provided evidence that IFN type II (i.e. IFN γ) also contributes to the IFN signature in SS glands, and therefore the role of IFN γ needs to be further investigated in humans and mice. Interestingly, Ro60 peptide immunized Balb/c mice significantly developed increased levels of IFN γ which correlated to decreased salivary flow [31], but the effect of increased IFN γ on a systemic IFN signature remains to be analyzed.



Autoantibodies and the BAFF/APRIL system in murine autoimmunity

IFN type I itself has vast effects on B cell survival, possibly perpetuating the pathogenic loop. IFNs induce BAFF- and APRIL- (A proliferation inducing ligand) expression in monocytes, hereby contributing to antibody-producing plasma cell survival resulting in prolonged pathogenic auto-antibody production. This further triggers IFN-signalling as well as increased IC deposition in target tissues leading to chronic inflammation, damage, and ultimately loss of function [32]. A recent study looking into the necessity of individual BAFF-receptors BCMA, TACI and BR3 in receptor-deficient NZM 2328 mice concluded any single BAFF-receptor to be dispensable for lupus-development in their model [33]. An earlier study by the same group however, found that BAFF-deficient NZM 2328 mice were largely spared from clinically overt disease. These mice only showed serological autoimmunity and renal pathology, whereas severe proteinuria and mortality were greatly reduced. Blocking BAFF might thus not fully reverse or eliminate underlying pathology, but seemingly leads to substantial clinical improvement in murine autoimmunity [34].

The study by Mackay and colleagues illustrates that our view on revisiting lupus-prone mouse models for SS pathology is a rewarding approach. In BAFF transgenic mice, considered a model for SLE, they observed SS-like disease characterized by severe sialadenitis, decreased saliva production and destruction of submandibular glands [35].

Supporting an important role for autoantibodies in pSS is a recent study performed in mice lacking the SLE- and pSS autoantigen Ro52/TRIM21. After local damage induction by ear tagging, Ro52/TRIM21^{-/-} mice develop a lupus-like phenotype. Ro52/TRIM21 interacts with IFN regulatory factors (IRFs), that play a role in tightly regulating IFN-signaling. In these mice, cellular damage drives pathology via potential triggering of endosomal TLRs through enhanced IFN production [36]. Ro52-targeted autoantibodies produced by autoreactive B cells might be interfering with the important regulatory role of Ro52/TRIM21 in maintaining balanced IFN-signaling.

Biologicals targeting the BAFF/APRIL system

Observations in lupus-prone mice led to rapid development of biologicals interfering in the BAFF /APRIL system such as Belimumab (anti-BAFF) and Atacicept (dual BAFF/APRIL-inhibitor) [37]. Treatment of mice with rapidly progressive glomerulosclerosis in both early and late stages of disease with BAFF-Ig or TACI-Ig, revealed that selective BAFF-blockade was sufficient to both prevent disease development and progression. This led to the conclusion that both treatments were equally effective in retaining remission by prolonged B cell depletion and a decrease in inflammatory response to renal IC deposition [38]. However when disease was accelerated by IFNs, BAFF blockade only proved beneficial in the initiation phase and did not prevent progression once autoantibodies were present. These biologicals might not be sufficiently effective in later disease stages in autoantibody-positive patients and additional therapies targeting the pathways activated by IFN might be essential additives. Presently, belimumab is tested in human

pSS, showing encouraging results. The belimumab in patients with pSS (BELLIS) trials justify future studies with the BAFF-targeting drug in the autoantibody positive subset of pSS [39, 40].

THE NOD MOUSE MODEL AS IFNNEGATIVE SS

Non-obese diabetic (NOD) and NOD-derived strains as spontaneous models for SS

Apart from spontaneously developing diabetes, female NOD mice recapitulate typical SS-like symptoms such as decreased salivary flow and lymphocytic infiltrates in salivary glands [41, 42]. In contrast to inducible models for autoimmunity, NOD mice follow a pattern of initial morphological changes in the salivary glands prior to the onset of focal infiltration and manifestation of clinical symptoms at about 16 weeks of age [43, 44]. Furthermore, the disease profile in NOD mice resembles human SS concerning composition of infiltrates in salivary glands and partially in terms of the autoantibody profile [45]. Additionally, NOD mice develop lymphocytic infiltrates in the lacrimal glands and interestingly, this dacryoadenitis develops more frequently in males. Microarray analysis on lacrimal glands of male NODs resulted in the identification of Cathepsins as candidate biomarkers for SS [46, 47]. Initial infiltrating immune cells were found to be responsible for this increased cathepsin expression, thereby initiating lacrimal gland remodeling and degradation [48]. Interestingly, cathepsin expression increased in parallel with proinflammatory cytokines during autoimmune development in the male NODs [46]. In general however, studies on lacrimal glands in NOD mice are rare and truly deserve more attention.

Autoimmune manifestations in NOD mice develop through a complex interplay of several factors composed of genetic predisposition and intrinsic immune dysfunctions which manifest under the influence of environmental conditions [49]. NOD mice develop diabetes in a major histocompatibility complex (MHC) class II dependent manner prior to autoimmune exocrinopathy. Changing MHC class II haplotype can protect NOD mice from developing diabetes but not SS-like disease, and affect the severity of sialadenitis [50, 51]. Association of SS with genes encoding for human leukocyte antigen (HLA) has been reported [52], and thus indicate the importance of MHC-haplotype.

Although T cells are the dominant type of lymphocytes found in the infiltrates, SS is thought to predominantly be a B cell-mediated disease. NOD-Igμ^{null} mice, which lack functional B cells, show the typical lymphocytic infiltrations in the salivary glands but do not develop hyposalivation until transfer of purified human SS-IgG or parental NOD-IgG, suggesting a crucial role for antibodies in an overt clinical stage of the disease rather than the initial phase of lymphocyte infiltration [53].

Transient depletion of regulatory T cells (Tregs) in NOD mice (performed at the age of 10 days) showed an accelerating effect on sialadenitis, while depletion in older mice did not



influence sialadenitis [54]. Moreover, depletion of Tregs in B cell-deficient^{-/-} NOD mice reversed resistance to an autoimmune phenotype and increased both the presence and size of salivary gland infiltrations compared to WT NOD mice [55]. These findings underline the importance of a balanced regulatory- and effector-cell population in different stages of disease. Nevertheless, how depletion of Tregs effects exocrine gland dysfunction remains to be established as the grade of infiltration does not correlate with actual dryness. This important point must be realized, as there is also no correlation between gland function and grade of infiltration in humans. Therefore studies combining analysis of gland function and infiltration pattern are warranted.

The development of diabetes and SS-like disease in NOD mice is accepted to occur as two separate events, after identification of Idd3 (also termed autoimmune exocrinopathy 1, Aec1) and Idd5 (also termed autoimmune exocrinopathy 2, Aec2) as the genetic risk regions being sufficient for manifestation of exocrine dysfunction in NOD mice [56]. Introduction of Idd3 and Idd5 loci from NOD in the non-autoimmune prone C57BL/6 strain resulted in the so-called C57BL/6.NOD-Aec1Aec2 strain, which develops SS-like disease but not diabetes. Comparable with human SS, SS-like disease in C57BL/6.NOD-Aec1Aec2 mice is initiated by a pre-clinical silent phase before the onset of overt disease [57, 58]. A study comparing human SS parotid gland tissue with salivary glands of Aec2/Aec2 mice revealed common dysregulated pathways - associated with leukocyte recruitment and germinal center formation [59] - underlining the relevance of the model for testing novel therapeutics.

The IFN signature in NOD and NOD-derived strains

As described above, upregulation of IFN inducible genes termed as the IFN signature has been observed both in target tissue and systemically in pSS patients [10, 60]. However, it is important to realize that observations in salivary glands do not imply that an IFN signature is systemically present.

The comparison of salivary gland IFN signatures in murine SS-like disease and human-SS has been recently reviewed and clearly reveals expression of IFN type I induced genes in human pSS and NOD salivary glands [61, 62]. Interestingly, microarray analysis of male NOD lacrimal glands did not reveal increased IFN type I expression [46]. However, it is debatable whether data from murine glands can be compared to human peripheral blood studies. It is thus peculiar that most murine SS-like studies are focused on the IFN signature in the exocrine glands, fully disregarding the systemic aspect of the disease. We evaluated the gene expression patterns of NOD monocytes at various time points of disease development and found no evidence for systemic IFN type I activation [McGuiness B, Beumer W *et al.*, unpublished work]. Hence, it should be mentioned that studies in human pSS simultaneously analyzing the IFN signature in salivary glands and peripheral blood are still lacking. Therefore, there may well be pSS patients with local IFN type I activation in the glands but lacking systemic IFN type I activity, similar to NOD mice.



The pathogenic effect of IFN type I was assessed in C57BL/6.NOD-Aec1Aec2 mice deficient for *Ifnar1* (B6.Aec1Aec2*Ifnar1*^{-/-}), thus unable to respond to IFN type I-mediated signaling [63]. B6.Aec1Aec2*Ifnar1*^{-/-} mice did not upregulate IFN-responsive genes in the submandibular glands in comparison to their WT counterparts, although only a limited number of targets were tested in this study. Most strikingly, these mice were protected from salivary gland dysfunction and showed reduced infiltration in salivary and lacrimal glands, even though they still generated a robust systemic autoantibody response. Taken together, these findings support the hypothesis that systemic and local IFN activation can occur as two separate events.

Although type I IFNs were initially thought to be the driving force for the IFN signature, both IFN type I and type II activity was detected in human pSS salivary gland biopsies [14], while in pSS a contribution of type II to the systemic IFN signature remains to be established. In submandibular glands of NOD mice it has clearly been demonstrated that IFN type II/γ participates in the early onset of SS-like disease. *NOD.IFNγ*^{-/-} and *NOD.IFN-γR*^{-/-}-mice fail to develop sialadenitis, though retaining lymphocytic infiltrates in the lacrimal glands [64]. Nevertheless, to date these findings remain limited to local similarities. Thus, although there are many overlapping features between human pSS and NOD mice, evidence for a systemic IFN type I signature as observed in more than 50% of the human pSS patients is lacking in NOD mice. We conclude that NOD and NOD-derived strains rather represent the subset of systemically IFNnegative SS patients, and could contribute significantly to insights into the pathogenesis of IFNnegative pSS patients.

Interventional studies in NOD and NOD-derived strains

Over the past years, several intervention studies were performed on NOD or NOD-derived models, most of them using C57BL/6.NOD-Aec1Aec2 as a model for SS. Due to space limitations, we selected from the large number of studies, those that are tested in clinical trials or are in our view interesting candidates for translation into clinics.

Development of malignant lymphomas is a risk in pSS and therefore studies on mechanisms of malignant transformation are important. Lymphoid structure formation was found to be predictive for lymphoma development in human pSS [65]. To evaluate the inhibitory effect on lymphoid structure formation in exocrine glands of NOD mice, blockage of the lymphotoxin-beta receptor (LTβR)-pathway by injection of LTBR-Ig was performed. LTBR-blockage ablated lymphoid structures and reduced salivary gland tissue degeneration and lacrimal gland pathology, whereas salivary flow was partially restored [66, 67]. Nevertheless, injection of LTβR-Ig was performed in an early disease stage, and thus the effect on disease progression when administered in a late, overt disease phase remains to be tested.

The manipulation of co-stimulatory mechanisms by local gene therapy gave less conclusive results. Attempts to inhibit CD40-ligation in NOD mice did not alter SS-like disease at all [68], whereas CTLA-4-IgG expression in C57BL/6.NOD-Aec1Aec2 mice improved sialadenitis and salivary gland function [69]. Presently CTLA-4-IgG is tested in clinical trials.

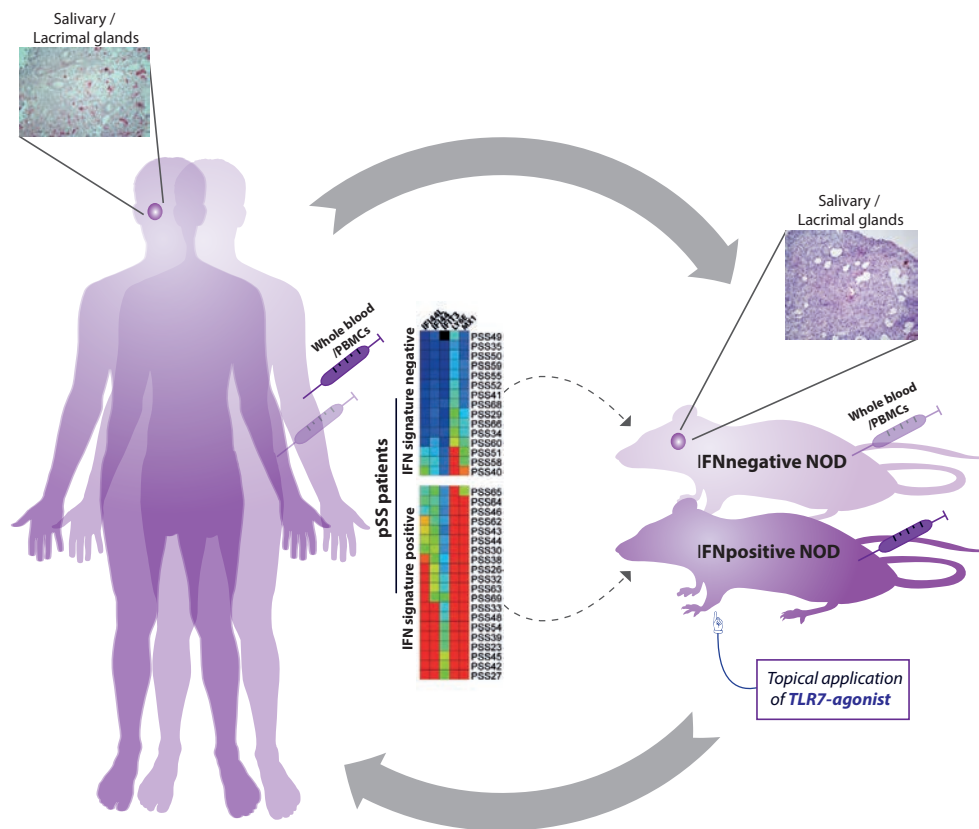
Several gene therapy-based studies targeting different SS-related cytokines were performed. In C57BL/6.NOD-Aec1Aec2 mice, targeting of interleukin (IL)-17 production by T-helper (Th)17 cells locally in the SGs led to improved SS-like disease, independent of disease-state at administration [70]. Accordingly, systemically administered gene-therapy using IL-27 to inhibit Th17-activity was performed in C57BL/6.NOD-Aec1Aec2 mice in early and advanced disease stages. The treatment did not alter lymphocytic infiltration but resulted in less severe clinical disease manifestation [71]. Overall, targeting Th17 networks might be a useful treatment option.

Recently the role of the cytokine IL-7, elevated in human-SS, has been analyzed using C57BL/6.NOD-Aec1Aec2 mice. IL-7 was found to enhance the Th1-response and promote the development of SS-like disease, whereas IL-7 blockade had a disease ameliorating effect. Furthermore, IL-7 was found to influence Th1-responses via IFN γ -activation [72]. Interestingly, IL-7 and TLR7 seem to act synergistically on T and B cell activation. Considering the importance of TLR7 activation in autoimmunity, IL-7 should be considered a future candidate for intervention studies [73]. Besides TLR7, TLR9 seems to be involved in the pathogenesis of SS-like disease in NOD mice. Activation of TLR9-dependent p38MAPK was recently found to occur in an early disease state [74]. However, agonistic TLR9 treatment, that activated the alternative NF κ B pathway, increased salivary flow [75]. These findings support a crucial role for the TLR7/9 balance, where increased TLR9 signaling potentially salvages autoimmunity, whilst a tilt towards TLR7 signaling actually exacerbates autoimmunity.

Taken together, it is important to note that the stage of SS-like disease in NOD mouse models strongly influence the outcome of different therapeutic interventions. The majority of treatments tested in mice were performed in an early disease stage. Intervening when overt disease is present in relevant animal models is required before these approaches are applicable in humans. Besides, human SS is mostly diagnosed in an overt phase.

PERSPECTIVES: FROM MOUSE TO MAN

When drawing conclusions from mouse models for clinical relevance in humans a proper understanding of both the similarities and differences in immune function between mouse and man remains crucial for adequate interpretation of mouse studies [4]. There is ongoing controversy when using murine models regarding appropriate extrapolation of obtained knowledge into a clinical setting. We therefore propose the best strategy to be “a back and forth interplay between mice and men”. Revisiting existing mouse models in a more disease-related way certainly might help to elucidate underrated aspects of the disease. In particular lupus-prone models that can be used for SS might reveal novel insights into SS pathogenesis. Here we aimed to highlight this approach, looping the circle from mouse to man and back (see Figure 2).



4

Figure 2. Primary Sjögren's syndrome: A back and forth interplay from Mouse to Man. In order to investigate the heterogeneous and complex pathogenesis of pSS, murine models such as NOD or NOD-derived-mice are indispensable. Knowledge-based implementation derived from mouse models are commonly implemented into human studies and potential clinical trials. As the heterogeneity of patients is often underestimated, we propose a back and forth interplay of knowledge between mouse and man, looping the circle from man to mouse and back. Hereby, models will further be improved to better resemble specific aspects of human-disease, essential in both therapeutic development and outcome-prediction. An important step will be to compare common deregulated pathways in both mouse and man to address therapeutic manipulations, by isolating whole blood/PBMCs or extracting target tissue biopsies from salivary and lacrimal glands. Patient selection and subgrouping according to their IFNsignatures, in IFNpositive and IFNnegative subgroups, will require separate mouse models per subgroup. As the NOD most likely represents the subset of systemically IFNnegative patients, we here propose the NOD-model revisited: TLR7-induced systemic IFN-signature in the NOD mouse as IFNpositive counterpart, by topical application of the TLR7-agonist imiquimod. Furthermore, comparing equal compartments in both subgroups of mice and men will give new insights into both the similarities and differences. Mouse models will remain crucial for pre-clinical exploration studies and will need continued revisiting and refining. TLR7, Toll-like receptor 7.

As SS in humans is very heterogeneous, multiple animal models are necessary to fully elucidate disease pathogenesis. Subtyping disease according to the prominent pathogenic player “IFN”, according to IFNsignature, is therefore a crucial first step. We highly recommend choosing appropriate mouse models for both IFNnegative as IFNpositive disease subtypes in future studies. In particular, we suggest the NOD as a model for systemically IFNnegative SS. Recently, evidence for distinct roles of IFN type II and III in autoimmunity has emerged [14, 15, 76], probably making subtyping even more complex in the near future but also much more effective for strategic therapeutic targeting.

In terms of developing new approaches for therapeutic interventions, the TLR-IFN network is a promising target and warrants more in depth investigation. The delicate balance between endosomal TLRs appears crucial to prevent autoimmunity, and a better understanding of these pathways and how they are balanced, will provide insight into specific targeted therapies [77]. Interestingly, tackling TLR7 will have effects on both pDCs and B cells simultaneously, and might prove beneficial by inhibiting multiple aspects of the disease with one compound. Whether TLR7 blockade alone or in combination therapies will prevent and/or ameliorate pSS pathogenesis remains to be investigated [13].

CONCLUSIONS

The heterogeneity of SS will most likely benefit from optimizing therapies, tailored to specific subgroups of the disease. Here we provide our perspective on the importance of subdividing SS patients according to their IFN signature, and recommend choosing appropriate mouse models for IFNpositive and IFNnegative SS-subtypes. Murine models better resembling human-disease phenotypes will be essential in this endeavour.

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Key messages:

- Simultaneous histopathology and functional analysis of salivary and lacrimal glands in relevant mouse models will result in more insight into SS pathogenesis.
- Lupus-prone mouse models should be revisited for salivary and lacrimal gland pathology and dysfunction.
- A distinction between local and systemic presence of IFN activity is essential, as local IFN activation does not directly imply the presence of systemic IFN activation.
- Therapeutic interventions should be tested when overt disease is present in relevant animal models, as pSS is diagnosed in this phase in humans.



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Chapter

5

RNA-sensing receptors TLR7, RIG-I and MDA5 in primary Sjögren's syndrome: a differential regulation in Interferon positive and Interferon negative patients

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ABSTRACT

Background/Purpose Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by autoantibodies targeting RNA-associated antigens, anti-SSA/SSB. The IFN type I signature is present in over half of pSS patients, associated with higher disease-activity and autoantibody presence. Plasmacytoid dendritic cells (pDCs) are considered to be the source of enhanced IFN type I signalling in these patients. To date the molecular pathways and processes of pDCs and their activation status in relation to IFN type I activation in pSS has not been characterized. The objective of this study was to unravel the molecular pathways underlying IFN type I bioactivity in pDCs of pSS patients. Since we found strong indications for an involvement and imbalance of nucleic acid sensing Pattern Recognition Receptors (PRRs) in a genome-wide expression analysis, we focused on abnormal expression levels of these receptors in circulating pDC. Since pDC are difficult to obtain from the circulation, monocytes were also studied.

Methods Blood samples were obtained from 42 Healthy controls (HC) and 120 pSS patients, stratified according to their IFN type I signature. Fluorescence-activated cell sorting was used to isolate CD123⁺BDCA4⁺ pDCs and CD14⁺ monocytes from peripheral blood mononuclear cells (PBMCs). Genome-wide microarray analysis was conducted on sorted pDCs in a small sample set and compared to a similar set obtained from the literature. mRNA expression of differentially expressed TLR and RLR related genes, as assessed by Ingenuity pathway analysis (IPA), was validated in sorted cell-suspensions using real-time quantitative PCR. To clarify a possible TLR7-driven activation of RLRs, PBMCs of HC were stimulated *in vitro* with Imiquimod, a TLR7 agonist, and inhibited with the specific TLR7 antagonist IRS661.

Results Confirming our microarray results we found an upregulation of TLR7, but not TLR9, in IFNpositive pDCs ($p < 0.05$) and monocytes ($p = 0.024$), as well as the further downstream signaling molecules MyD88, RSAD2 and IRF7. We also observed the upregulation of intracellular RNA-sensing receptors RIG-I (DDX58) and MDA-5 (IFIH1). *In vitro* PBMC studies revealed that triggering of the TLR7 pathway caused upregulation of RIG-I and MDA5, which could be blocked by specific TLR7-inhibition. The widespread upregulation of TLR7, of its downstream signaling pathway and of RIG-I and MDA5 was confined to IFNpos pSS patients. IFNneg patients had a contrasting pattern of PRR expression (TLR7 normal, TLR9, RIG-I and MDA5 decreased).

Conclusion Taken together, we conclude an imbalanced expression pattern of the RNA-sensing receptors TLR7, TLR9, RIG-I and MDA5 in pDCs and monocytes of IFNpos pSS patients. This profile could explain the pathogenic IFN production with downstream systemic inflammatory effects in these patients. We propose that the largely reduced expression of nucleic acid sensing receptors in IFNneg pSS patients explains in part their IFN negativity.

Key words: Toll-like receptor 7; RIG-I-like receptors; RNA-sensing; Interferon; plasmacytoid DCs

INTRODUCTION

A pivotal role for Interferons (IFN) has been extensively described in systemic autoimmune diseases, such as primary Sjögren's syndrome (pSS) and Systemic lupus erythematosus (SLE). The IFN type I signature, assessed by measuring a set of IFN type I inducible genes (IFIGs), is present in over half of patients and is associated with higher disease activity [1, 2]. To date, it is however not clear what causes this marked over activation of IFNs.

Plasmacytoid Dendritic Cells (pDCs) represent a small but distinct DC subset, comprising approximately 0.2 to 0.8% of total peripheral blood mononuclear cells (PBMCs). pDCs are the major source of IFN type I during antiviral immunity [3-8]. pDCs also produce IFN type I in response to self-derived nucleic acids originating from damaged tissues, and are thought to be the most likely cause of IFN type I overexpression in systemic autoimmunity [9, 10], and have been suggested to play a distinct role in pSS pathogenesis [11].

pSS is characterized by autoantibodies against ribonucleic acid (RNA)-associated antigens, anti-SSA (Ro52/Ro60) and -SSB [12]. Previously we described the IFN type I signature in pSS to be associated with higher autoantibody presence [2, 13], supporting a possible role for RNA and RNA-containing immune complexes (ICs) in the induction of IFN type I expression. The present concept on the induction of IFN type I is that self-nucleic acids or ICs containing self-nucleic acids enter the pDC, the latter via FcγRIIIa receptors, and are detected by endosomal TLRs followed by the induction of IFN gene expression.

After having taken up exogenous self-nucleic acids, endosomal Toll-like receptors (TLR), TLR7 and TLR9 are crucial for IFN production by pDCs in autoimmunity [14]. TLRs are a highly conserved transmembrane receptor family of pattern recognition receptors (PRR), playing a crucial role in host-cell defence against microbial pathogens [15, 16]. Negative feedback mechanisms of TLR-signalling is crucial in preventing overactivation and chronic inflammation, and loss of these tight regulatory mechanisms can result in autoimmunity [16]. Recently, opposing effects were described for TLR7 and TLR9 in lupus-like murine models. Interestingly TLR7 deletion limited autoimmunity, while TLR9 deletion paradoxically exacerbated disease [17, 18].

In addition to this exogenous route, an endogenous activation route of cytoplasmic innate sensors – the so-called RIG-I like receptors (RLRs) also leading to IFN production – has recently been suggested to play a role in IFN positive autoimmune diseases (Figure 1).

TLRs and the cytoplasmic RLRs both elicit proinflammatory- as well as IFN-responses upon activation. The RIG-I like receptors (RLRs), consist of retinoic acid inducible gene-I (RIG-I/DDX58), melanoma differentiation associated gene-5 (MDA-5/IFIH1), and 'laboratory of genetics and physiology' (LGP2/DHX58). In contrast to monocytes, pDCs express only marginal levels of RLRs in steady-state [19], however, expression of these receptors can rapidly be induced by TLR7 and TLR9 stimulation [20]. Interestingly, pDCs are sensitized to recognize specific RLR-agonists, only after prior endosomal TLR-stimulation. Furthermore, in pDCs TLR-ligands were found to



upregulate RIG-I expression in an IFN type I-independent manner [20], indicating a close collaboration between TLRs and RLRs [15, 21]. The relevance of the RLRs to autoimmunity has been shown by the detection of specific mutations in IFIH1 (MDA5) that are associated with enhanced susceptibility to autoimmunity. A gain-of-function mutation in the IFIH1 gene supports a role for these cell intrinsic sensors as culprits in IFN-driven autoimmunity [22, 23].

A third interesting PRR with regard to stimulation by self-nucleic acids is the endogenous cytoplasmic sensor protein kinase R (PKR), also known as eukaryotic translation initiation factor 2- α kinase 2 (EIF2AK2) or interferon-induced double stranded (ds)RNA-activated protein kinase. PKR is activated by auto-phosphorylation after binding of dsRNA and is a critical mediator of anti-proliferative and antiviral effects exerted by IFNs [24].

pDCs have been suggested to play a distinct role in pSS pathogenesis [11]. Infiltrates of pDCs are found in appreciable numbers in the salivary glands of pSS patients, concurrent with increased IFN type I expression in the glands [25, 26]. The low frequency of these cells in the peripheral blood, with even lower numbers in pSS, has however limited extensive studies on understanding their possibly pathogenic role [11]. To date the molecular pathways and processes of circulating pDCs and their activation status in relation to IFN type I activation in pSS has not been characterized. Therefore, the objective of this study was to unravel the molecular pathways underlying IFN type I bioactivity in pSS using whole gene (Affymetrix) expression analysis in a highly purified pDC population from pSS patients, in comparison to that of healthy controls. We found in particular upregulation of TLR7 in pDCs of IFNpositive (IFNpos) pSS patients, and downregulation of TLR9 (both expression levels were verified with qPCR).

To identify crucial genes in the TLR7-signalling pathway, we compared our genome analysis data to publicly available data on TLR7-stimulated pDCs [27]. In the study by Birmachu and colleagues pDCs were extrinsically stimulated with a TLR7-specific ligand (3M-852A) followed by microarray analysis using a similar method as used in our study. This resulted in the identification of the RLRs as crucial molecules, and in further qPCR studies we observed the upregulation of intracellular RNA-sensing receptors RIG-I and MDA5 amongst others in IFNpos patients. *In vitro* studies with PBMCs of healthy volunteers revealed that triggering of the TLR7 pathway caused upregulation of RIG-I and MDA5.

Since monocytes are innate immune cells expressing PRRs (RLRs and TLRs) and have previously been reported to respond to RNA by producing IFN type I [15], and the number of circulating pDC is small and experiments with this population are therefore limited, we also studied the PRRs of interest in peripheral blood monocytes of a large cohort of IFNpos and IFNnegative (IFNneg) pSS patients. In essence, we found the same results in monocytes as in pDCs. The upregulation of TLR7, of its downstream signalling pathway and of RIG-I, MDA5 and PKR appeared confined to the monocytes of IFNpos pSS patients. The IFNneg patients were – in contrast – characterized by a normal expression of the RNA-sensing receptors TLR7 and PKR, a stronger downregulation of TLR9 and a downregulation of the RLRs RIG-I and MDA5.

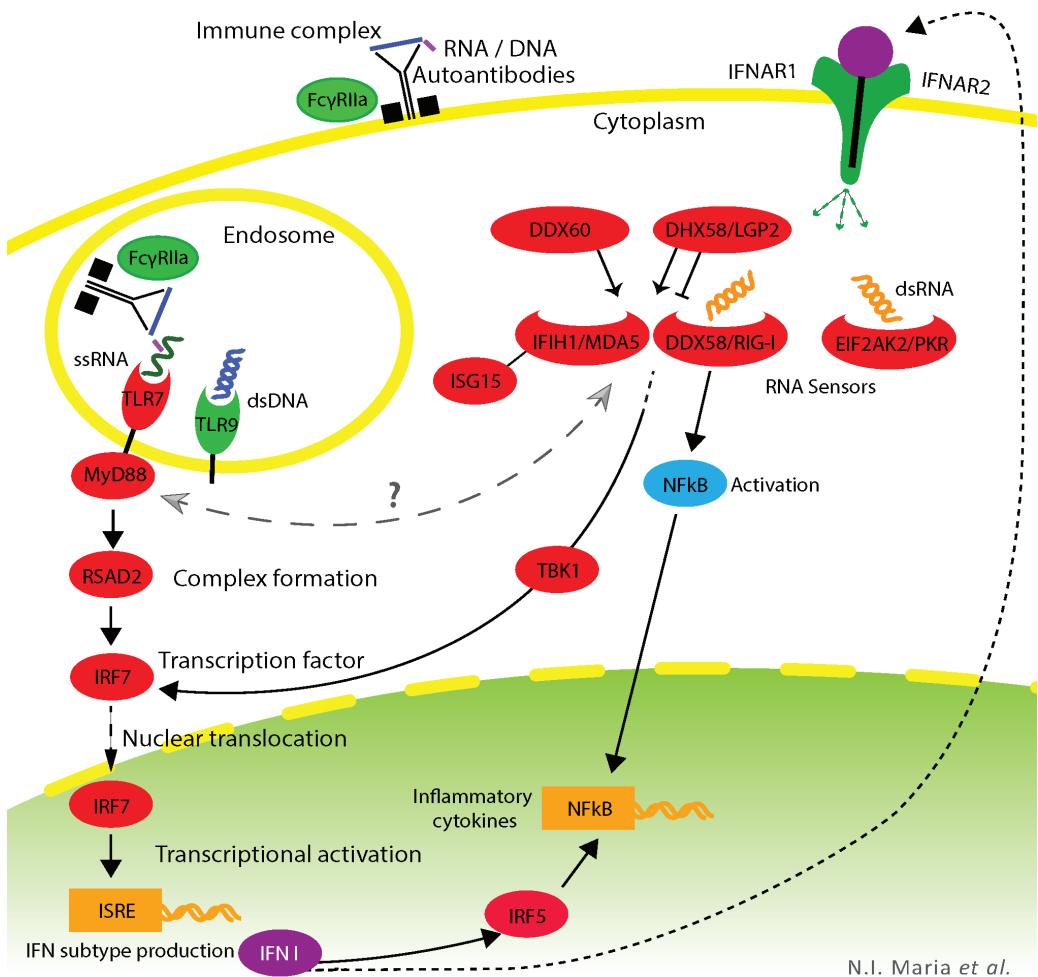


Figure 1. Schematic representation of overactive TLR7-induced signalling in IFN type I positive pSS. On the left, the pathway induced in response to RNA/DNA containing-immune complexes by FcγRIIa binding and internalization is shown. TLR7, expressed in the endosome, becomes activated when a RNA-containing immune complex is internalized and crosslinked with TLR7. Activation of TLR7 induces the signalling cascade leading to production of IFN type I subtypes (IFN I) and inflammatory cytokines. The possible collaboration between TLR7 and RLRs is depicted by the grey dotted line. TLR7-signalling might induce overexpression and downstream signalling of the RNA-sensing receptors DDX58/RIG-I and IFIH1/MDA5, as well as EIF2AK2/PKR. Furthermore expression of the RLRs is also directly inducible by IFN-I (green dotted lines), thus amplifying the TLR7-induced loop. Gene expression levels for genes depicted were measured by RT-qPCR in peripheral whole blood of IFN type I positive pSS patients compared to HCs. Red: upregulated, green: similar gene expression, blue: gene expression not determined. Here, the pathways were simplified to depict only essential signalling of interest.

Taken together, we found an imbalance in the expression of the exogenous and endogenous RNA-sensing receptors in IFN positive pSS patients (TLR7 up, TLR9 down, RIG-I up and MDA5 up), a profile that can explain the pathogenic IFN production in these patients. In IFNneg patients we found TLR7 normal, TLR9 down, RIG-I down and MDA5 down, and we propose that this reduced expression of RNA sensing receptors explains in part the IFN negativity of these pSS patients.

MATERIALS AND METHODS

Patients

Patients positively diagnosed with pSS, according to the 2002 American-European criteria were recruited [28]. 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) [29]. Healthy controls (HC), neither suffering from autoimmune diseases nor using corticosteroids, were included. Study subjects (see Table 1) were screened to be free of symptoms of underlying viral infections at inclusion. The Medical Ethical Review Board of the Erasmus MC Rotterdam approved the study and written informed consent was obtained.

Blood collection, preparation and 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, peripheral blood mononuclear cells (PBMCs) were isolated by low-density gradient centrifugation within 1-4 hours after sample collection to avoid cell activation (stored in liquid Nitrogen).

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 [2].

Flow cytometric cell sorting

pDCs can be identified by the expression of BDCA2 and BDCA4, as well as high expression of CD123 [30]. Here, BDCA4 (CD304/Neuropilin1) was used to characterize pDCs, as BDCA2 (CD303) is known to have inhibitory effects on IFN type I production in pDCs (Supplemental Figure S1) [31, 32]. PBMCs were thawed, centrifuged 5 min (1500 rpm, 4 °C) and resuspended on ice in 100 µl Sort-buffer (PBS pH 7.4, 2mM EDTA 1M, 0.5% BSA). FACS (fluorescence-activated cell sorting) was used to sort PBMC cell suspensions in steady state. In order to achieve highest purity of pDCs, PBMC-membrane staining was performed with fluorescently labeled antibodies: anti-BDCA-4 (PE; Milteny Biotec), anti-CD123 (PE-Cy7; eBioscience, CA, USA), anti-CD3 (PerCP-Cy5;

Table 1. Patient and Control characteristics.

		pSS	
	HC (n=42)	IFNneg (n=58)	IFNpos (n=62)
Cohort Demographics			
Female (%)	37/42 (88.1%)	54/58 (93.1%)	56/62 (90.3%)
Mean age (years)	52.5 ± 9.67	54.7 ± 13.3	54.7 ± 13.3
Patient characteristics			
Disease duration (years)	-	13.2 ± 8.0	12.0 ± 7.6
Laboratory parameters			
Anti-SSA	-	39/50 (78)	56/57 (98)
Anti-Ro52	-	15/51 (29)	43/55 (78)
Anti-Ro60	-	23/47 (49)	48/51 (94)
Anti-SSB	-	19/48 (40)	49/51 (96)
Medication status (%)			
Pilocarpine	-	19/51 (37)	21/56 (38)
Hydroxychloroquine	-	37/51 (73)	32/57 (56)
Corticosteroids	-	8/51 (16)	7/56 (13)

Data are presented as mean ± SD, or as number (%)

Becton Dickinson Biosciences (BD), CA, USA), anti-CD14 (APC/Cy7; BD) and anti-CD19 (APC; BD), 15 min in the dark. Cells were sorted using a FACSaria II cell sorter (BD). Reanalysis of sorted cells confirmed a >98% purity.

Real-time quantitative PCR

Total RNA was isolated from purified CD14⁺ sorted cell-populations using RNeasy 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 on a 7900HT Fast Real-Time PCR System, all according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). For calculation of relative expression, all samples were normalized to expression of the housekeeping gene ABL [33]. Fold change values were determined from normalized CT values using $2^{-\Delta\Delta CT}$ method (User Bulletin, Applied Biosystems) [34].

Subtyping pSS patients according to the monocyte IFN type I signature

The monocyte IFN type I signature was defined by the relative expression of 5 IFIGs – IFI44L, IFI44, IFIT3, LY6E and MxA, as previously described. 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 [35, 36]. pSS patients were stratified according to IFNscore into patients positive for the IFN type I signature (*IFNpos*; IFNscore \geq 10) and patients negative for the signature (*IFNneg*; IFNscore $<$ 10) [2, 13].

Microarray Genome-wide expression profiling of CD123⁺BDCA4⁺ pDCs

Genome-wide transcriptome data were collected from sorted CD123⁺BDCA4⁺ pDC of 6 pSS patients (3 IFNpos and 3 IFNneg) and 4 HC.

CD123⁺BDCA4⁺ pDC-RNA isolation, amplification and gene expression analysis

Total RNA was isolated from purified CD123⁺BDCA4⁺ sorted cell-populations with the PicoPure RNA isolation kit (Arcturus®, Applied Biosystems, CA, USA) according to the manufacturers protocol, including a DNase I treatment (Qiagen, Venlo, The Netherlands) to remove genomic DNA contamination. RNA quality was assessed on a Bioanalyzer (Aligent Technologies Inc, CA, USA) to ensure good quality RNA: RIN \geq 8. The RNA was reverse transcribed, amplified, biotinylated and fragmented with the Ovation® Pico WTA system and Encore™ Biotin Module (NuGEN Technologies, Leek, The Netherlands) and subsequently hybridized on Human Genome U133 plus 2.0 Array Gene Chips (Affymetrix, High Wycombe, UK) according to the manufacturers protocols. The dataset will be located at NIH, gene expression omnibus (GEO), when published. Accession number of repository for expression microarray data: GSExxx. To increase quality control, all pDC samples were handled on the same day.

Microarray analysis: data normalization, statistical analysis and functional pathway analysis

Quality analysis of the CEL data was assessed by running a standardized workflow developed at the BiGCaT department of Maastricht University - The Netherlands (www.arrayanalysis.org). The expression data containing .CEL files were imported and processed further with Partek Genomics Suite 6.6 (Partek Inc., Saint Louis, MO, USA). pDCs obtained from 4 HC, 3 IFNneg and 3 IFNpos pSS patients were analyzed. After quality control analysis of the gene chip arrays, 2 HC, 2 IFNneg and 3 IFNpos pSS samples remained for further analysis. Data were normalized as previously described [37]. Briefly, raw intensity values of all samples were normalized by background correction and quantile normalization using Partek GC Robust Multichip Analysis. To visualize the correlation between the samples, principal component analysis (PCA) was used. The normalized data file was transposed and fold changes (FC) with associated p-values were calculated for the comparison of the pSS patient group to the HC group using ANOVA statistics in Partek.

Gene annotation and functional pathway analysis was performed in Ingenuity pathway analysis (IPA) (Qiagen, Redwood City, CA, USA), by uploading the FC and p-value data into IPA, and applying a cutoff of $-2 \leq \text{FC} \leq 2$ and $\text{p-value} < 0.05$. Probes were mapped to the gene level and

averaged based on the median FC-values. A core analysis on the data was performed in IPA. Upstream regulators were analyzed by selection on the basis of z-score (>2 or >-2).

Hierarchical clustering

Hierarchical clustering was performed in Partek 6.6. In brief, the quantile normalized values of each probe were used to calculate the relative values of each sample compared to the average of all samples for each probe. These relative ratio values were modified to a relative fold change scale. For graphical display purposes the maxima of the data range were set from -3 to +3. Hierarchical clustering was performed both for the rows and the columns. The complete linkage clustering method was used for both rows and columns and Euclidean clustering was used to determine both row and column dissimilarity.

Quantitative PCR validation of CD123⁺BDCA4⁺ pDC Microarray

CD123⁺BDCA4⁺ pDCs were sorted as described above, and stored in Ambion RNA/later solution to ensure RNA stabilization. Total RNA was isolated with the Ambion RNAqueous-Mirco Kit, including a DNase I treatment (Qiagen, Venlo, The Netherlands) to remove genomic DNA contamination, subsequently reverse-transcribed to cDNA using the SuperScript[®] VILO[™] cDNA Synthesis Kit (Invitrogen, CA, USA). cDNA was subjected to preamplification (14 cycles) using the TaqMan PreAmp master mix kit and predesigned primer/probe sets in order to specifically amplify genes of interest in all samples. RQ-PCR analysis was then performed on preamplified samples using the predesigned primer/probe sets, all according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

Immunofluorescence and confocal microscopy

Sorted CD14⁺ monocytes were fixed with 4% paraformaldehyde onto poly-L-lysine (0.1 % w/v in water; Sigma P8920) coated slides (Nutacon, Leimuiden, Holland), seeding 2×10^4 cells/eyeglass. Slides were preincubated with normal chicken and goat serum (NCHs, NGS) at RT for 10 min before incubation with either polyclonal TLR7, (Rabbit, Life technologies Bleiswijk, the Netherlands) or polyclonal MDA5 (Rabbit, Abcam, Cambridge, UK), and monoclonal phospho (p)IRF7 (Mouse IgG1, BD bioscience Breda, the Netherlands) for 1hr, followed by staining with AlexaFluor-labeled chicken-anti-rabbit AF488 and goat-anti-mouse AF594 (IgG1, Molecular Probes, Bleiswijk, Netherlands) with 2% normal human serum (NHS). Slides were mounted in Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) for 10 min. Appropriate isotype controls were used to confirm antibody-specificity. Microscopic analysis was performed on a ZEISS LSM700-confocal (63x objective). ZenSoftware 2009 was used for confocal image acquisition and processing, and ImageJ software for analysis.



Immunohistochemistry

Paraffin embedded and formalin-fixed 5 μm thick sections of minor salivary gland (SG) tissue from pSS patients were incubated overnight at 58°C. Deparaffinization and heat-induced epitope retrieval (HIER) was performed with a PT link (Dako A/S, Glostrup, Denmark) for 20 min at 98°C using either Dako EnVision Target retrieval solution pH 6 or pH 9. Endogenous peroxidase was quenched for 10 or 30 min with Dako REAL Peroxidase-Blocking solution depending on primary antibody (Ab), sections were blocked for 30-60 min with Protein Block. All primary Abs were incubated overnight at 4°C diluted in Dako Antibody Diluent. Secondary HRP-coupled antibodies and/or polymer HRP conjugates were incubated for 30-120 min at RT, followed by 4-10 min incubation with diaminobenzidine (DAB) as peroxidase substrate for color development. Finally, sections were counterstained using Dako Hematoxylin, dehydrated and mounted with VectaMount Permanent Mounting medium (Vector Laboratories, Peterborough, UK). The following anti-human antibodies were used in indicated dilutions: rabbit anti-MDA-5 (1:1000; Thermo Fisher Scientific, Rockford, USA), mouse anti-CD4 clone 4B12 (1:20; Dako), mouse anti-RIG-I clone 2M6F10 (1:100; Thermo Fisher Scientific), rabbit anti-TLR7 clone EPR2088(2) (1:100; Abcam, Cambridge, UK), sheep anti-BDCA-4 (1:10; R&D systems, Abingdon, UK), mouse anti-CD19 clone LE-DC19 (1:100; Dako) and rabbit anti-MxA (Proteintech, Manchester, UK). For rabbit and mouse primary Abs: EnVision™ Detection system (Dako), for sheep antibodies: rabbit anti-sheep (1:10; Dako). For negative isotype control stains, sections were incubated with either rabbit IgG, mouse IgG, sheep serum, or secondary reagent only.

In vitro stimulation bioassays

HC PBMCs were seeded at a concentration of $2 \times 10^6/250 \mu\text{l}$, and starved during 1 hour at 37° (in starvation medium: RPMI- with 0.5% FKS and 0.05% P/S). Cells were subsequently stimulated for 5 hours with 1.0 $\mu\text{g/ml}$ Imiquimod (R837, IQ; InvivoGen, San Diego, USA), in the presence or absence of the specific TLR7 inhibitor (IRS661, TIB MOLBIOL, Berlin, Germany) at increasing concentrations (0.5, 1.0, 2.5 and 5.0 nM) in polypropylene tubes.

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 $p < 0.05^*$ or $p < 0.01^{**}$ were considered statistically significant. Statistical analysis was performed using IMB SPSS 20.0 (SPSS, Chicago, IL, USA). Graphs were designed with Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA).

RESULTS

Comparative analysis of gene expression profiles in CD123⁺BDCA4 pDCs of IFNpos and IFNneg pSS

To characterize pDCs in pSS in relation to the IFN signature, a microarray analysis was conducted on FACS-sorted BDCA4⁺CD123⁺ pDCs obtained from HCs, IFNneg and IFNpos pSS patients without any *ex vivo* stimulation (Supplemental Figure S1) [30-32]. pDCs were analyzed for differences in genome-wide gene expression profiles between the groups (Figure 1). Group Class-Comparisons (CC) were evaluated for all 3 groups – IFNpos vs. HC – IFNneg vs. HC – and – IFNpos vs. IFNneg. When comparing IFNpos to HCs 1234 differentially expressed genes (DEGs) were identified using a multivariate permutation test, where 865 DEGs remained significant at $p\text{-value} < 0.05$ and Fold change (FC) ≥ 2.00 . The majority of DEGs (642; 74.2%) were upregulated in the IFNpos pDCs compared to HC. Figure 2 shows a Venn diagram of all significant DEGs between the groups and hierarchically clustered heatmaps illustrating the differences between groups. Ingenuity pathway analysis (IPA) of the data in the context of Gene Ontology and gene regulatory network analysis enabled us to identify molecular and cellular pathways of interest. We focused on the endogenous PRR pathways (TLRs, RLRs and PKR) involved in RNA handling.

IFNpos pSS patients overexpress the TLR7-signalling pathway and downregulate TLR9 in pDCs

IPA analysis of the TLR-signalling pathway revealed a significant upregulation of TLR7 [$\uparrow 3.53$ fold up; $p=0.015$], and not of TLR9, in pDCs of IFNpos pSS compared to HC pDCs (Figure 3A). In contrast, TLR9 was two-fold down regulated in pDCs of IFNpos pSS patients compared to HCs, however this difference did not reach statistical significance in the microarray comparison [$\downarrow 2.12$ fold down; $p=0.28$]. A significant upregulation of several TLR7 downstream signalling molecules including MyD88, encoded by the myeloid differentiation primary response protein 88 gene [$\uparrow 2.4$ fold up; $p=0.03$] was also observed. The endogenous PRR receptor PKR was significantly upregulated in IFNpos pSS [$\uparrow 5.4$ fold up; $p=0.013$]. Additionally, PKR (EIF2AK2) was one of the top predicted upstream regulators ($p=4.44\text{E-}05$, Z-score=2.89; data not shown) in IFNpos pDCs.

To validate these micro-array results, top-ranking (lowest p-value, largest fold-up/down) and additional differentially expressed genes (DEGs) of interest were validated by qPCR using newly sorted CD123⁺BDCA4⁺ pDCs in a cohort of HC (n=10), IFNpos (n=9) and IFNneg (n=7) patients.

Confirming our microarray results, TLR7 ($p=0.02$), MyD88 ($p=0.03$) and PKR ($p=0.002$) were up regulated in pDCs of IFNpos pSS compared to HC. With regard to TLR9, this receptor was significantly downregulated in both IFNpos and IFNneg patients, this difference reaching significance in IFNneg pSS compared to HC pDCs ($p=0.02$) (Figure 3B).

In Figure 3C and 3D additional qPCR validated DEGs of interest are shown, such as RSAD2 (encoding Radical S-Adenosyl methionine Domain Containing 2), also known as Viperin,



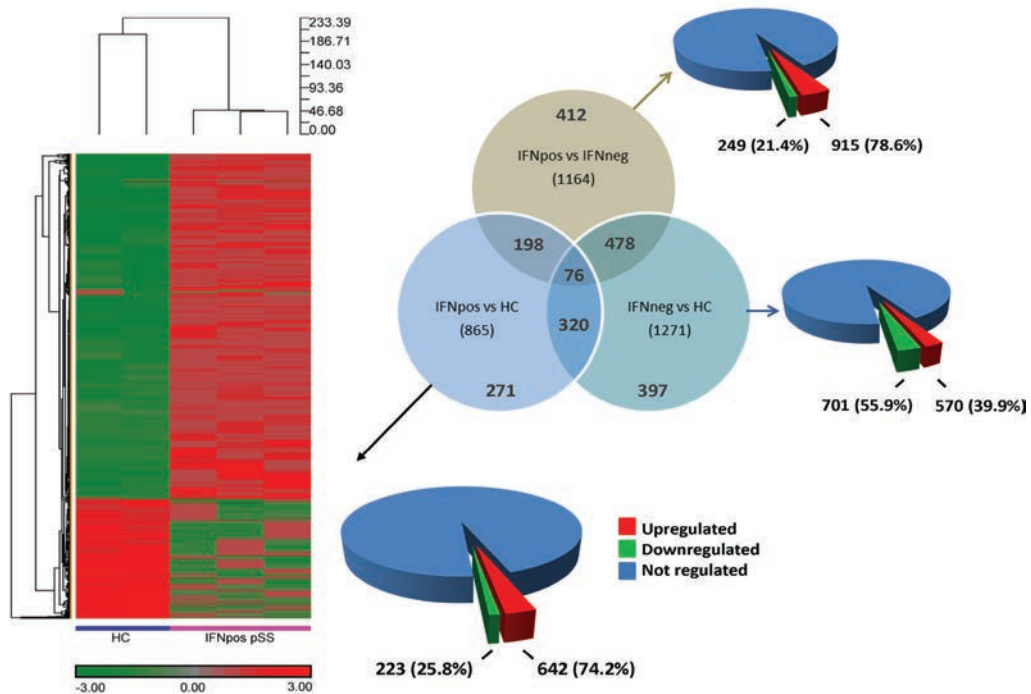


Figure 2. Genome wide gene expression profiles in pDCs of IFNpositive and IFNnegative pSS patients. Group Class-Comparisons (CC) for – IFNpositive pSS (IFNpos) vs Healthy Control (HC) – IFNnegative pSS (IFNneg) vs HC – and – IFNpos vs IFNneg – were evaluated in circulating BDCA4⁺CD123⁺ pDCs. Venn Diagram shows total number of differentially expressed genes (DEGs) between groups; p-value<0.05, Fold Change (FC) ≥ 2 , using a multivariate permutation test. In brackets the total amount of DEGs is shown for each comparison. For IFNpos vs HC, a heatmap with hierarchical clustering (euclidean distance with average linkage) of the DEG genes between groups is shown. Normalized 2 log-transformed probeset- expression values are visualized as a gradient from low (green) to high (red) expression.

which was significantly upregulated confined to IFNpos pDCs ($p < 0.01$). Viperin is an IFIG that was recently shown to promote TLR7/9-dependent IFN type I production in pDCs [38, 39]. Additionally, genes of the previously described monocyte IFN type I signature such as MxA ($p < 0.001$) [2, 13] and other IFIGs such as STAT1 (encoding signal transducer and activator of transcription 1) ($p < 0.001$) were upregulated in IFNpos pDCs. When subjecting the validated DEGs to a hierarchical pathways analysis, STAT1 was observed to connect all DEGs (Figure S2). STAT1 is a crucial transcription factor in the IFN signalling pathways, downstream of the IFNAR [40, 41].

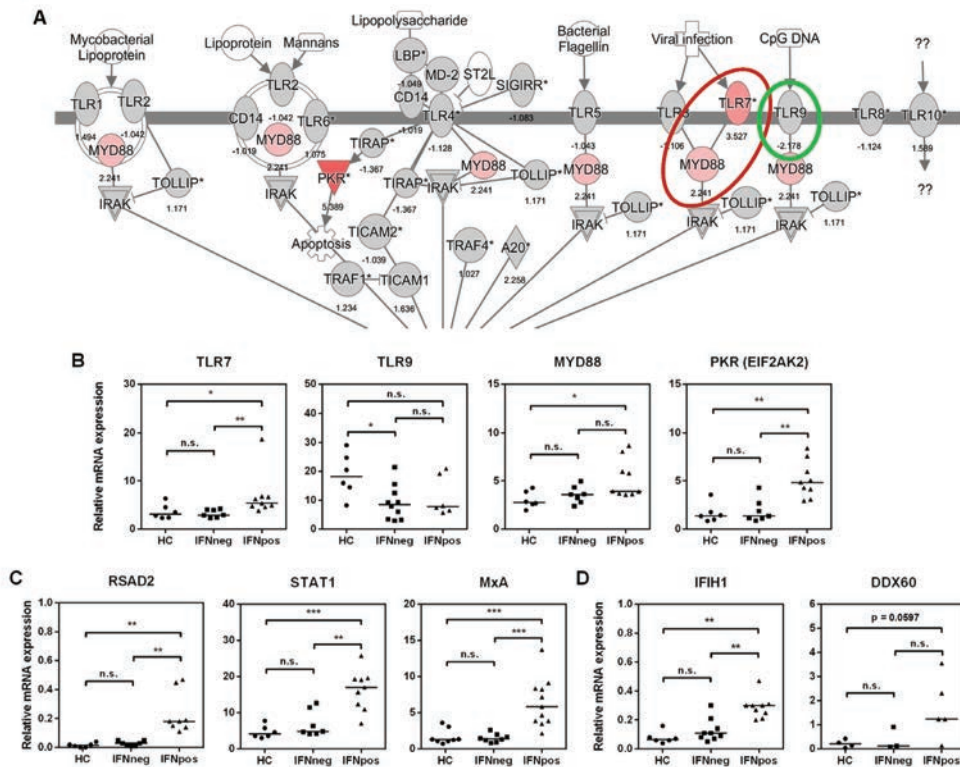


Figure 3. TLR7 - but not TLR9 - is significantly upregulated in circulating BDCA4⁺CD123⁺ plasmacytoid DCs of IFN type I signature positive pSS patients. A) Ingenuity pathway analysis (IPA) network of the TLR7-induced IFN pathway in IFNpos pDCs compared to HC. The TLR signalling pathway shows sole upregulation of TLR7 [\uparrow 3.53 fold up; $p=0.015$] in IFNpos pDCs, compared to HC (and to IFNneg pSS [\uparrow 3.23 fold up; $p=0.019$]; data not shown). Furthermore the key adaptor protein for downstream signal transduction, MyD88 (myeloid differentiation primary response gene 88), was also significantly upregulated [\uparrow 2.4 fold up; $p=0.03$], confined to IFNpos pDCs. Plotted graphs show the differentially expressed genes (DEGs) of interest, validated by RQ-PCR in a validation cohort of HC (n=10), IFNneg (n=7) and IFNpos (n=9) pSS, in sorted BDCA4⁺CD123⁺ pDCs to confirm mRNA upregulation of **B)** TLR7 and downstream DEGs in the IPA analysis, such as MyD88 and PKR, the downregulation of TLR9, and **C)** other DEGs of interest, involved in the TLR- and IFN-signalling pathways such as RSAD2 (encoding Radical S-Adenosyl methionine Domain Containing 2), also known as Viperin, signal transducer and activator of transcription 1 (STAT1), Mx α , and **D)** IFIH1 and DDX60. Each symbol represents an individual sample; horizontal lines represent the median. To compare medians the Mann-Whitney U test was used. n.s., not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Similar expression patterns for TLR7, TLR9 and the TLR7-signalling pathway in monocytes of pSS patients as in pDCs

To further assess the extent of the imbalanced expression of TLR7 and TLR9 in other immune cells taking up antigens in pSS, and more abundantly present in the circulation, we measured

mRNA expression levels of these receptors and the key downstream genes MyD88 and IRF7 in the TLR7-signalling pathway in CD14⁺ monocytes of a large set of HC (n=41), IFNneg (n=50) and IFNpos (n=50) patients (Figure 4A). In addition, RSAD2 levels were measured as this gene is involved in translocation of IRF7 to the nucleus in pDCs [38].

Overall, the data showed that in CD14⁺ monocytes of IFNpos pSS patients TLR7 was significantly upregulated compared to HC and to IFNneg pSS patients, and that TLR9 was significantly downregulated in monocytes of IFNpos compared to HC (although the expression levels of TLR9 in CD14⁺ monocytes were very low) (Figure 4). In IFNneg pSS patients TLR7 expression was equal to healthy controls, whereas TLR9 expression was significantly reduced as compared to HC.

The genes involved in the TLR7-signalling pathway, MyD88, RSAD2 and IRF7 were all significantly upregulated in CD14⁺ monocytes of IFNpos pSS as compared to IFNneg pSS and HC (Figure 4A). In IFNneg patients only MyD88 expression was significantly higher as compared to HC. Figure 1 shows a schematic overview of the TLR7 pathway leading to the nuclear translocation of IRF7.

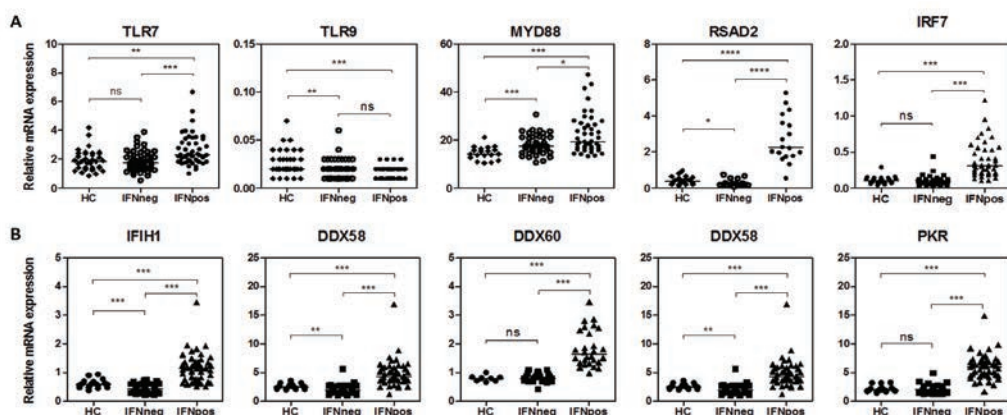


Figure 4. Upregulation of the TLR7-signalling pathway and RNA-sensing receptors is confined to IFNpositive pSS patients. Relative mRNA gene expression levels in isolated CD14⁺ monocytes of healthy controls (HC, n=41), IFNnegative (IFNneg, n=50) and IFNpositive (IFNpos, n=50) pSS patients are depicted for (A) the endosomal TLR7/9-signalling pathway and, (B) the RLR family of receptors as well as PKR. 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. Ns, not significant; *, *p*<0.05; **, *p*<0.01; ***, *p*<0.001; ****, *p*<0.0001.

Intracytoplasmic RNA-sensing PRRs upregulated in IFNpos pSS

To further identify important genes in the TLR7 pathway in pDCs of IFNpos pSS patients we performed an IPA comparison analysis between the Birmachu data set of TLR7-stimulated vs. unstimulated HC pDCs [27] and our pSS genome-wide expression data set specifically of pDCs of

IFNpos pSS vs. those of HC. This analysis resulted amongst others in the identification of IFIH1 and DDX60 as important TLR7-driven DEGs overlapping between TLR7-triggered pDCs and pDCs of IFNpos pSS patients (see Table S1). DDX60 is a recently identified helicase, found to promote RLR-mediated signalling during viral infections [42]. We were able to validate the higher expression of these 2 RLRs in pDC of IFNpos pSS patients in qPCR (see Figure 3D; DDX60 in fewer pDC samples due to lack of material). We further assessed the mRNA expression levels of the RLRs, IFIH1, DDX58, DHX58 and DDX60 in CD14⁺ monocytes (Figure 4B). The RLR-family members were all significantly upregulated in IFNpos monocytes. Interestingly, IFIH1 and DDX58 were significantly downregulated in IFNneg pSS monocytes compared to HC. The RLR-downstream signalling molecule TBK1 (TANK-binding kinase 1) was upregulated in monocytes of IFNpos pSS compared to IFNneg pSS (Figure S3).

Cytoplasmic sensor PKR expression was assessed in pDCs (Figure 3B) and monocytes (Figure 4B). PKR was upregulated in monocytes and pDCs of IFNpos pSS patients compared to IFNneg pSS and HC. In IFNneg patients PKR was expressed at the same level as in HC in both cell types.

Protein expression of TLR7, MDA5 and phosphorylated IRF7 in monocytes of pSS patients

To assess protein expression of TLR7 and MDA5 in monocytes of IFNpos pSS patients, FACS-sorted CD14⁺ monocytes were co-stained with antibodies specific for TLR7 or MDA5 and phosphorylated (p)IRF7. Confocal microscopy showed TLR7 (green) and pIRF7 (red) co-expression in IFNpos pSS, which was mostly absent in HC and IFNneg monocytes (Figure 5; upper panel). MDA5 (green) was expressed in HC and IFNneg pSS at comparable levels, whereas much more prominent in IFNpos pSS. Furthermore clear nuclear staining of pIRF7 was observed (red dots) in MDA5⁺ IFNpos pSS monocytes (Figure 5; lower panel).

Protein expression of TLR7, RIG-I and MDA5 in monocytes minor pSS salivary glands

Salivary glands are a characteristic target organ of the autoimmune attack in pSS and were previously shown to exhibit IFN positive pDC infiltrations. As we observed marked overexpression of TLR7 and the RLRs, IFIH1/MDA5 and DDX58/RIG-I in circulating monocytes and pDC in IFNpos pSS, we assessed the local expression of these receptors in pSS SG biopsies (Figure 6). MxA (an IFIG and biomarker of IFN bioactivity) showed strong expression within the lymphocytic foci but was also characterized by diffuse staining of ductal and acinar gland cells (Figure 6). TLR7 was present within the foci, however expressed by fewer cells. RIG-I and MDA5 were strongly expressed by cells within the lymphocytic foci.



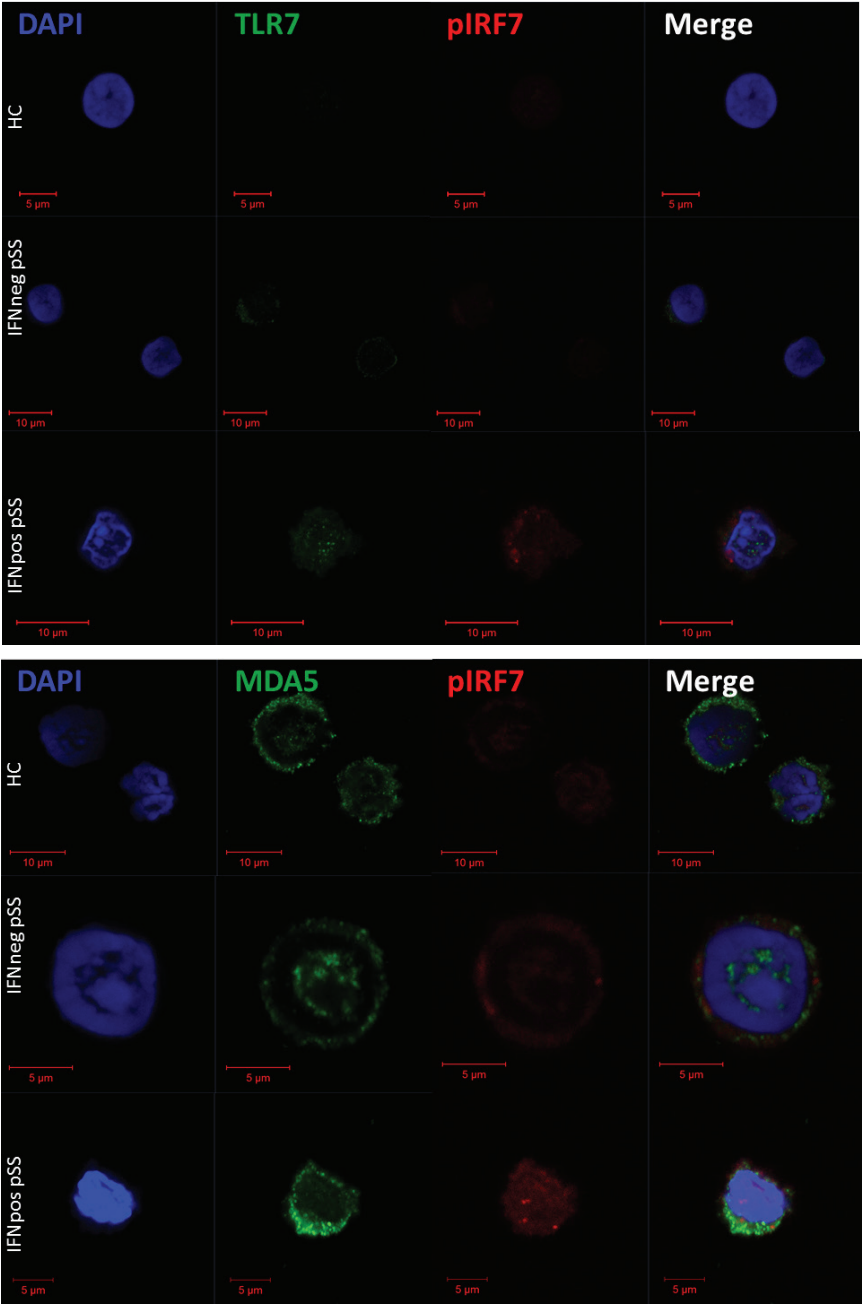


Figure 5. TLR7 and MDA5 overexpression in IFNpos pSS monocytes. Monocyte TLR7 (upper panel) and MDA5 (lower panel) expression (green), co-stained for phosphoIRF7 (pIRF7; red) in HCs, IFNneg and IFNpos. Representative images are shown. Nuclei were stained with DPI (blue). Appropriate isotype control stainings were used.

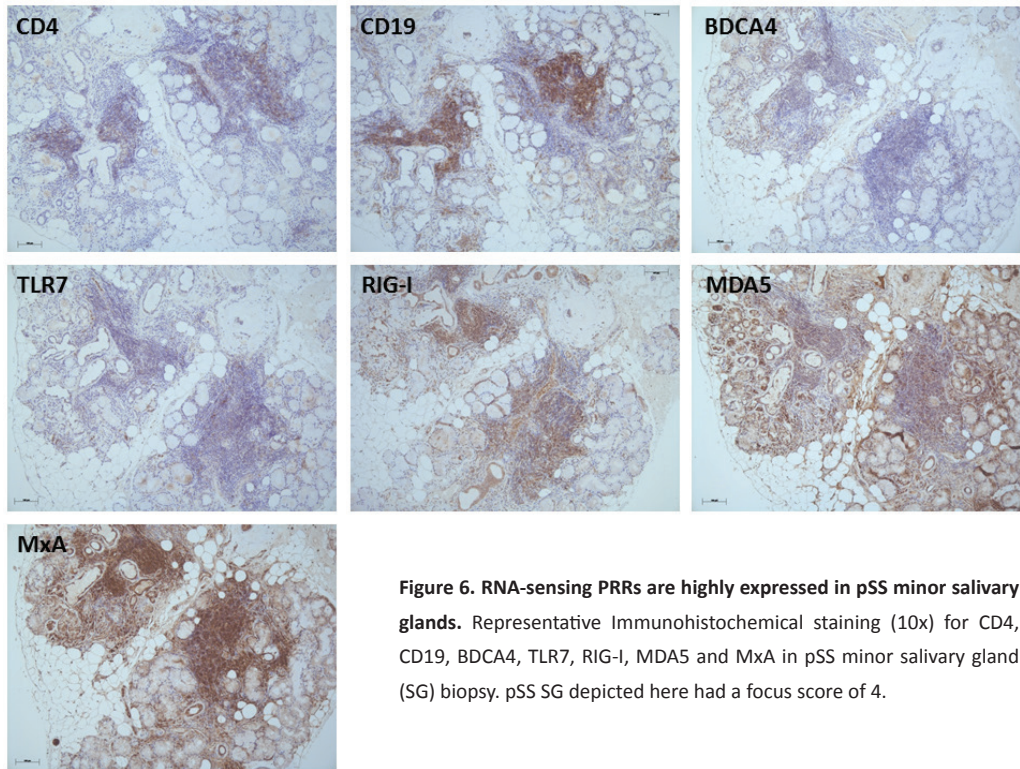


Figure 6. RNA-sensing PRRs are highly expressed in pSS minor salivary glands. Representative Immunohistochemical staining (10x) for CD4, CD19, BDCA4, TLR7, RIG-I, MDA5 and MxA in pSS minor salivary gland (SG) biopsy. pSS SG depicted here had a focus score of 4.



The RIG-I like family of receptors are induced by TLR7-mediated signalling

To verify the positive interaction between the TLR7 pathway and the RLRs described in the Birmachu experiments and suggested by our genome-wide expression analyses, PBMCs of a set of HCs were stimulated *in vitro* with the TLR7 agonist imiquimod (IQ) in the presence or absence of the specific TLR7 antagonist IRS661. TLR7 mRNA expression was significantly induced by IQ, whereas TLR9 expression was significantly downregulated (Figure 7). Furthermore, TLR7 triggering significantly induced expression of downstream signalling molecules MyD88 (Figure 7), IRF7 and RSAD2 (data not shown). The RLRs (IFIH1, DDX58, DHX58 and DDX60), which were expressed at relatively low levels at baseline were also induced by TLR7 triggering (Figure 7). Additionally the RNA-sensing receptor PKR showed a similar expression pattern. The TLR7 induced gene expression (+/-) was inhibited dose-dependently by IRS661 (0.1, 0.25, 0.5 and 1.0 nM), with exception of TLR9 expression, which was upregulated by TLR7 antagonism (Figure 7).

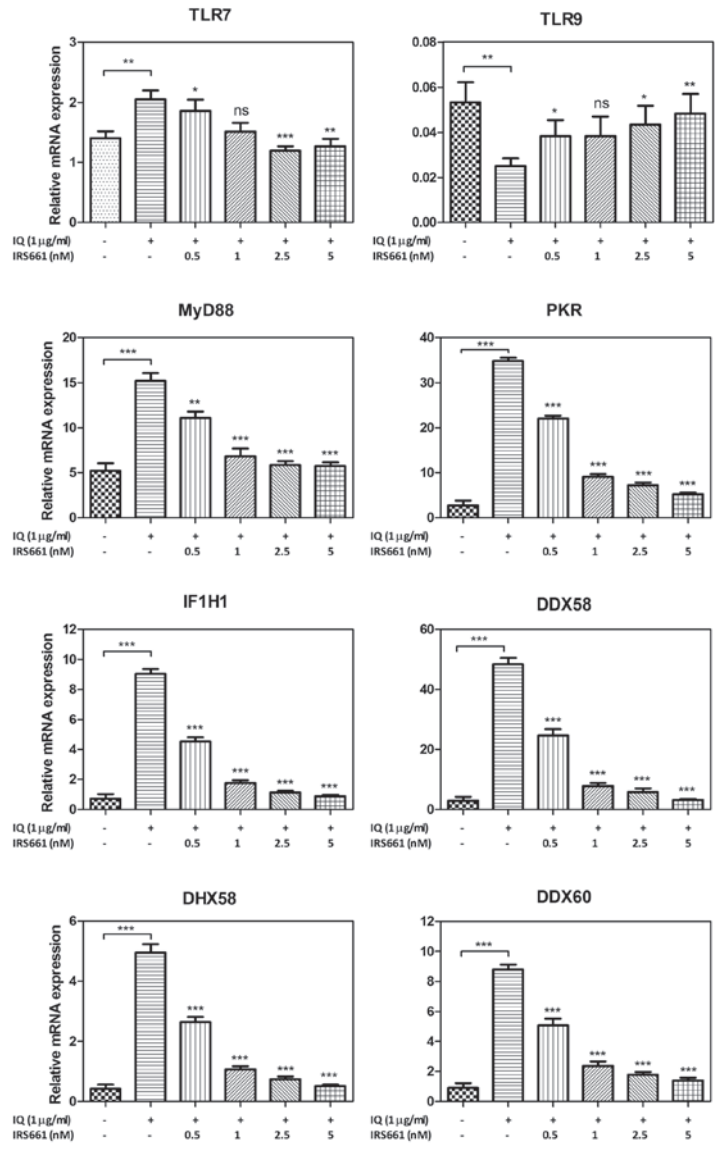


Figure 7. RIG-I like receptor expression induced after specific TLR7-triggering in healthy control PBMCs, and blocked by specific small molecule TLR7 inhibitor IRS661. Relative mRNA gene expression levels shown of genes related to the TLR7 pathway after 5 hours of culturing HC-PBMCs with the optimal concentration TLR7 agonist imiquimod (1.0 µg/mL), with or without the addition of the specific TLR-7 antagonist 'IRS661' in dose dependent manner (0.5, 1.0, 2.5 and 5.0 nM); control condition is solely cells cultured in starvation medium (-/-). Gene expression data are presented as means ± SEM of 3 independent experiments (in duplo), and means were compared using the paired t-test. Ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

DISCUSSION

This study shows an aberrant pattern of expression of exogenous- and endogenous-pathway RNA-sensing receptors in pDC and monocytes of IFNpos and IFNneg pSS patients. In IFNpos pSS patients we observed TLR7, but not TLR9, to be upregulated. Downstream signalling molecules such as MyD88, RSAD2 and IRF7 were also upregulated. Endosomal TLR7 recognizes single-stranded RNA and its activation in pDCs is resulting in abundant IFN type I production [8, 9]. A two-fold TLR7 overexpression induces autoimmunity in susceptible mouse strains and influences the stringency of the autoreactive B cell repertoire as well as spontaneous germinal center-formation in these mice, pointing towards the importance of tight TLR7-regulation in maintaining immune tolerance [43]. Opposing effects were recently described for TLR7 and TLR9, where TLR7 deletion limited systemic autoimmunity, whereas TLR9 deletion paradoxically exacerbated disease [17, 44]. Therefore imbalances between TLR7/TLR9 expression and the stringent control of TLR7 expression and signalling, seem to be crucial to restrict systemic autoimmunity [17, 45-47]. The aberrant profile of TLR7 and TLR9 expression, as found in this study in IFNpos pSS patients, is in accord with such a view.

Next to TLRs, PKR (EIF2AK2) was one of the top predicted upstream regulators ($p=4.44^{E-05}$, Z-score=2.89; data not shown) in IFNpos pDCs, and appeared upregulated in pDCs and monocytes of IFNpos pSS patients. PKR is a known IFIG often described upregulated in pSS, however, apart from being a RNA-sensing receptor involved in TLR-signalling, its exact role in pSS pathogenesis remains elusive [48-50].

Another IFIG we observed to be significantly upregulated in IFNpos pDCs and monocytes was RSAD2 (Viperin). In pDCs, Viperin has been shown to facilitate IRF7 translocation to the nucleus, and loss of Viperin reduced TLR7/9-mediated IFN type I production in pDCs [38, 39]. It is however not known if RSAD2 is also involved in TLR7/9-dependent signalling in other cells. Interestingly, a strong correlation between RSAD2 and IFIH1 ($r=0.903$; $p<0.0001$, data not shown) in monocytes of pSS patients was observed. A role for Viperin in the RLR pathway has to our knowledge not been described to date, however, the compound might facilitate downstream IRF translocation in these cells, resulting in more efficient IFN type I production.

In a comparison of extrinsically TLR7-triggered pDCs of healthy controls (Birmachu *et al.*) and pDCs of IFNpos patients of this study we found the RLRs IFIH1 and DDX60 and their known regulators LGP2 (DHX58) and DDX60 among the top common genes upregulated [51]. We verified the upregulation of these endogenous intracellular RNA-sensing receptors and their regulators in pDC and monocytes of IFNpos pSS patients. The RLR family of receptors RIG-I and MDA5 has recently been described to collaborate with the TLRs [15, 20, 21] in inducing IFN type I expression in pDCs and monocytes. The following observations of our study support this view: 1) *In vitro* studies with PBMCs of healthy controls revealed triggering of the TLR7 pathway to cause an upregulation of the RNA-sensing receptors RIG-I and MDA5. Specific TLR7 antagonism



subsequently inhibited the TLR-induced genes. A similar experiment in the CAL-1 pDC cell-line [52] showed comparable results (data not shown). *II*) In immunocytochemistry, monocytes with high MDA5 protein expression showed clear nuclear expression of pIRF7, indicating TLR pathway activation combined with MDA5 expression in monocytes of IFNpos pSS patients. Interestingly, MDA5 upregulation has previously been described in glands of an SS-like murine model (C57BL/6.NOD-Aec1Aec2) [53]. Although only a limited number of biopsies were studied, we observed TLR7, RIG-I and MDA5 expression in salivary gland infiltrates indicating that in addition to a possible role in the systemic activation of IFN type I these molecules also could contribute to the local IFN activation in the glands. *III*) Monocytes of IFNpos pSS patients express the RLR-downstream signalling molecule TBK1 at an increased level compared to IFNneg pSS patients. Taken together, the RNA-sensing receptors – TLR7, RIG-I and MDA5 – seem to act together in amplifying the pathogenic IFN-driven loop in IFNpos pSS patients.

In pDCs and monocytes of IFNneg pSS patients the expression profile of nucleic acid sensing receptors was completely different from that of IFNpos patients. Monocytes and pDCs of IFNneg pSS patients had a decreased TLR9 expression compared to HCs, whereas TLR7 expression was normal with downstream molecules also virtually normal (yet MyD88 was slightly raised and RSAD2 slightly down in monocytes). Interestingly, the RLRs RIG-1 and MDA5 were significantly downregulated in the antigen presenting cells in IFNneg pSS patients. Apparently this profile of exogenous- and endogenous-pathway RNA-sensing receptors prevents an IFN response, with potential downstream consequences for autoantibody production and development of systemic manifestations. The altered expression of these nucleic acid sensing receptors in IFNneg pSS patients as compared to HCs in addition indicates, that - similar as observed in the NOD mouse model for sialoadenitis - a downregulation of RNA-sensing receptors might coincide with a particular disease phenotype [53], although different from that in the IFNpos pSS patients. This idea is in accord with the concept that a perfect balance of these sensing receptor systems is essential for preservation of tolerance, and that dysregulation can result in systemic autoimmunity.

Our observation of similar patterns of expression dysregulation of RNA-sensing receptors in monocytes and pDCs points to an intrinsic aberrancy in the expression of these sensing receptors in innate immune cells in pSS patients. The cause of this intrinsic aberrancy remains to be established, but several mechanisms can be deduced from the literature and our own body of work. The abundant presence of the ligands of these PRRs has been shown to contribute to the increased expression of for instance TLR7. We also showed that *in vitro*, that the TLR7 agonist Imiquimod induced expression of TLR7 as well as of RLRs in PBMC, while downregulating TLR9. In pSS, the abundance of nucleic acid-containing ICs and apoptotic cell debris are likewise to contribute to increased TLR7 expression. Interestingly an increased presence of autoantibodies against ribonucleic proteins (RNA associated antigens) potentially resulting in RNA-containing ICs is associated with IFN positivity [54]. For the RLRs endogenous intracytoplasmic nucleic acids as a result of chronic damage or the aberrant intracellular processing of nucleic acids can

potentially activate the RLR induced IFN production. Also mutations in the RLR genes, such as observed in Aicardi-Goutieres syndrome and some cases of SLE, can result in the sustained activation or enhanced sensitivity to triggering leading to IFN production [22, 23]. In this respect it is also of interest to note that small molecule endosomal TLR-inhibitors, such as the specific TLR7 antagonist IRS661, are gaining increased attention in autoimmunity as potential therapeutics [55-58]. Such inhibitors might be a potential novel therapy for pSS.

Limitations of this study are amongst others, the small sample for the pDC microarray analysis. Also the low number of arrays used decreases the power of the comparisons. However our genome-wide expression analysis has been verified in qPCR for the genes of interest, but we have to take into account that there might be some amplification bias, however minimal, due to the multiple amplification steps used for the small number of pDCs isolated from peripheral blood.

Despite these limitations we are able to conclude that there is an imbalance in the expression of the nucleic acid sensing receptors (TLR7 up, TLR9 down, RIG-I up and MDA5 up, and PKR up) in pDC and monocytes of IFNpos pSS patients, a profile which can explain the increased inflammatory pathogenic IFN production with downstream systemic effects in these patients. In IFNneg pSS patients the expression of TLR7 and PKR was normal in pDC and monocytes, that of TLR9 down, and that of both RIG-I and MDA5 down. We propose that this largely reduced expression of RNA-sensing receptors in these pSS patients explains in part their IFN negativity.

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SUPPLEMENTAL DATA

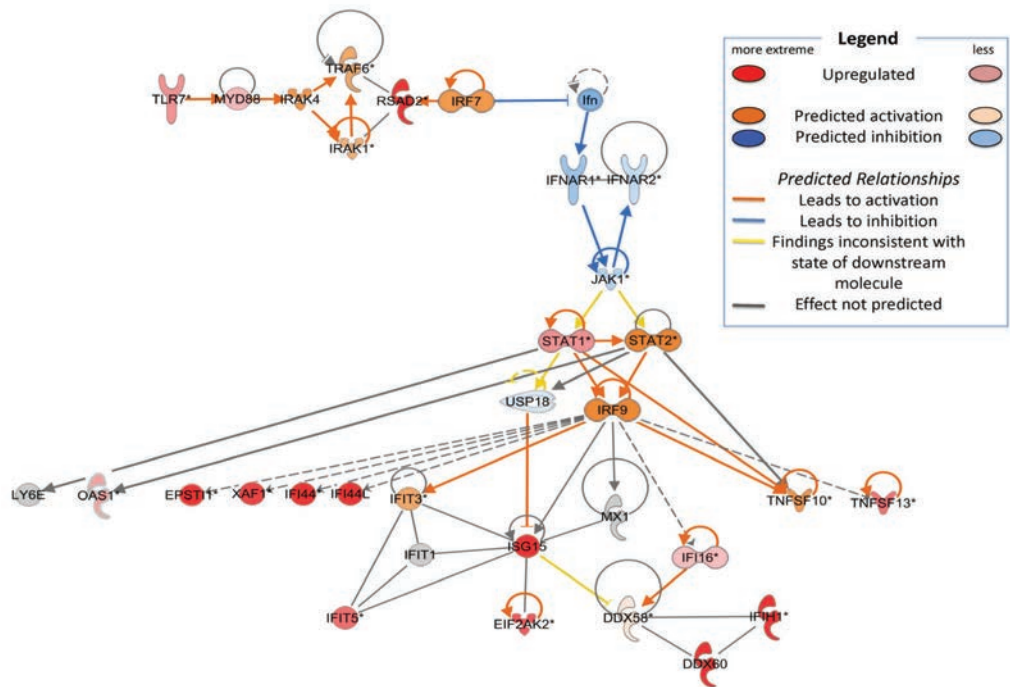


Figure S1. TLR7-induced pathway in IFNpositive pSS pDCs. Network depicted of the TLR7-induced IFN pathway in IFNpos pDCs compared to HC, built in Ingenuity pathway analysis (IPA). A schematic representation of the TLR7-induced network and downstream IFIGs is shown for the IPA comparison between IFNpos pSS and HC pDCs. Genes depicted in various shades of pink-to-red are significantly upregulated, depending on degree of upregulation and the (according to IPA), predicted upregulated (orange) and downregulated (blue) genes. Genes in grey did not reach significance according to subjected cut-off ($p\text{-value} < 0.05$; $FC \geq 2.00$). Interactions in this network are based on IPA-predicted relationships between the depicted genes. Dotted lines represent indirect interactions.

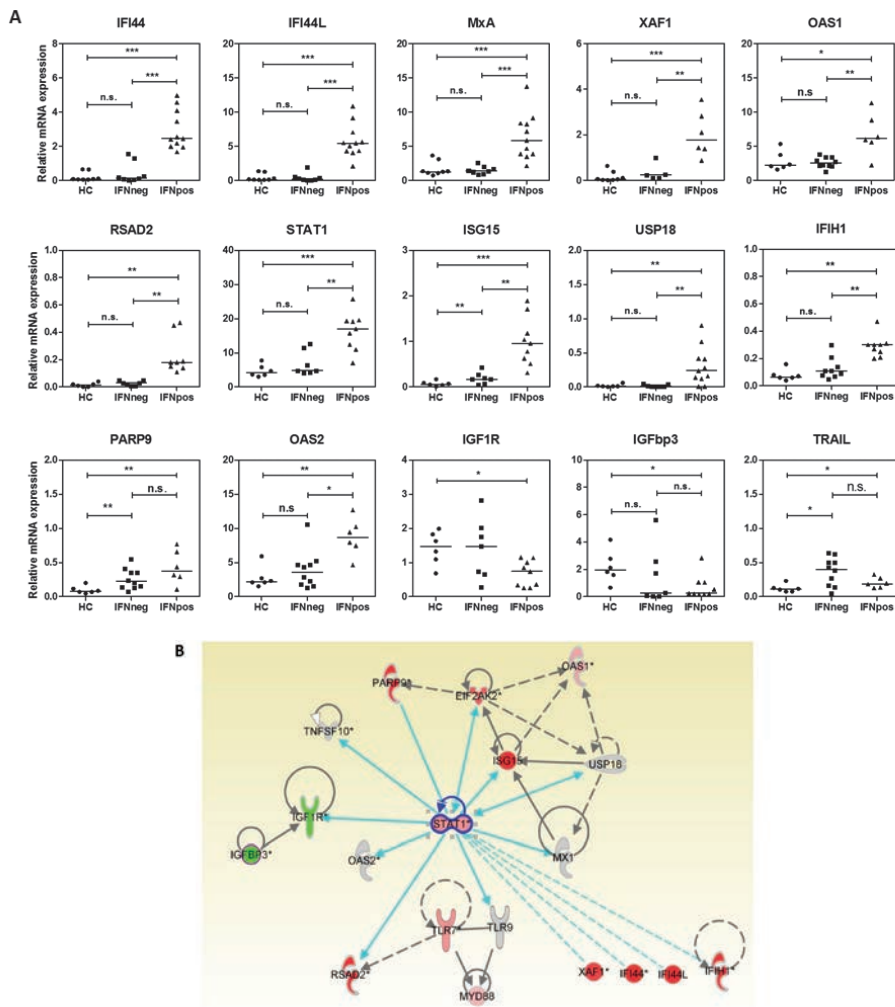


Figure S2. Validation DEGs significantly upregulated in circulating BDCA4⁺CD123⁺ plasmacytoid DCs of IFN type I signature positive pSS patients in steady state. **A)** Differentially expressed genes (DEGs) previously described as being IFN type I inducible genes (IFIGs) were also validated. Upper panel shows DEGs often used to calculate the IFN type I signature in pSS: IFI44 ($p < 0.001$), IFI44L ($p < 0.001$), MxA ($p < 0.001$), XAF1 ($p < 0.01$) [2, 12] and other IFIGs such as OAS1 ($p < 0.01$), STAT1 ($p < 0.001$), ISG15 ($p < 0.001$), USP18 ($p < 0.01$), and the nucleic acid sensor IFIH1 (also MDA5; $p < 0.01$), were significantly upregulated confined to IFNpos pDCs. Middle and lower panels show other IFIGs of interest, involved in the TLR- and IFN-signalling pathways: RSAD2 (encoding Radical S-Adenosyl methionine Domaining Containing 2), also known as Viperin, was significantly upregulated confined to IFNpos pDCs ($p < 0.01$). Viperin is an IFIG that regulates viral replication, and was recently shown to promote TLR7/9-dependent IFN type I production in pDCs [38, 39]. The genes PARP9 (poly ADP-ribose polymerase family member 9; $p < 0.01$) and TRAIL (also tumor necrosis factor apoptosis-inducing ligand or TNFSF10; $p < 0.05$) were significantly upregulated in both IFNneg and IFNpos pDCs compared to HC, and not significantly different within the pSS subgroups (Figure S3A). The insulin-like growth factor (IGF) genes, IGF1R ($p = 0.02$) and IGFbp3 ($p = 0.02$), were significantly downregulated in IFNpos pDCs compared to HC. **B)** When subjecting the validated DEGs to a hierarchical pathways analysis in IPA, STAT1 resulted as the central gene, connecting all other validated DEGs. STAT1 (signal transducer and activator of transcription 1) is a crucial transcription factor in the IFN signalling pathways, downstream of the IFNAR [40, 41].

Table S1. TLR7-driven DEGs (n=44) overlapping between TLR7-triggered pDCs and IFNpos pDCs.

IFNpos vs HC pDC				Birmachu TLR7 stim vs HC pDC		
Gene Symbol	Gene ID	p-value	FC	Gene Symbol	Gene ID	FC
ADAM9	1570042_a_at	9.06E-04	12.138	ADAM9	202381_at	3.524
ASNS	205047_s_at	2.52E-02	2.495	ASNS	205047_s_at	2.558
CD83	204440_at	2.83E-02	7.485	CD83	204440_at	3.803
CFLAR	208485_x_at	7.39E-03	3.457	CFLAR	210563_x_at	8.375
CHMP5	218085_at	3.19E-02	4.162	CHMP5	219356_s_at	2.573
CPD	229600_s_at	4.22E-02	3.643	CPD	201942_s_at	5.994
CSNK2A1	212072_s_at	1.81E-03	2.738	CSNK2A1	206075_s_at	2.219
DDX60	218986_s_at	1.95E-02	11.305	DDX60	218986_s_at	14.156
EIF5	241843_at	3.22E-02	2.44	EIF5	208708_x_at	2.937
EXT1	230183_at	1.16E-02	6.296	EXT1	201995_at	32.449
GCLC	202923_s_at	1.40E-02	2.482	GCLC	202923_s_at	5.513
GTPBP4	218239_s_at	1.30E-02	2.658	GTPBP4	218239_s_at	2.82
IFI44L	204439_at	1.46E-02	24.235	IFI44L	204439_at	21.22
IFIH1	219209_at	4.22E-03	7.657	IFIH1	219209_at	3.922
IFIT5	203595_s_at	1.77E-02	4.669	IFIT5	203595_s_at	9.036
ISG15	205483_s_at	4.65E-03	9.768	ISG15	205483_s_at	24.157
MCL1	227175_at	1.69E-02	6.795	MCL1	200798_x_at	2.573
MNDA	204959_at	1.21E-02	16.352	MNDA	204959_at	21.765
MYD88	209124_at	4.18E-02	2.241	MYD88	209124_at	2.608
NAMPT	217739_s_at	2.90E-02	3.14	NAMPT	217739_s_at	7.382
NFYA	204107_at	5.16E-03	3.704	NFYA	204109_s_at	4.001
NOTCH2	212377_s_at	2.33E-02	2.102	NOTCH2	210756_s_at	3.839
OAS1	205552_s_at	4.97E-03	3.053	OAS1	205552_s_at	12.461
PICALM	212511_at	5.11E-04	2.399	PICALM	215236_s_at	9.087
PIM2	204269_at	4.14E-02	4.196	PIM2	204269_at	3.519
PIP5K1A	207391_s_at	1.29E-02	2.848	PIP5K1A	211205_x_at	3.369
PLSCR1	202446_s_at	1.28E-03	5.671	PLSCR1	202446_s_at	9.911
PSMA2	201316_at	4.17E-02	2.377	PSMA2	201317_s_at	2.486
RAB8B	219210_s_at	4.35E-02	2.985	RAB8B	219210_s_at	7.662
REL	235242_at	1.97E-02	3.198	REL	206036_s_at	7.627
RSAD2	213797_at	2.88E-02	11.922	RSAD2	213797_at	79.45
SENP5	222110_at	2.52E-02	4.387	SENP5	57703_at	2.506
SOD2	216841_s_at	3.41E-02	2.658	SOD2	215223_s_at	3.805
SP100	202863_at	1.70E-02	2.949	SP100	202863_at	3.448
SPTLC2	216202_s_at	6.00E-03	4.281	SPTLC2	203127_s_at	2.17
SRSF10	206095_s_at	5.39E-04	2.278	SRSF10	210178_x_at	2.174
STK4	225364_at	2.82E-02	2.252	STK4	211085_s_at	7.031
TFEC	206715_at	1.21E-02	2.826	TFEC	206715_at	3.718
TMEM194A	212621_at	1.95E-02	7.099	TMEM194A	212619_at	5.94
WDR74	221712_s_at	2.25E-03	2.974	WDR74	221712_s_at	2.926
XAF1	228617_at	2.80E-02	6.245	XAF1	206133_at	11.986
ZBTB43	204180_s_at	4.07E-02	4.835	ZBTB43	204182_s_at	2.95
ZCCHC2	222816_s_at	3.93E-02	5.197	ZCCHC2	219062_s_at	2.948
ZNF107	243312_at	6.14E-04	6.245	ZNF107	205739_x_at	2.343

Grey genes are the top 10 overlapping DEGs between datasets

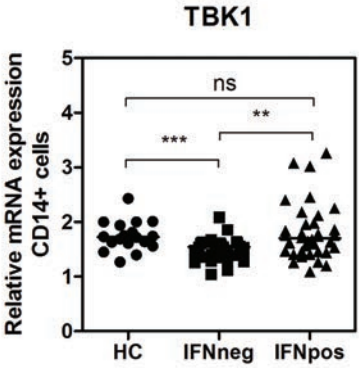


Figure S3. TBK1 is upregulated in CD14⁺ monocytes of IFN-positive pSS patients. TANK-binding kinase 1 (TBK1) is a crucial downstream signalling molecule in the RLR signaling pathway, showing a similar pattern in CD14⁺ monocytes as observed for RIG-I (DDX58) and MDA5 (IFIH1).





Maria '12

Chapter

6

Increased Tregs associated with elevated indolamine-2,3,-dioxygenase activity and an imbalanced kynurenine pathway in Interferon positive primary Sjögren's syndrome

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ABSTRACT

Introduction Indoleamine-2,3-dioxygenase (IDO), the rate-limiting enzyme converting tryptophan (TRP) to kynurenine (KYN), is driven in part by type I/II IFNs. Naïve T cells are polarized into FoxP3⁺ regulatory T cells (Tregs) upon exposure to either IDO⁺ cells or KYN. Recent studies suggest the KYN pathway to reflect a crucial interface between the immune and nervous system. Here we hypothesized an increase in Tregs, in concordance with increased IDO activity, in IFN signature positive primary Sjögren's syndrome (pSS) patients and investigated the downstream KYN pathway.

Methods Serum of 71 Healthy controls (HC), 58 IFNnegative (IFNneg) and 66 IFNpositive (IFNpos) pSS patients was analyzed using HPLC, for TRP and KYN levels. CD14⁺ monocyte mRNA-expression of IDO and downstream enzymes in the KYN pathway were assessed using real-time quantitative PCR. CD4⁺CD45RO⁺T helper (Th) memory cell populations were analyzed by flow cytometry.

Results IDO activity (KYN/TRP-ratio; $p=0.0054$) and percentage of CD25^{hi}FoxP3⁺Tregs ($p=0.039$) were significantly increased in IFNpos pSS, and correlated significantly ($p=0.002$; $r=0.509$). Circulating IFNpos pSS monocytes upregulated IDO₁-expression ($p<0.0001$), which correlated with the IFNscore ($p<0.0001$; $r=0.816$). Interestingly, the pro-apoptotic and neurotoxic downstream enzyme KMO ($p=0.0057$) was upregulated, whereas KAT_I ($p=0.0003$), KAT_{III} ($p=0.016$) and KAT_{IV} ($p=0.04$) were downregulated in IFNpos pSS compared to HC.

Conclusion Here we find enhanced IDO activity in coherence with increased CD25^{hi}FoxP3⁺Tregs, and identify an imbalanced KYN pathway with evidence for a shift towards potentially more pro-apoptotic and neurotoxic metabolites in IFNpos pSS patients. Intervening in these IFN and IDO-induced imbalances offers a new array of possibilities for therapeutic interventions in pSS.

Keywords: Sjögren's syndrome, Interferon, Indoleamine-2,3-dioxygenase, Treg Cells

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by functional impairment of the salivary and lacrimal glands, causing ocular (keratoconjunctivitis sicca) and oral dryness (xerostomia), with frequent presence of multiple extraglandular manifestations, such as vasculitis, severe fatigue and multi-organ involvement (1-3). The systemic interferon (IFN) type I signature, as assessed by expression of multiple IFN-inducible genes, is present in over half of pSS patients and identifies a subgroup of patients with more active disease (4-6). Several relevant pathways downstream of IFN type I activation, such as the induction of B-cell activating factor (BAFF) have been identified (4, 7, 8). Interestingly in addition to IFN type I, recent findings provided evidence for a role of IFN type II (IFN γ) in the activation of IFN signature related genes in pSS salivary glands (9). A better understanding of the downstream effects induced by enhanced IFN activity in pSS is crucial in the development of new selective therapeutic interventions.

In this study we focus on the kynurenine (KYN) pathway, which can be rapidly induced by both type I and type II IFNs (10). The KYN pathway is the major metabolic pathway in mammals to catabolize tryptophan (TRP), tightly regulated by the immune system. Indoleamine-2,3-dioxygenase (IDO) is the rate limiting enzyme in this tryptophan catabolism (TRP-cat), degrading TRP to KYN (11). KYN can be converted into immunomodulatory, potentially neuroactive metabolites collectively termed "kynurenines" through two main routes: conversion by 1) the inflammatory-driven enzyme Kynurenine-3-Monooxygenase (KMO) into the pro-apoptotic metabolite quinolinic acid (QUIN), known to be a potent neurotoxic *N*-methyl-*D*-aspartate (NMDA) receptor agonist and 2) by kynurenine aminotransferases (KATs) into the immunosuppressive metabolite kynurenic acid (KYNA), a potentially neuroprotective NMDA-receptor antagonist. The conversion into either route is highly balanced under healthy, non-inflammatory conditions (see Figure 1A) (11-14).

IDO is a critical participant in the maintenance of peripheral immune tolerance. Enhanced IDO activity results in depletion of TRP, an essential amino acid required for T cell proliferation, thus limiting T cell responses. Additionally, IDO is able to induce the development of T regulatory (Treg) cells, necessary to preserve tolerance (15-17). CD4⁺ naïve T cells are converted into FoxP3⁺ Tregs upon exposure to either IDO⁺ dendritic cells (DCs) or KYN (13, 18-20). CD25^{hi}FoxP3⁺ Tregs represent a distinct subset of T cells that play a crucial role in maintenance of self-tolerance, by repressing immune responses to self (21-23). Consequently IDO functions at the interface between inflammation and tolerance, regulating and fine-tuning immune homeostasis whilst establishing tolerance to self.

The KYN pathway has been extensively studied in relation to neurodegenerative disorders, where KYNA plays a potentially neuroprotective role and QUIN can act as a neurotoxin (11, 14). There is increasing interest in the role of kynurenines in regulation and maintenance of immune



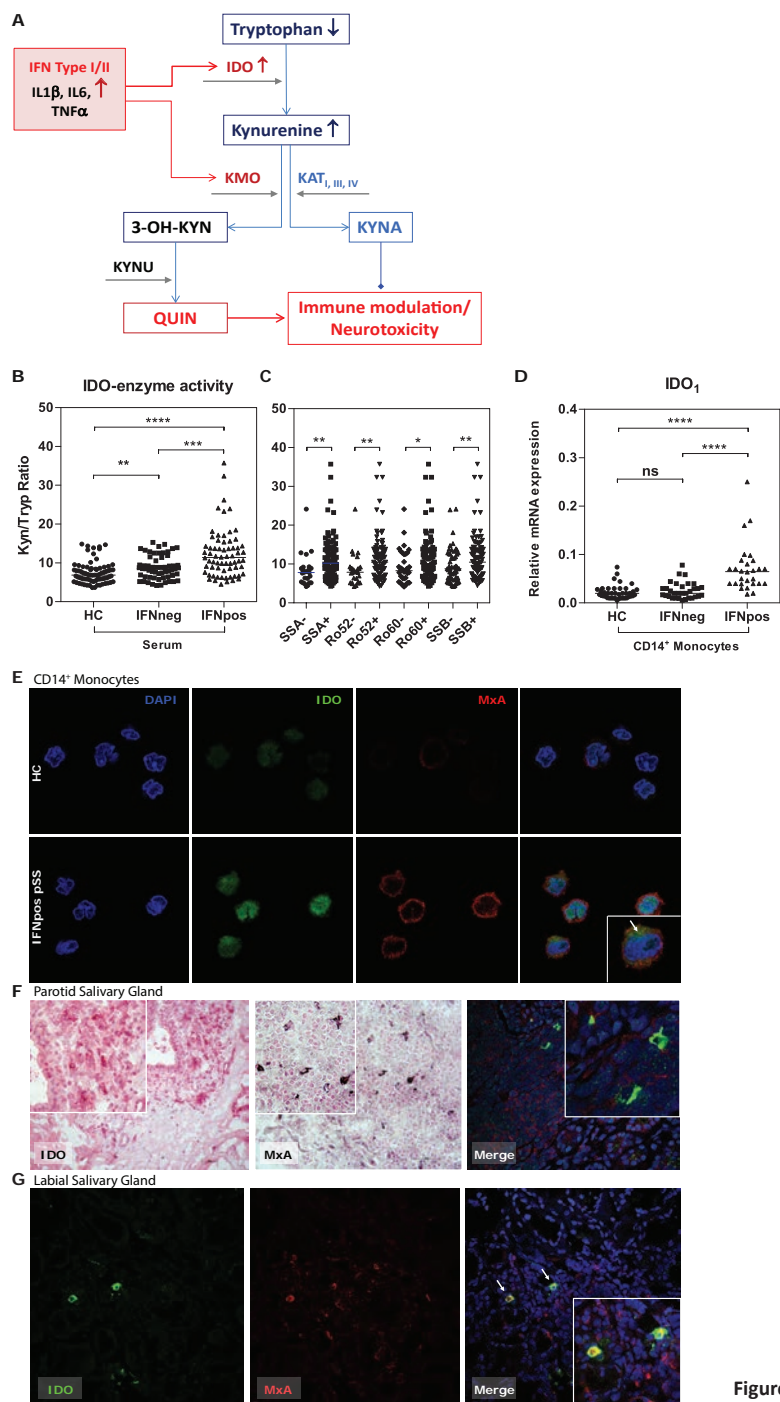


Figure 1.

homeostasis, and recent studies suggest this pathway to reflect a crucial interface between the immune and nervous system (13). The exact role of IDO and the regulatory kynurenine pathway in autoimmunity has, however, remained elusive.

We hypothesized enhanced IFN activity in pSS patients to result in higher IDO expression, and investigated the possible downstream effects of IDO activity on tolerance induction and the immunomodulating KYN pathway. In this study we stratified patients according to their IFN signature. To investigate the possible effects of IDO activity on tolerance induction and effects on T cell imbalances, we assessed T-helper (Th) memory populations in a subset of our pSS cohort. Here we find enhanced IDO activity in coherence with increased CD25^{hi}FoxP3⁺Tregs, and evidence for an imbalanced production of downstream immunomodulating metabolites, related to the IFN signature.

Figure 1 (see left). Enhanced IDO activity and expression in IFNpositive pSS. A) Schematic representation of tryptophan (TRP) catabolism into Kynurenine (KYN), driven by the key enzyme indoleamine-2,3-dioxygenase (IDO). IDO is induced by interferon (IFN) Type I/II and inflammatory cytokines. KYN can be converted into immuno- and potentially neuroactive downstream metabolites: 1) the pro-apoptotic and potentially neurotoxic metabolite quinolinic acid (QUIN), converted by Kynurenine-3-Mono oxygenase (KMO), or 2) the neuroprotective and immunosuppressive metabolite kynurenic acid (KYNA), converted by the kynurenine aminotransferases (KATs). *Red*; inflammatory-driven or potentially neurotoxic, *Light blue*; immunosuppressive and potentially neuroprotective. **B)** Serum concentrations of TRP and its metabolite KYN were measured by reversed-phase HPLC. The Kyn/Tryp-ratio reflecting IDO-enzyme activity depicted for Healthy controls (HC), IFNnegative (IFNneg) and IFNpositive (IFNpos) pSS patients, and **C)** pSS patients in the presence (+) or absence (-) of autoantibodies. **D)** IDO₁ mRNA-expression in CD14⁺ monocytes is depicted. *P* values are shown; **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.001. Horizontal lines indicate the median. To compare medians the Man Whitney-U test was used. **E)** Representative immunofluorescence images of IDO₁ (green) and MxA (red) in CD14⁺ monocytes of HC and IFNpos pSS. **F)** Immunohistochemical staining for IDO₁ and MxA in pSS *parotid* salivary gland (SG) biopsy, and confocal merge of IDO₁ (green) and MxA (red). **G)** Confocal zoom onto IDO₁ and MxA double-positive cells in *labial* SG biopsy (white arrows). Nuclei are stained with DAPI (blue).



PATIENTS AND METHODS

Patients

Patients positively diagnosed with pSS, according to the 2002 American-European criteria were recruited (24). Disease activity was assessed using the European league against rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) (25). Healthy controls (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 & Supplemental Table S1). The Medical Ethical Review Board of the Erasmus MC Rotterdam approved the study and written informed consent was obtained.

Blood collection, preparation and 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, peripheral blood mononuclear cells (PBMCs) were isolated using low-density gradient centrifugation within ≤4 hours (Ficoll; stored in liquid nitrogen).

Flow cytometric cell sorting

PBMCs were thawed, centrifuged 5 min (1500 rpm, 4 °C) and resuspended in 100 µl sort-buffer (PBS pH 7.4, 2mM EDTA 1M, 0.5% BSA). FACS (fluorescence-activated cell sorting) was used to sort PBMC cell suspensions. PBMC-membrane staining was performed with fluorescently labeled antibodies: anti-CD3 (PerCP-Cy5; Becton Dickinson Biosciences (BD), CA, USA), anti-CD14 (APC/Cy7; BD) and anti-CD19 (APC; BD), for 15 min in the dark. Cells were sorted using a FACSaria III cell sorter (BD). Reanalysis of sorted CD14⁺ monocytes confirmed >98% purity.

Measurement of complement, immunoglobulin levels and autoantibodies

C3 and C4 complement, IgG, IgA, IgM immunoglobulin and anti-SSA/B autoantibody levels were measured as described previously (4).

Analysis of tryptophan and kynurenine concentrations

Tryptophan and kynurenine were measured in serum samples using high-performance liquid chromatography (HPLC) coupled to UV/Fluorescence detection as previously described (26). Chromatographs were generated by CLASS-VP software (Shimadzu, UK). Results are expressed as ng of analyte per ml of serum. See supplemental files for full method description.

Real-time quantitative PCR

Total RNA was isolated from purified CD14⁺ monocytes using RNeasy columns (Qiagen, Hilden, Germany), subsequently reverse-transcribed to cDNA using a High-Capacity cDNA

Table 1. Demographics and clinical characteristics.

		pSS		
	HC (n=71)	IFNneg (n=58)	IFNpos (n=66)	
Cohort Demographics				
Female (%)	66/71 (93)	57/58 (98)	59/66 (89)	
Mean age (years)	52 ± 11.8	61 ± 9.9	56 ± 14.1	
IDO activity				One-Way ANOVA*
Kynurenine (ng/ml)	583.5 (254.4)	539.7 (191.8)	677.3 (295.1)	p=0.016
Tryptophan (ng/ml)	8201.4 (2920.2)	6451.1(1230.7)	5878.5(1475.1)	p<0.0001
KYN/TRYP ratio	7.4 (2.9)	8.8 (3.0)	12.4 (6.2)	p<0.0001
Patient characteristics				IFNneg vs IFNpos#
Disease duration (years)	-	11.0 ± 8.4	11.3 ± 7.8	n.s.
ESSDAI	-	2.7 ± 3.3	5.63 ± 6.9	p=0.019
Laboratory parameters				
Anti-SSA	-	36/58 (62)	63/66 (95)	p<0.0001
Anti-Ro52	-	24/51 (47)	47/51 (92)	p<0.0001
Anti-Ro60	-	31/51 (61)	46/51 (90)	p<0.0001
Anti-SSB	-	15/58 (26)	51/66 (77)	p<0.0001
IgG (g/l)	-	10.9 (4.6)	15.2 (4.0)	p<0.0001
IgA (g/l)	-	2.1 (1.5)	3.1 (2.1)	p<0.0001
IgM (g/l)	-	1.1 (0.7)	1.4 (1.0)	p=0.061
C3 (g/l)	-	1.19 (0.44)	1.07 (0.30)	p=0.21
C4 (g/l)	-	0.20 (0.09)	0.19 (0.1)	n.s.
Rf (IE/ml)	-	0.0 (25.0)	25.0 (100.0)	p=0.007
CRP (mg/l)	-	1.5 (3.8)	1.5 (6.8)	n.s.
Hb (mmol/l)	-	8.2 (0.90)	8.0 (1.1)	P=0.03
Medication status (%)				
Pilocarpine	-	22/58 (38)	23/66 (35)	n.s.
Hydroxychloroquine	-	21/58 (36)	37/66 (56)	n.s.
Fluconazol	-	6/58 (10)	9/66 (14)	n.s.
Corticosteroids	-	9/58 (16)	7/66 (11)	n.s.

Data are presented as mean ± SD, median (IQR) or as number (%) according to data distribution.

Groups were compared with *One-way ANOVA (all three groups) or #Man-Whitney U test (two pSS groups).

HC, Healthy controls; IFNneg, IFN type I signatur negative; IFNpos, IFN type I signature positive pSS, primary

Sjögrens syndrome; Ig, immunoglobulins; C, complement factor; Rf, rheumatoid factor; CRP, C-reactive protein; Hb, haemoglobin



Reverse Transcription Kit and RQ-PCR analysis using predesigned primer/probe sets, all according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). For calculation of relative expression, all samples were normalized to expression of the housekeeping gene *Abl* (27). Fold change values were determined from normalized CT values using $2^{-\Delta\Delta CT}$ method (User Bulletin, Applied Biosystems)(28).

IFN score

Monocyte IFN type I signature was defined by the relative expression of the 5 indicator genes – IFI44L, IFI44, IFIT3, LY6E and MxA (4). $Mean_{HC}$ 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 (29, 30). pSS patients were stratified according to their IFNscore in pSS patients positive for the IFN type I signature (*IFNpos*; IFNscore \geq 10) and patients negative for the signature (*IFNneg*; IFNscore $<$ 10) (4).

Immunohistochemistry

Labial and parotid salivary gland (SG) biopsies were obtained from pSS patients from University Medical Center Utrecht and University Medical Center Groningen, The Netherlands, respectively. SG biopsies, were cut into 6 μ m sections on a freezing microtome and mounted in a serial manner on Star Frost adhesive glass slides (Knittergläser, Braunschweig, Germany). See supplemental files for full MxA (ProteinTech group, IL, USA) and IDO₁ (kB9-1, kindly donated by Dr. Osamu Takikawa, Ōbu, Japan) staining protocols. The IDO₁ MoAb is highly specific and does not cross-react with IDO₂, as its epitope is localized at an N-terminus of the IDO₁ protein where there is no homology between IDO₁ and IDO₂. Slides were counter stained with hematoxylin (Merck, Darmstadt, Germany) and imbedded in Kaiser's glycerol gelatin (Merck). Appropriate isotype controls were used to confirm antibody-specificity.

Immunofluorescence

Sorted CD14⁺ monocytes were fixed with 4% paraformaldehyde onto poly-L-lysine (0.1 % w/v in water; Sigma P8920) coated slides (Nutacon, Leimuiden, Holland), seeding 1.5 x 10⁶ cells/eyeglass. SG biopsy-slides were fixed with acetone, air-dried and washed with PBS (as for immunohistochemistry). Slides were preincubated with normal chicken and goat serum (NChs, NGS) at RT for 10min before incubation with polyclonal MxA (ProteinTech) and IDO₁ MoAb (kB9-1) for 1hr, followed by staining with AlexaFluor-labeled chicken-anti-rabbit AF594 and goat-anti-mouse AF488 (Molecular Probes, Bleiswijk, Netherlands) with 2% normal human serum (NHS). Slides were mounted in Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) for 10 min. Appropriate isotype controls were used to confirm antibody-specificity. Microscopic analysis was performed on a ZEISS LSM700-confocal (63x objective). ZenSoftware 2009 was used for confocal image acquisition and processing, and ImageJ software for analysis.

Flow cytometry of CD14⁺ monocytes

Flow cytometric analysis of intracellular MxA protein expression in peripheral blood CD14⁺ monocytes (of 2 pSS patients with paired parotid biopsy material at time of sample collection) was performed as described previously (5).

Flow cytometry of T-helper cell populations

MoAb-preparations, intracellular cytokine detection and flow cytometry were performed as described previously (31). 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). 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.

Multiplex bead cytokine array

Serum-cytokine levels were assessed using a 27-MultiPlex cytokine, chemokine and growth factor magnetic bead-assay (Bio-Plex Pro M50-OKCAF0Y, Bio-Rad Laboratories, Inc., Veenendaal, The Netherlands), according to manufacturer's protocol. Serum samples were diluted 1:4 (serum was not thawed \geq 1). Samples were analyzed with the Luminex MAGPIX® Analyzer.

Statistical analysis

Comparisons were analyzed using the non-parametric Mann-Whitney U test to compare medians, and 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. Multiple group comparisons were analyzed using the Kruskal-Wallis or One-Way ANOVA test; Statistical analysis was performed using IBM SPSS 21.0 (SPSS, Chicago, IL, USA). Graphs were designed with Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA).

RESULTS

Enhanced IDO activity in relation to the IFN signature

To assess systemic blood levels of TRYP and KYN, HPLC analysis of these metabolites was performed simultaneously in sera of 71 HC, 58 IFN type I signature negative (IFNneg) and 66 IFNpositive (IFNpos) pSS patients (Table 1). Peripheral KYN levels were significantly increased in



IFNpos pSS, compared to HC ($p < 0.05$) and to IFNneg pSS ($p < 0.01$). In contrast, TRP levels were significantly decreased in both IFNneg ($p < 0.001$) and IFNpos pSS ($p < 0.0001$) compared to HC, showing significant TRP-depletion in pSS (see supplemental figure S1). Interestingly, TRP levels were significantly lower in IFNpos compared to IFNneg pSS ($p < 0.001$; see Table 1). The KYN/TRP-ratio – reflecting IDO enzyme activity (32) – was significantly increased in total pSS patients compared to HC ($p < 0.0001$), which is in line with previous findings (Figure S1) (33). Stratifying patients according to their IFNscore showed IDO activity (KYN/TRP-ratio) to be significantly increased in IFNneg pSS compared to HC ($p < 0.01$), reaching even higher significance in IFNpos pSS ($p < 0.0001$; see Figure 1B). Additionally, IDO activity was significantly higher in IFNpos compared to IFNneg pSS patients ($p < 0.001$).

We subsequently determined whether the KYN/TRP-ratio was associated with classical aberrant immune parameters of pSS, such as anti-SSA/SSB autoantibodies, rheumatoid factor (Rf), immunoglobulin (Ig) and C-reactive protein (CRP) levels (Table 1). KYN/TRYP-ratios were significantly higher in anti-SSA (divided in Ro52 & Ro60) and anti-SSB positive pSS patients (Figure 1B&S1), also positively correlating with other features of active disease; i.e. Rf ($p = 0.002$; $r = 0.359$), IgG ($p = 0.001$; $r = 0.297$), IgA ($p = 0.007$; $r = 0.308$), IgM ($p = 0.002$; $r = 0.359$) and CRP ($p = 0.001$; $r = 0.320$) (data not shown). ESSDAI disease activity scores were significantly increased in IFNpos compared to IFNneg pSS (Table 1), however, there was no significant correlation between total ESSDAI scores and the KYN/TRP-ratio.

Increased IDO expression in IFNpositive pSS

IDO is mainly expressed by cells of the myeloid lineage e.g. monocytes, dendritic cells (DCs) and macrophages (34, 35). Therefore, we assessed IDO expression in circulating CD14⁺ monocytes, as largest myeloid cell population in the blood and one of the precursors for tissue DCs. IDO consists of two gene paralogues, IDO₁ and IDO₂, IDO₂ being a recently discovered IDO₁-related gene lacking signaling activity (36). Enhanced IDO₁ mRNA expression in CD14⁺ monocytes was confined to IFNpos patients ($p < 0.0001$; Figure 1C). There was no detectable mRNA expression of IDO₂ (data not shown). A significant positive correlation was observed between IDO₁ mRNA expression in circulating monocytes and IDO enzyme activity, as reflected by the serum KYN/TRYP-ratio ($p = 0.008$; $r = 0.2861$, data not shown).

To assess IDO expression on protein level, FACS-sorted CD14⁺ monocytes were co-stained with antibodies specific for IDO₁ and Myxovirus-resistance protein A (MxA). MxA is an IFN-inducible protein, previously identified by us as a biomarker for systemic IFN activity in pSS (5). Herein MxA was used as a marker for IFN activity. Confocal microscopy revealed clear cytoplasmic MxA expression (red) in IFNpos pSS, which was mostly absent in HC monocytes (Figure 1D). Elevated IDO₁ expression (green) was observed in IFNpositive pSS, and occasional co-localization was observed for IDO₁ and MxA (white arrow). IDO₁ showed mainly a nuclear localization in CD14⁺ monocytes.

We next analyzed IDO₁ and MxA expression in parotid and labial SG biopsies. Immunohistochemical staining of pSS parotid SG revealed co-expression of these two proteins, which was confirmed by immunofluorescent co-staining (Figure 1E). Moreover, IDO₁ and MxA double positive cells were also observed in labial SG biopsies, particularly in the infiltrates (white arrows show co-localization; Figure 1F). Analysis of intracellular MxA protein expression in paired parotid SG biopsy- (immunohistochemistry) and peripheral blood monocyte-samples (using flow cytometry) of 2 pSS patients revealed both local and systemic overexpression of MxA (Figure 1D&S2).

Increased expression of immunomodulatory KMO, and decreased KAT expression in IFNpositive pSS

As described, KYN is metabolized through two main routes (Figure 1A). The QUIN-KYNA balance is regulated by KMO and the KAT enzymes respectively. Similar to IDO, KMO is also driven by pro-inflammatory cytokines such as Type I/II IFNs (11, 14, 20, 37). The KAT enzymes, a family comprising four isoforms (KAT_{I-IV}), have recently been shown to play a crucial role in mediating resilience to stress-induced depression (38). Here we find higher KYN levels in serum of IFNpos pSS patients (Figure S1B). These findings warranted further investigation of the downstream metabolism of KYN in circulating monocytes, as these downstream metabolites could have important immunomodulating properties. In a genome-wide microarray gene expression analysis from sorted CD14⁺ peripheral blood monocytes, we previously found KMO to be significantly upregulated (Fold Change (FC)=2.629) in IFNpos compared to IFNneg pSS [unpublished data; N.I. Maria *et al.*]. Confirming our microarray results, here we found increased mRNA expression of KMO in IFNpos pSS, compared to both HC (p=0.006) and IFNneg pSS (p<0.0001) (Figure 2D). Interestingly, KMO expression was in fact downregulated in IFNneg pSS compared to HC (p=0.014). In contrast to KMO, the enzymes KAT I, III and IV, catalyzing the conversion of KYN to the potentially immunosuppressive metabolite KYNA (13), were downregulated in IFNpos pSS (Figure 2A-C; KAT_I (p=0.0003), KAT_{III} (p=0.016) and KAT_{IV} (p=0.04)). KAT_{II}-expression could not be detected in isolated monocytes. These same KAT-isoforms were recently found upregulated in muscle of stress-resistant mice (38). Furthermore expression of the enzyme Kynureninase (KYNU), downstream of KYN, was significantly upregulated in both IFNpos and IFNneg pSS patients, compared to HC (Figure 2E). These findings indicate an imbalanced production of downstream immunomodulating tryptophan metabolites in IFNpositive pSS, and a shift towards more pro-apoptotic and neurotoxic QUIN in IFNpos pSS as shown in the schematic representation in figure 2F.



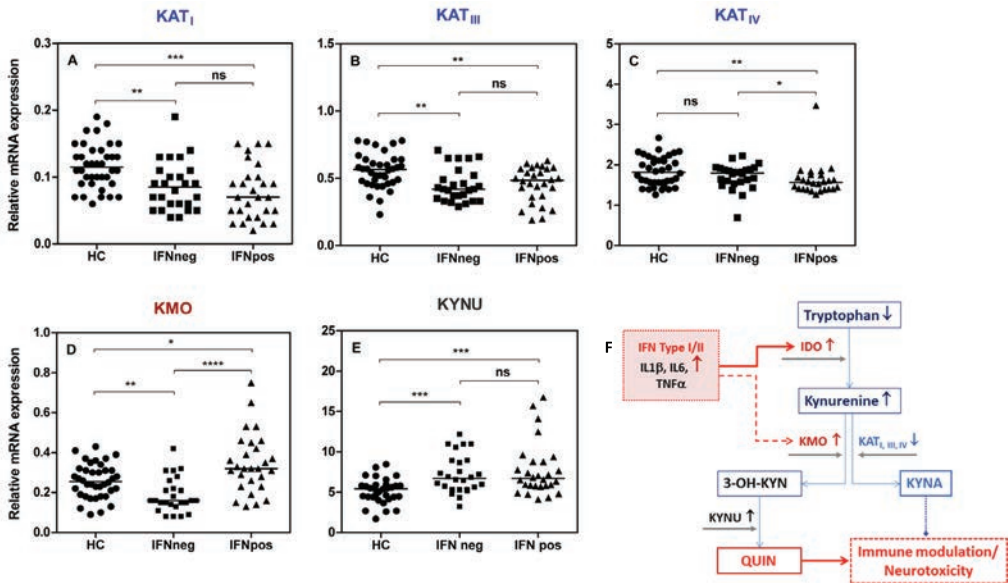


Figure 2. Potentially neuroprotective KAT-enzymes downregulated and potentially neurotoxic KMO-enzyme upregulated in CD14⁺ monocytes of IFNpositive pSS patients. Relative mRNA gene expression in CD14⁺ monocytes of pSS patients (n=54) and healthy controls (HC) (n=32). Patient group is stratified in patients negative for the IFN type I signature (IFNneg) (n=26) and patients positive for the signature (IFNpos) (n=28). The potentially neuroprotective KAT (Kynurenine aminotransferase)-enzymes **A)** KAT_I, **B)** KAT_{III}, and **C)** KAT_{IV}, (blue), **D)** The potentially neurotoxic KMO (Kynurenine-3-mono-oxygenase; red) and **E)** KYNU (Kynureninase). Each symbol represents an individual sample; To compare medians the Mann-Whitney U test was used; **p<0.01, ***p<0.001, ****p<0.0001. **F)** Schematic representation of the aberrant tryptophan catabolism in IFNpositive pSS patients, showing enhanced conversion of kynurenine and a skew towards the conversion of more quinolinic acid (QUIN), the pro-apoptotic and potentially neurotoxic metabolite, and less kynurenic acid (KYNA), the neuroprotective and immunosuppressive metabolite.

Enhanced IDO activity is associated with increased CD25^{hi}FoxP3⁺ Tregs in IFNpositive pSS

Under normal conditions IDO-induced TRP catabolism is a tolerogenic effector system, suppressing Teff-function and promoting Treg-differentiation (Figure 3) (18, 35). Here we investigated this possible role, and hypothesized an increase in Tregs in IFNpos pSS in concordance with increased IDO activity. Th cell populations were analyzed in peripheral blood of 20 HC, 18 IFNneg and 21 IFNpos pSS patients (Table S1). CD4⁺CD45RO⁺ Th memory cell populations were defined by chemokine receptor expression: CD25^{hi}FoxP3⁺ Tregs, and the CD25⁺ subsets CCR6⁺CCR4⁺CXCR3⁺CCR10⁺ Th17, CCR6⁺CCR4⁺CXCR3⁺CCR10⁺ Th22, CCR6⁺CXCR3⁺CCR4⁺ Th1 and CCR6⁺CXCR3⁺CCR4⁺ Th2-cells were analyzed by flow cytometry. To distinguish Th-subsets the gating strategy was used as previously described (Figure S3) (39). Interestingly,

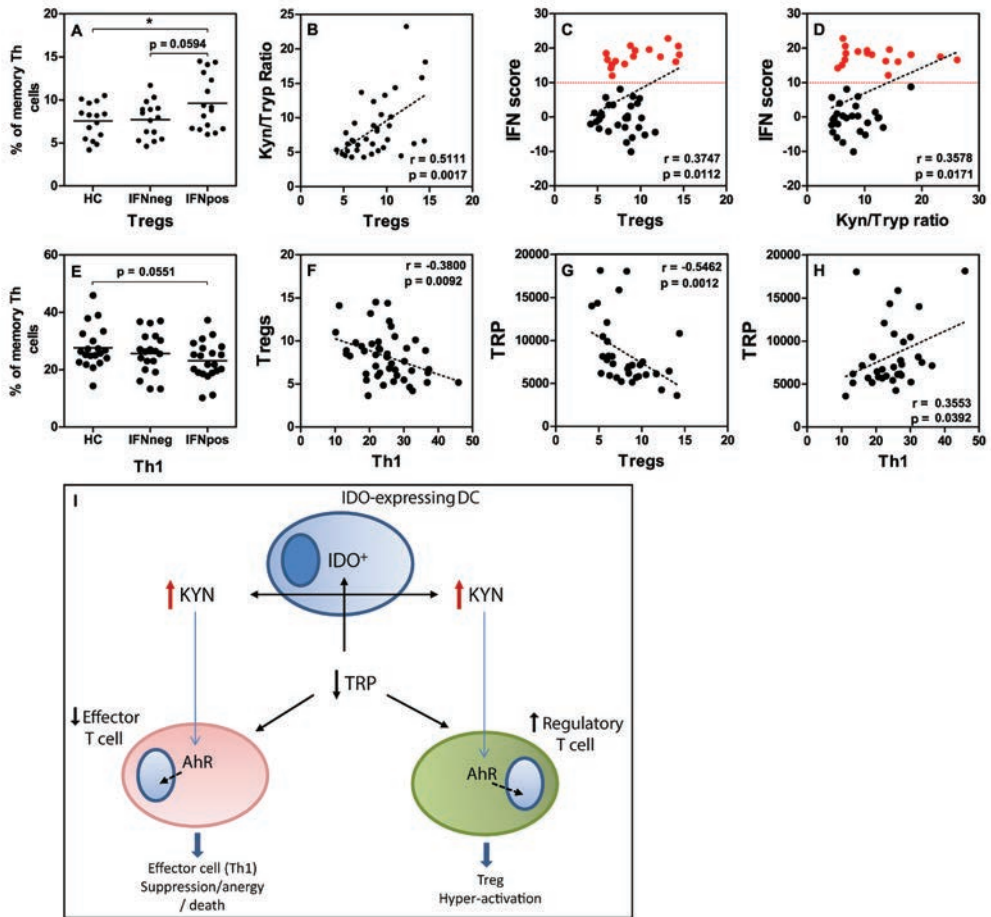


Figure 3. Percentage of CD25^{hi}FoxP3⁺ Tregs are increased in IFNpositive pSS and correlate with IDO activity. T helper memory (CD4⁺CD45RO⁺) cell populations were analyzed by flow cytometry in peripheral blood. **A)** Percentage of memory Th cells is depicted for CD25^{hi}FoxP3⁺ regulatory T (Treg) cell populations, stratified in healthy controls (HC; n=15), IFNnegative (n=15) and IFNpositive (n=16) pSS patients. Correlation between **B)** Tregs and the Kynurenine/Tryptophan (Kyn/Trp) ratio, reflecting IDO-enzyme activity, **C)** Tregs and the IFNscores and **D)** the Kyn/Trp ratio and IFNscores. **E)** Percentage of memory Th-cells is depicted for Th1 cell populations. Correlation between **F)** Treg and Th1 cell percentages, **G)** tryptophan (TRP; ng/ml) and Tregs **H)** TRP (ng/ml) and Th1 cells. Correlation coefficients (r) and P values are shown ($*p < 0.05$). Each symbol represents an individual sample; Pearson correlation test was used for normally distributed data (C), and the Spearman's rho for data not normally distributed. **I)** Tryptophan depletion particularly affects Th1 cells (suppression/energy/death) and induces Treg differentiation in a balance between immunity and tolerance. The Aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor, which regulates T cell balances and is thought to be a crucial factor in the regulation of immune responses; *Adapted from (35)*.

CD25^{hi}FoxP3⁺ Tregs were significantly increased in IFNpos pSS patients ($p=0.039$), also significantly correlating with IDO activity, as assessed by the KYN/TRP-ratio ($p=0.002$; $r=0.5111$), and the IFNscore ($p=0.011$; $r=0.375$) (Figure 3A-D). For the CD25⁺ Th-subsets no significant differences were observed (Figure S3), however Treg and Th1 ($p=0.009$; $r=-0.3800$) cell percentages negatively correlated, where Th1 positively correlated with TRP serum levels ($p=0.039$; $r=0.3553$) and Tregs negatively correlated with TRP ($p=0.001$; $r=-0.5462$) (Figure 3E-I).

In this smaller T cell cohort (Table S1), the disease activity as assessed by the ESSDAI significantly correlated with the IFNscore ($p=0.035$; $r=0.515$), in line with our previous findings (4, 5). For other clinical parameters, the IFNscore also correlated with serum IgG levels ($p=0.004$; $r=0.465$) and rheumatoid factor ($p=0.006$; $r=0.565$). Interestingly, serum IgG levels (g/l) significantly correlated with both IDO₁ ($p=0.01$; $r=0.392$) and KMO ($p=0.002$; $R=0.536$) mRNA expression in this cohort, and KMO highly correlated with the IFNscore ($p<0.0001$; $r=0.679$) (data not shown). Furthermore, Treg percentages correlated with CRP levels ($p=0.0254$; $r=0.422$), thrombocyte counts ($p=0.009$; $r=0.466$) and the rheumatoid factor (Rf; $p=0.0352$; $r=0.562$). There was however no statistical significance observed for Tregs and pSS autoantibody profile, or other clinical parameters measured in this study.

Elevated IDO-related inflammatory and immune-regulating serum cytokine levels in IFNpositive pSS

The capacity of DCs to initiate either tolerance or immunity, aside from cell intrinsic properties, is for a large part driven by their surrounding cytokine environment (20). We therefore assessed the inflammatory cytokine profile in serum of pSS patients and HC. Interleukin (IL)-1 α , IL-1 β , IL-2, IL-6 and tumor necrosis factor (TNF)- α are, next to IFN type I/II, known to enhance IDO enzyme activity both in the periphery and the central nervous system (CNS) (14, 40, 41). IL-1 α ($p<0.01$), IL-1 β ($p<0.05$), IL-6 ($p<0.05$) and TNF- α ($p<0.05$) were upregulated in IFNpos pSS compared to HC and IFNneg pSS (Figure 4). IL-2 ($p<0.05$) was only significantly upregulated between IFNpos and IFNneg pSS. IFN γ (IFN type II) showed a trend for upregulation, but did not reach significance. The regulatory cytokines IL-4 and IL-10, which are known to inhibit IDO activity as well as that of KMO (41), did not show a significant difference. The chemokine monocyte chemoattractant protein 1 (MCP-1/CCL2), involved in recruitment of inflammatory myeloid cells to target tissue such as the CNS (42, 43), was highly upregulated in IFNpos pSS patients compared to both HC ($p<0.0001$) and IFNneg pSS ($p=0.006$, Figure 4).

T cell functioning and plasticity is also highly regulated by the surrounding cytokine environment. IL-7 was previously shown to abrogate the suppressive capacity of Tregs, which was restored when IL-7 was removed (44). IL-7 overexpression has been described in labial SGs of pSS patients, correlating with both local and peripheral disease parameters (45). Here we found IL-7 ($p<0.05$) significantly elevated confined to IFNpos pSS (Figure 4).

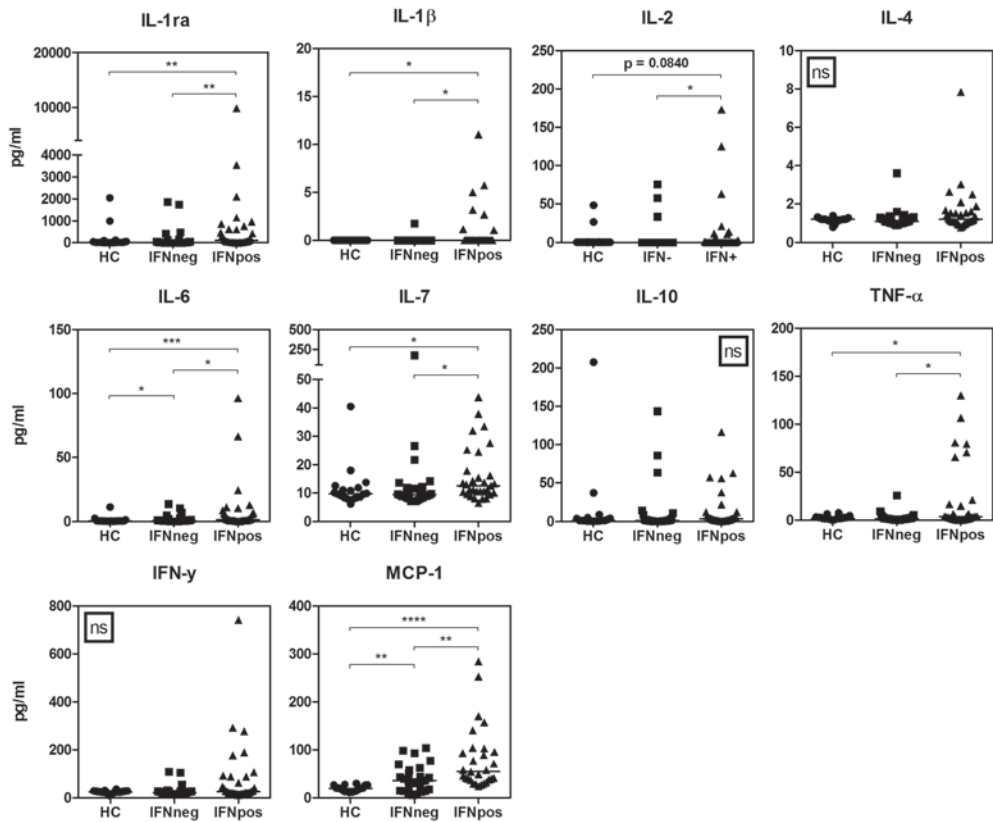


Figure 4. Elevated inflammatory and immune-regulating cytokines in serum of IFNpositive pSS patients. Serum cytokine and chemokine levels (pg/ml) were assessed in healthy controls (HC; n=18), IFNnegative (IFNneg; n=28) and IFNpositive pSS patients (IFNpos; n=31) with a multiplex bead-assay, and analyzed with the Luminex MAGPIX® Analyzer. *P* values are shown; **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001. Horizontal lines indicate the median. To compare medians the Man Whitney-U test was used. IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MCP-1 (CCL2), monocyte chemoattractant protein 1.

DISCUSSION

The exact role of IDO and the regulatory KYN pathway in autoimmunity has remained elusive, where IDO is placed at the interface between inflammation and tolerance induction. In this study, we identified an imbalanced KYN pathway in pSS. We found enhanced IFN activity to be associated with significantly higher IDO expression and activity, in coherence with increased numbers of circulating CD25^{hi}FoxP3⁺ Treg cells, and evidence for a shift towards potential overproduction of the metabolite QUIN downstream of KYN (Figure 5). Systemic KYN levels were

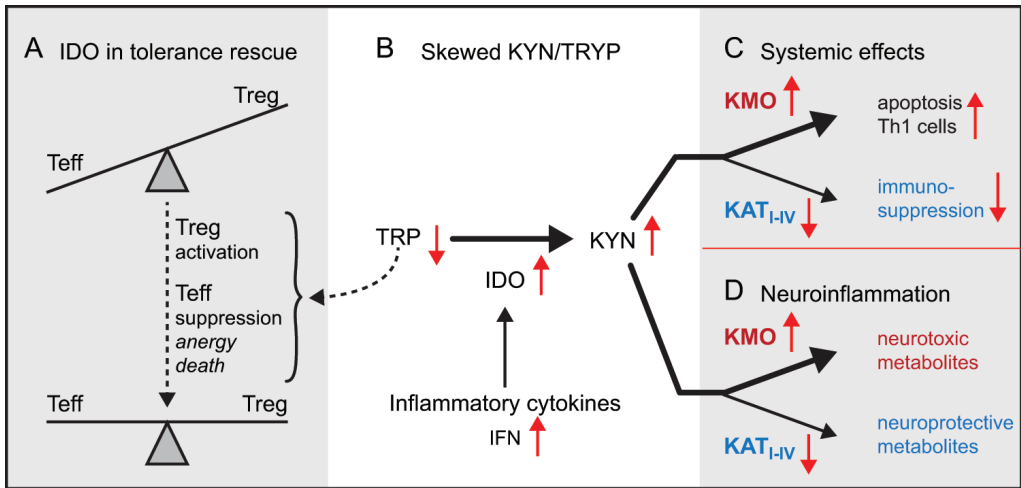


Figure 5. Hypothetical model of IDO-driven imbalances in IFNpositive pSS. **A)** Increased percentages of CD25^{hi}FoxP3⁺ Tregs as potential immune rescue-mechanism to increase “tolerance to self”, driven by tryptophan (TRP) depletion and directly by increased indoleamine-2,3-dioxygenase (IDO) expressing cells. **B)** Skewed kynurenine/tryptophan (KYN/TRYP)-ratio, reflecting enhanced IDO activity, is driven by increased IFN type I/II activity and other inflammatory cytokines. Imbalances in downstream enzymes KMO (Kynurenine-3-Mono oxygenase) ↑, and KAT_{I,II,IV} (kynurenine aminotransferases) ↓ causing **(C) Systemic effects:** increased Th1 apoptosis due to accumulation of pro-apoptotic and potentially neurotoxic metabolite quinolate (QUIN), while there is potentially less immunosuppression due to decreased neuroprotective and immunosuppressive metabolite kynurenic acid (KYNA). **(D) Neuroinflammation:** increased KMO ↑ and decreased KAT_{I,II,IV} ↓ expression potentially results in a skew towards more neuroinflammatory metabolites in IFN signature positive pSS.

found to be significantly increased in IFNpos pSS patients compared to HC whereas TRP levels were significantly decreased, resulting in an increased KYN/TRP-ratio, the measure reflecting systemic IDO enzyme activity.

In CD14⁺ monocytes, increased IDO₁ mRNA expression was correspondingly confined to IFNpos patients and highly correlated to the level of IFN activity, calculated as an IFNscore. There was no detectable mRNA expression of IDO₂, a recently discovered IDO₁-related gene lacking signaling activity (36). Recent findings indicate that in mice, IDO₂ gene-deletion leads to defects in autoimmune responses affecting the ability of IDO₁ to influence Treg generation, indicating a role for IDO₂ in controlling tolerance. The role of IDO₂ in loss of tolerance clearly warrants further investigation.

Increased IDO serum activity has previously been described in pSS and was associated with disease severity (33), however, the association with the IFN signature in pSS has not been studied before. Here we observe IDO₁ and MxA protein co-expression in pSS SGs. Furthermore we

describe simultaneous detection of MxA in SG and peripheral blood monocytes of the same pSS patient. Interestingly, IDO₁ showed mainly nuclear localization in CD14⁺ monocytes, although it was previously described to be recruited to and activated in the cytoplasm for catabolization of TRP into KYN (15). The observed nuclear localization might be consistent with the non-enzymatic nuclear function of IDO as a signaling molecule. This pathway contributes to IDO self-amplification in an IFN-dependent loop and results in a tolerogenic phenotype (17, 36).

To investigate the possible effects of IDO activity on tolerance induction, we assessed T-helper memory populations in a subset of our pSS cohort. CD25^{hi}FoxP3⁺ Tregs were significantly increased in IFNpos pSS and significantly correlated with IDO activity, indicating that increased IDO activity might be promoting Treg-differentiation in these IFNpos patients. Furthermore, the Treg/Th1 imbalance found here is in line with previous findings, that TRP-depletion particularly affects Th1 cells and promotes Treg-differentiation (Figure 3) (35). Tregs are suppressor T-cells, maintaining peripheral tolerance by inhibiting the activation and expansion of self-reactive T-cells. Expansion of these autoreactive T-cells occurs in an IL-7-rich environment. IL-7 was previously shown to abrogate the suppressive capacity of Tregs, which was restored when IL-7 was removed (44). The IL-7 increase observed here in serum of IFNpos pSS patients, could thus inhibit Treg-cell function, possibly resulting in loss of tolerance. Interestingly, a recent study investigating IFN α -influences on the suppressive activity of CD25^{hi}FoxP3⁺ Tregs, showed IFN α to abolish the suppressive function of Tregs without affecting their differentiation program (23). Further studies addressing the role of IDO and IL-7 on Treg-functionality in an IFN-rich environment are clearly warranted.

A limitation of this study is that it was not possible to quantify other downstream metabolites in our serum samples, besides TRYP and KYN, due to lack of HPLC-sensitivity. In addition to our observations of increased KMO expression in IFNpos pSS which could lead to QUIN accumulation, and decreased KATs possibly leading to less KYNA, directly measuring KYNA and QUIN levels could give further insight into the downstream effects of the observed imbalances. Furthermore, the detection of IDO₁ protein expression in salivary glands is limited to a small sample. Larger studies on local and systemic IDO expression are warranted.

The KYN pathway represents a crucial interface between the immune and nervous system (20). Firstly, depletion of TRP via enhanced IDO activity, affects bio-availability of serotonin, another product from TRP and an important neurotransmitter for mood regulation (11). Increased systemic IDO activity also leads to high levels of KYN, which in contrast to its downstream metabolites, can readily cross the blood-brain barrier (BBB) (12). Peripheral-blood-derived KYN accounts for roughly 60% of total brain-KYN, thereby highly contributing to the kynurenine pathway within the CNS (12, 38, 46, 47). Moreover, systemic blood level fluctuations of TRP and KYN directly affect the kynurenine pathway in the brain (11). Interestingly, reducing systemic KYN levels has recently been shown to protect the brain from stress-induced changes associated with depression (38). Particularly under inflammatory or stress conditions the bioavailability of KYN



in the brain determines the rate of neurotoxic QUIN production in the brain. Here, an increased expression of the enzyme KMO together with a decreased expression of the KAT enzymes was observed in IFNpos pSS monocytes, indicating higher QUIN production. Interestingly, decreased KMO expression was observed in IFNneg pSS, indicating potential overproduction of KYNA in these patients. Whether these imbalances in the downstream kynurenine pathway in circulating monocytes reflects the situation in the brain, is however not known but the effect of similar imbalances in microglia, the myeloid cells of the brain, can be envisioned. It is therefore tempting to speculate that the here described imbalances in TRP-cat also affects brain function, next to their effect on the immune system. pSS patients often experience disabling fatigue and a depressed mood as their most severe symptoms, reducing overall health-related quality of life (1-3). To date it is however largely unknown what factors cause these disabling symptoms in pSS. Our further studies will focus on the role of the imbalanced KYN pathway in pSS patients on symptoms of fatigue and depression. Furthermore, systemic immune-induced IDO activation has previously been shown to increase QUIN in both plasma and cerebrospinal fluid (CSF) with high correlation between the two, supporting the hypothesis that peripherally induced IDO can cause neurotoxic effects (48, 49).

We previously identified IFNpos pSS as a subgroup of patients with more active disease (4, 5). Enhanced IDO activity in IFNpos pSS is associated with more active biological aspects of pSS such as autoantibody positivity and Immunoglobulins, however, also with higher Treg percentages. Whether the shift in Tregs reflects an immune rescue-mechanism to increase “tolerance to self” in these patients, or rather represents a subset of non-functional cells remains to be established.

In conclusion, we find enhanced IDO activity in coherence with increased CD25^{hi}FoxP3⁺ Tregs, and aberrant downstream KYN metabolism with evidence for a shift towards potentially more QUIN and less KYNA production in IFNpos pSS patients. Intervening in these IFN and IDO-induced imbalances offers a new array of possibilities for therapeutic interventions in pSS.

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SUPPLEMENTAL FILES AND FIGURES

Analysis of tryptophan and kynurenine concentrations

Tryptophan and kynurenine were measured in serum samples using high- performance liquid chromatography (HPLC) coupled to UV/Fluorescence detection as previously described (26). The mobile phase contained 50 mM glacial acetic acid, 100 mM zinc acetate (Sigma, Ireland) and 3% acetonitrile dissolved in double-distilled NANOpure water HPLC grade H₂O (Sigma). The pH was adjusted to 4.9, using 5 M NaOH. Serum was diluted 1:1 in mobile phase containing 6% perchloric acid spiked with 200 ng/20 µl of *N*-methyl 5-HT (Sigma) as internal standard. Diluted serum samples were centrifuged at 20 000 rpm for 20 min and supernatants were filtered into new eppendorf tubes, using a 0.45 µm filter-fitted syringe (Phenomenex, UK). 20 µl of the filtered supernatant was injected using a Waters autosampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology C18 column with specific area of 100 mm × 4.6 mm and particle size of 2.6 µm, Phenomenex, UK) for separation of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP) was calibrated to analyze UV-spectra from 240-370 nm, and a fluorescent detector (Shimadzu RF-20A XS prominence fluorescence detector) set to excitation wavelength 254 nm; emission wavelength 404 nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu, UK). Results are expressed as ng of analyte per ml of serum.

Immunohistochemistry

Labial and Parotid Salivary gland (SG) biopsies were obtained from pSS patients from University Medical Center Utrecht and University Medical Center Groningen, The Netherlands. SG biopsies, were cut into 6µm sections on a freezing microtome and mounted in a serial manner on Star Frost adhesive glass slides (three sections per slide; Knittergläser, Braunschweig, Germany). Sections were fixed with acetone, air-dried and washed with phosphate-buffered saline (PBS) pH 7.8.

For MxA staining, tissue sections were incubated with a rabbit-anti-human polyclonal MxA antibody (ProteinTech group, IL, USA), followed by a secondary swine-anti-rabbit antibody labeled with horse radish peroxidase (DAKO, Glostrup, Denmark) with 2% normal human serum (NHS) to prevent non-specific binding. Development was performed by exposure to 0.01% di-amino-benzidine (DAB) in acetate buffer (pH 6.0) containing 1% NiSO₄ and 0.02% H₂O₂. The slides were counter-stained with nuclear fast red, dehydrated by an ethanol/xylene series and embedded with Depex mounting medium (BDH, Poole, England).

For IDO staining, tissue sections were incubated with a mouse-anti-human monoclonal antibody (MoAb) IDO₁ (kkB9-1, kindly donated by Dr. Osamu Takikawa, Ōbu, Japan), blocked with a avidine/biotine blocking-kit (Vector, Burlingame, UK), and incubated with secondary rabbit-anti-mouse biotinylated antibody (DAKO Glostrup, Denmark) with 2% NHS. Slides were

incubated with Alkaline Phosphatase-conjugated avidin/biotin-complex (StreptAB-Complex, DAKO). Development was performed by exposure to a phosphatase-new fuchsin substrate (Sigma-Aldrich, St. Louis, USA). The IDO₁ MoAb is highly specific and does not cross-react with IDO₂, as its epitope is localized at an N-terminus of the IDO₁ protein where there is no homology between IDO₁ and IDO₂.

Slides were counter stained with hematoxylin (Merck, Darmstadt, Germany) and imbedded in Kaiser's glycerol gelatin (Merck). Appropriate isotype controls were used to confirm antibody-specificity.

Table S1. Demographics and clinical characteristics of Tcell cohort.

		pSS	
	HC (n=20)	IFNneg (n=18)	IFNpos (n=21)
Demographics			
Female (%)	20/20 (100)	18/18 (100)	19/21 (91)
Mean age (years)	55 ± 11	59 ± 12	60 ± 13
Disease duration (years)	-	10.83 ± 6.9	12.64 ± 8.3
ESSDAI	-	2.75 ± 3.3	5.24 ± 5.8
Laboratory parameters			
Anti-SSA (n)	-	7/18 (39)	21/21 (100)
Anti-Ro52 (n)	-	7/18 (39)	21/21 (100)
Anti-Ro60 (n)	-	6/18 (33)	20/21 (95)
Anti-SSB (n)	-	4/18 (22)	16/21 (76)
IgG (g/l)	-	12.1 (8.15)	17 (4.65)
IgA (g/l)	-	2.05 (1.38)	4.2 (3.95)
IgM (g/l)	-	1.69 (3.73)	1.20 (2.41)
C3 (g/l)	-	1.41 ± 0.42	1.2 ± 0.28
C4 (g/l)	-	0.24 (0.12)	0.19 (0.05)
Rf (IE/ml)	-	0 (9.0)	0 (150)
CRP (mg/l)	-	7.5 (18.5)	6.0 (18.0)
Medication status (%)			
Pilocarpine	-	9/18 (50)	10/21 (48)
Hydroxychloroquine	-	12/18 (67)	15/21 (71)
Corticosteroids	-	1/18 (6)	2/21 (10)

Data are presented as mean ± SD, median (IQR) or as number (%) according to data distribution.

HC, Healthy controls; IFNneg, IFN type I signature negative; IFNpos, IFN type I signature positive; pSS, primary Sjögrens syndrome; Ig, immunoglobulins; C, complement factor; Rf, rheumatoid factor; CRP, C-reactive protein.



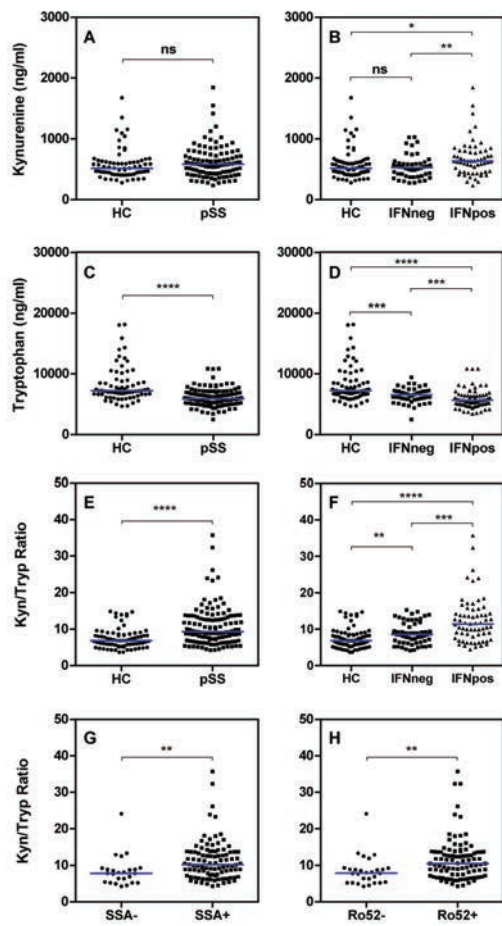


Figure S1. Enhanced Tryptophan catabolism in pSS.

Serum concentrations of Tryptophan and its metabolite Kynurenine were measured by reversed-phase HPLC simultaneously. **A)** Kynurenine, **C)** Tryptophan levels, and **E)** the Kynurenine per Tryptophan Ratio (reflecting IDO-enzyme activity) are shown in pSS (n=124) compared to Healthy controls (HC) (n=71). **B)** Kynurenine, **D)** Tryptophan levels, and **F)** the Kyn/Tryp ratio when stratifying pSS patients according to their IFNsignature, in IFNnegative (n=58) and IFNpositive pSS (n=66), compared to HC. Results are expressed as ng of analyte per ml of serum (ng/ml). The Kyn/Tryp ratio in the presence (+) or absence (-) of autoantibodies **G)** anti-SSA, **H)** anti-Ro52, **I)** anti-Ro60 and **J)** anti-SSB. Each symbol represents an individual sample; horizontal lines represent the median. To compare medians the Mann-Whitney U test was used; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

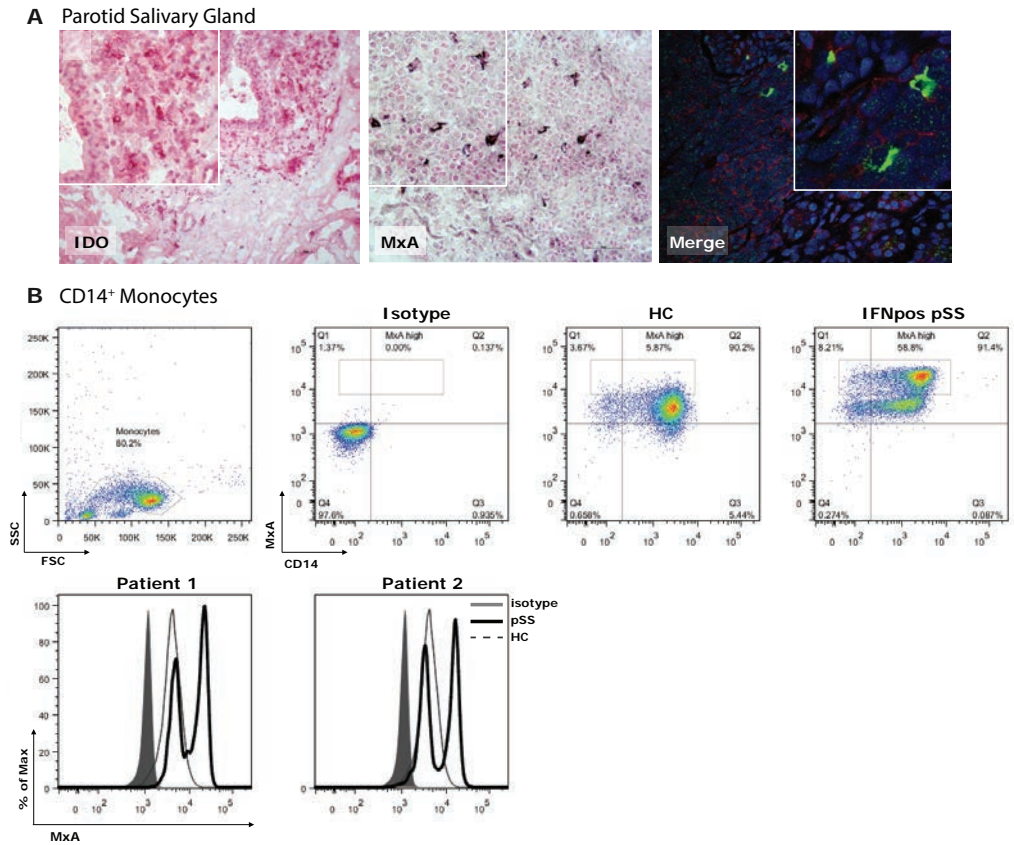


Figure S2. MxA expression in salivary glands and monocytes of pSS. A) Representative immuno-histochemical staining for IDO₁ (left) and MxA (middle) in pSS parotid salivary gland (SG) biopsy; (right) confocal staining of IDO₁ (green) and MxA (red) depicted as a merged image. **B)** Flow cytometric analysis of intracellular MxA protein expression in peripheral blood CD14⁺ monocytes of 2 pSS patients, whom also had IDO₁ and MxA-positive parotid SG biopsies at time of sample collection (as shown in A). In upper panel representative dotplots show CD14 and MxA protein expression in circulating blood monocytes for HC and IFNpositive (IFNpos) pSS patient, with appropriate isotype control. In lower panel histograms are depicted for MxA expression (% of maximum (max) mean fluorescence intensity), wherein shaded histogram represents the isotype control, solid lines represent the 2 MxA-positive pSS patients (patient 1 (left) and 2 (right)) and dotted line represents HC. Flow cytometric analysis was performed as previously described (5).

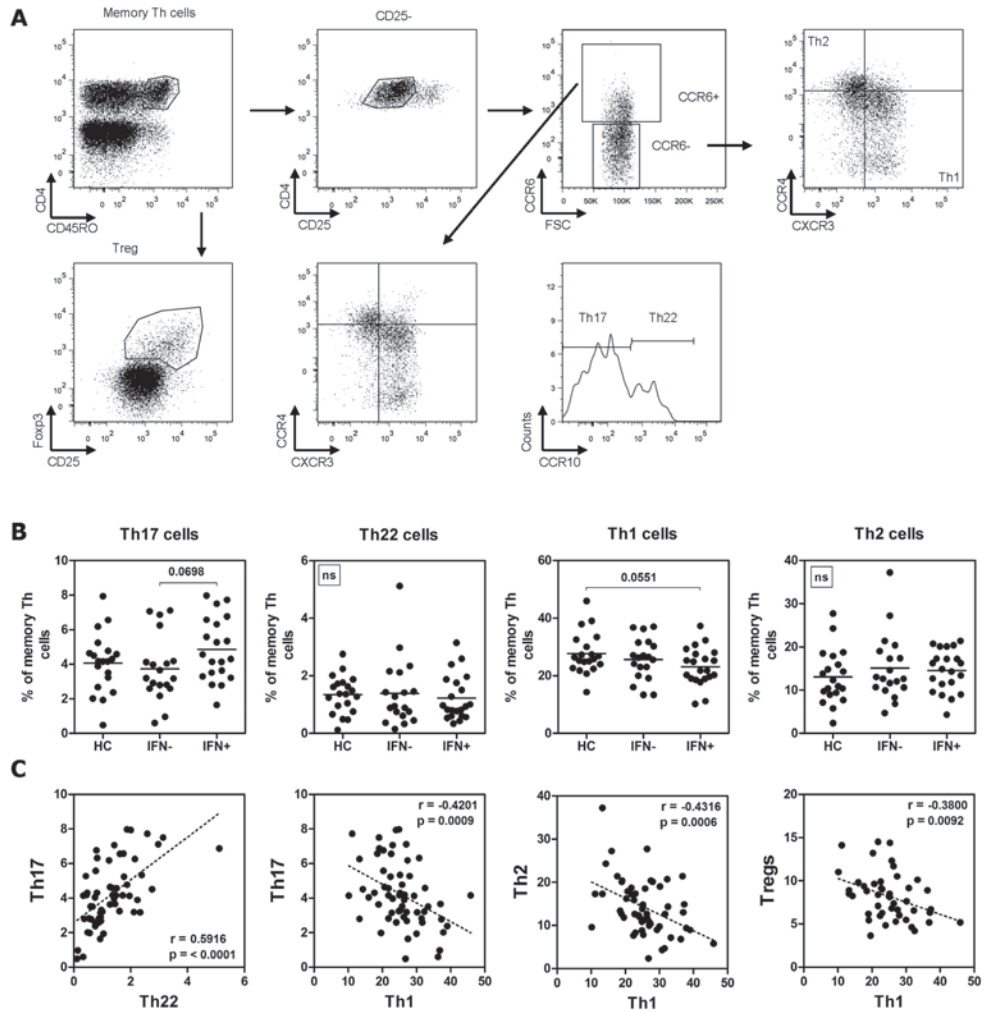


Figure S3. Memory T helper subsets in pSS, in relation to the IFN type I signature. (A) FACS analysis of CD4⁺CD45RO⁺ cells to obtain memory T helper (Th) subset populations; Th1, Th2, Th17 and Th22 cells were distinguished using chemokine receptor-expression, excluding CD25⁺ cells. These CD25⁺ cells were further gated to enriched for CD25^{hi}FoxP3⁺ Tregs. **(B)** Percentage of memory Th-cells, as defined by chemokine receptor expression, are depicted for Th17, Th22, Th1 and Th2- Th cell populations, stratified in healthy controls (HC; n=20), IFNnegative (IFN-; n=18) and IFNpositive (IFN+; n=21) pSS patients. **(C)** Significant correlations between Thelper populations.





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Chapter

7

Fatigue and depression in primary Sjögren's syndrome are not associated with an Interferon-driven production of tryptophan catabolites

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ABSTRACT

Introduction Primary Sjögren's syndrome (pSS) patients often experience disabling fatigue and depressed mood as their most severe symptoms. Indoleamine-2,3-dioxygenase (IDO), the rate-limiting enzyme converting tryptophan (TRP) to kynurenine (KYN), is driven in part by type I/II IFNs and systemic overactivation of IFN signaling is evident in primary Sjögren's syndrome (pSS). Interestingly aberrant systemic TRP catabolism (TRP-cat) resulting in a shift from neuroprotective towards neurotoxic downstream metabolites, collectively called kynurenines (kyns), has been associated with mood disturbances, and possibly contributes to symptoms of fatigue and depression in pSS. Here we investigate the possible association between enhanced IDO activity and an imbalanced KYN pathway and symptoms of fatigue and depression in pSS. We hypothesized that IFN overactivation in IFNpos pSS patients activates IDO, depletes TRP, increases KYN levels and skews the KYN pathway towards neurodegenerative components, resulting in increased fatigue and symptoms of depression.

Methods In serum of 54 Healthy controls (HC), 29 IFNneg and 30 IFNpos pSS patients analysis of TRP and Kynurenine (KYN) was performed simultaneously using HPLC. CD14⁺ monocyte mRNA-expression of IDO₁ and downstream KMO and KAT enzymes was assessed. Furthermore, pSS patients were characterized for disease activity (ESSDAI), fatigue (MFI-20) and depressive symptoms (CES-D).

Results In contrast to our hypothesis, 4 out of the 5 dimensions of fatigue showed a significant *negative* correlation with IDO activity (the KYN/TRP-ratio): physical fatigue ($r=-0.35$; $p=0.02$), reduced activity ($r=-0.32$; $p=0.04$), reduced motivation ($r=-0.35$; $p=0.02$) and mental fatigue ($r=-0.34$; $p=0.02$). Unexpectedly, symptoms of fatigue and depression did not correlate with the IFN signature. TRP serum levels positively correlated with 4 of the 5 dimensions of fatigue, with physical fatigue ($r=0.35$; $p=0.02$), reduced activity ($r=0.37$; $p=0.04$), reduced motivation ($r=0.43$; $p=0.004$) and mental fatigue ($r=0.45$; $p=0.002$). Furthermore TRP significantly correlated with CES-D depression scores in pSS ($r=0.34$; $p=0.03$).

Conclusion In this study, we were unable to find evidence that fatigue and depression are due to an IFN- driven higher production of kyns from TRP-cat. It is possible that alternative TRP-cat routes to serotonin, as well as other non-IFN related mechanisms, play a role in symptoms of fatigue and depression in pSS. Better understanding the role of increased TRP serum levels and symptoms of fatigue and depression in pSS might give new possibilities for specific targeting of these debilitating symptoms in pSS.

Key words: Depression, Fatigue, IDO, Interferon, primary Sjögren's syndrome, tryptophan

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by functional impairment of the salivary and lacrimal glands, causing ocular and oral dryness, as well as various systemic manifestations. pSS patients often experience disabling fatigue and a depressed mood as their most severe symptoms, reducing overall health-related quality of life. This extensively effects physical, psychological and social functioning, being a source of major disability in these patients [1-3]. To date it is however largely unknown what factors cause these disabling symptoms in pSS.

Several physiological models of fatigue have been proposed but the exact nature and pathophysiology of fatigue remain largely elusive [4]. The constant production of pro-inflammatory cytokines due to peripheral immune activation, can act on the brain causing so called sickness behavior [5]. Development of symptoms of fatigue and depression in vulnerable individuals suffering from systemic infections, cancer or autoimmune diseases, could in part be caused by a sustained systemic inflammatory state. A role for type I IFNs in fatigue and depression is supported by the induction of these symptoms in subjects receiving IFN-treatment for infectious diseases [6, 7]. On occasion these IFN-treated patients also reported characteristic pSS-like symptoms, such as dry eyes (keratoconjunctivitis sicca) and mouth (xerostomia) [8-10], supporting a role of IFN type I in pSS pathogenesis. We previously identified the presence of the IFN type I signature in over half of pSS patients. This subgroup of patients had higher disease activity and more systemic manifestations [11, 12].

Dysregulation of the kynurenine (KYN) pathway is linked to neuro-inflammation and has been associated with psychiatric diseases like depression [13]. The KYN pathway (Figure 1) is the major metabolic pathway in mammals to catabolize tryptophan (TRP), and is tightly regulated by the immune system. Indoleamine-2,3-dioxygenase (IDO) is the rate limiting enzyme in the KYN pathway, degrading TRP – an essential precursor of the neurotransmitter serotonin – to KYN [13]. Interestingly, IDO expression is regulated by both type I and type II IFNs [14]. In contrast to its downstream metabolites, KYN can readily cross the blood-brain barrier (BBB) [15]. In the brain, KYN is converted into neuroactive compounds through two possible routes: conversion of 1) the neurotoxic metabolite quinolinic acid (QUIN) by Kynurenine-3-Monooxygenase (KMO) and 2) the neuroprotective metabolite kynurenic acid (KYNA) by kynurenine aminotransferases (KATs), a family of potentially neuroprotective enzymes (Figure 1) [13, 15].

Systemic blood level fluctuations of TRP and KYN directly affect the KYN pathway in the brain [13]. Peripheral-blood-derived KYN accounts for roughly 60% of total brain KYN, thereby highly contributing to the KYN pathway within the central nervous system (CNS) [15-18]. KYN is metabolized to QUIN under inflammatory- and stress-conditions [19], and the reduction of systemic KYN levels has recently been shown to protect the brain from stress-induced changes associated



with depression [16]. The possible contribution of IDO and aberrancies of the KYN pathway to fatigue in pSS patients is unknown and here we address this item.

We recently identified an altered KYN pathway in pSS patients. Enhanced IFN activity was associated with significantly higher IDO expression and activity, and systemic KYN levels were significantly increased in IFN type I positive pSS patients compared to HC, whereas TRP levels were significantly decreased [N.I Maria *et al*, submitted; **Chapter 6**]. This resulted in an increased KYN/TRP-ratio, which reflects systemic IDO enzyme activity [20]. Furthermore we found evidence for a shift towards potential overproduction of the metabolite QUIN downstream of KYN, since the enzyme KMO was raised in circulating monocytes of IFNpos pSS patients, while the KAT enzymes were downregulated (Figure 1). Whether these imbalances in the downstream kynurenine pathway, as measured in the periphery, reflect the situation in the brain is however not known. Systemic immune-induced IDO activation has previously been shown to increase QUIN in both plasma and cerebrospinal fluid (CSF) with high correlation between the two, supporting the hypothesis that peripherally induced IDO can cause neurotoxic effects [21, 22].

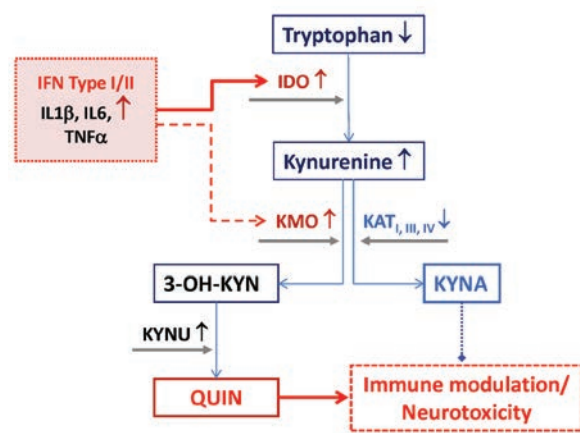


Figure 1. Enhanced IDO activity and an imbalanced Kynurenine pathway in IFNpositive primary Sjögren's syndrome. Schematic representation of the aberrant tryptophan catabolism into Kynurenine (KYN), driven by the key enzyme indoleamine-2,3-dioxygenase (IDO). IDO is induced by interferon (IFN) Type I/II and inflammatory cytokines. KYN can be converted into immuno- and potentially neuroactive downstream metabolites: 1) the pro-apoptotic and potentially neurotoxic metabolite quinolinic acid (QUIN), converted by Kynurenine-3-Mono oxygenase (KMO), or 2) the neuroprotective and immunosuppressive metabolite kynurenic acid (KYNA), converted by the kynurenine aminotransferases (KATs). In IFNpositive pSS patients, there is enhanced conversion of kynurenine and a skew towards the conversion of more QUIN and less KYNA, possibly leading to more immune modulation and neurotoxicity in IFNpositive pSS patients. *Red*; inflammatory-driven or potentially neurotoxic, *Light blue*; immunosuppressive and potentially neuroprotective.

In this study we further characterize pSS patients, stratified according to their IFN signature, for symptoms of fatigue and depression, and investigate the association between these symptoms and IDO-activity. We postulate the following mechanism: IFN overactivation in IFN positive pSS patients activates IDO, depletes TRP, increases KYN levels and skews the KYN pathway towards neurodegenerative components, resulting in increased fatigue and symptoms of depression in IFN positive pSS patients.

PATIENTS AND METHODS

Patients

Patients positively diagnosed with pSS, according to the 2002 American-European criteria were recruited [23]. Disease activity was assessed using EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI). The biological component of the ESSDAI entails a clonal component and/or hypocomplementemia (low C3 or C4) and/or hypergammaglobulinemia or high IgG levels, amongst others [24]. Healthy controls (HC), neither suffering from autoimmune diseases nor using corticosteroids, were included. Study subjects (see Table 1) were screened to be free of symptoms of underlying viral infections at inclusion. The Medical Ethical Review Board of the Erasmus MC Rotterdam approved the study and written informed consent was obtained.

Assessment of Fatigue and Depressive symptoms

pSS patients were characterized using validated questionnaires. Dimensions of fatigue were assessed in n=59 pSS patients using the Dutch version of the multidimensional fatigue inventory (MFI). The MFI is a 20-item self-report assessing five dimensions of fatigue: General Fatigue, Physical Fatigue, Mental Fatigue, Reduced Motivation and Reduced Activity [25]. Each dimension consists of 2 items indicating and 2 items contraindicating fatigue, in order to limit bias of answering tendencies. The MFI is constructed as a short, easy to administer questionnaire providing information on the nature of the experience and its intensity [25], previously used in pSS [1]. A higher score indicates higher levels of fatigue, ranging from 4-20 [1].

Depressive symptoms were assessed using the Dutch-validated Center for Epidemiologic Studies Depression (CES-D) self-report 20-item scale, considered to cover the most important components of depressive symptomology as experienced by the patient over the last week (reactive depression) [26]. The scale was assessed according to the guidelines described by Bouma J. *et al.*, 2012.

Blood collection, preparation and isolation

Blood was collected in clotting tubes for serum preparation (stored at -80°C), in PAXgene RNA tubes (PreAnalytix) for whole blood RNA analysis (stored at -80°C), and in sodium-heparin



tubes (Greiner Bio-One, Germany). From heparinized blood, peripheral blood mononuclear cells (PBMCs) were isolated using low-density gradient centrifugation within ≤ 4 hours (Ficoll; Stored in liquid nitrogen).

Analysis of tryptophan and kynurenine concentrations

Tryptophan and kynurenine were measured in serum samples using high performance liquid chromatography (HPLC) coupled to UV/Fluorescence detection as previously described [27]. The mobile phase contained 50 mM glacial acetic acid, 100 mM zinc acetate (Sigma, Ireland) and 3% acetonitrile dissolved in double-distilled NANOpure water HPLC grade H_2O (Sigma). The pH was adjusted to 4.9, using 5 M NaOH. Serum was diluted 1:1 in mobile phase containing 6% perchloric acid spiked with 200 ng/20 μ l of *N*-methyl 5-HT (Sigma) as internal standard. Diluted serum samples were centrifuged at 20 000 rpm for 20 min and supernatants were filtered into new eppendorf tubes, using a 0.45 μ m filter-fitted syringe (Phenomenex, UK). 20 μ l of the filtered supernatant was injected using a Waters autosampler and a Reverse Phase analytical column (Kinetex™ Core Shell Technology C18 column with specific area of 100 mm \times 4.6 mm and particle size of 2.6 μ m, Phenomenex, UK) for separation of metabolites. A PDA-UV detector (Shimadzu SPD-M10A VP) was calibrated to analyze UV-spectra from 240-370 nm, and a fluorescent detector (Shimadzu RF-20A XS prominence fluorescence detector) set to excitation wavelength 254 nm; emission wavelength 404 nm, were used to detect the metabolites. Chromatographs were generated by CLASS-VP software (Shimadzu, UK). Results are expressed as ng of analyte per ml of serum.

Measurement of complement, immunoglobulin levels and autoantibodies

C3 and C4 were measured using an Immage® nephelometer (Beckman Coulter, Woerden, The Netherlands). IgG, IgA, IgM were measured via turbidimetry using a Modular P800 (Roche, Almere, The Netherlands). Anti-SSA and anti-SSB were determined by EliA (Thermo Scientific), confirmed with ANA profile immunoblot (EuroImmun) and when discrepant, re-confirmed by QUANTA Lite ELISA-kit (INOVA).

Real-time quantitative PCR

Total RNA was isolated from purified CD14⁺ monocytes using RNeasy 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, all according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

Total RNA from PAXgene RNA tubes (PreAnalytix) was isolated according to manufacturer's protocol. For calculation of relative expression, all samples were normalized to expression of the household gene Abl [28]. Fold change values were determined from normalized CT values using $2^{-\Delta\Delta CT}$ method (User Bulletin, Applied Biosystems) [29].

Subtyping pSS patients according to the IFN type I signature

The Monocyte IFN type I signature

The Monocyte IFN type I signature was defined by the relative expression of the 5 indicator genes – IFI44L, IFI44, IFIT3, LY6E and MxA, as previously described. 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 [30, 31]. pSS patients were stratified according to IFNscore into patients positive for the IFN type I signature (*IFNpos*; IFNscore \geq 10) and patients negative for the signature (*IFNneg*; IFNscore $<$ 10) [12, 32].

Statistical analysis

Comparisons were analyzed using the non-parametric Mann-Whitney U test to compare medians (for skewed distributions) and independent T-test 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 ($n=5$ for the 5 dimensions of fatigue). Multiple group comparisons were analyzed using the Kruskal-Wallis or One-Way ANOVA test; Statistical analysis was performed using IBM SPSS 21.0 (SPSS, Chicago, IL, USA). Graphs were designed with Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA).

RESULTS

Fatigue and depressive symptoms are not significantly different between IFNpos and IFNneg pSS

The 59 pSS patients were stratified into IFNneg ($n=29$) and IFNpos ($n=30$) according to the monocyte IFN signature (Table 1). In this pSS cohort, CES-D depression scores highly correlated with all 5 dimensions of fatigue ($r \geq 0.50$; $p < 0.001$). CES-D showed the strongest correlation with the dimension of mental fatigue ($r=0.639$; $p < 0.0001$) and reduced motivation ($r=0.548$; $p < 0.0001$, data not shown).

All separate dimensions of fatigue, as well as the total MFI, were not significantly different between IFNpos and IFNneg pSS (Table 1). Moreover, total CES-D scores were also not significantly different between IFNpos and IFNneg pSS (Table 1). The 5 dimensions of fatigue were significantly higher in both the IFNneg and IFNpos pSS patients, compared to a “historic” group of healthy individuals (Table 2) [1]. In this comparison we used publically available data from 32 healthy women, age 26-82 (median 57) to visualize the increased dimensions of fatigue in total pSS. We however have to take into account that these acquired MFI-data from healthy



Table 1. Demographics and clinical characteristics.

		pSS		
	HC (n=54)	IFNneg (n=29)	IFNpos (n=30)	
Demographics				
Female (%)	50/54 (93)	26/29 (90)	28/30 (93)	
Mean age (years)	54.2 ± 9.8	61.5 ± 9.2	56.2 ± 11.9	
Disease duration (years)	-	14.1 ± 8.8	12.1 ± 8.2	n.s.
ESSDAI	-	9.5 ± 7.8	10.8 ± 8.2	n.s.
CES-D	-	10 (14)	14 (12)	n.s.
MFI				
General fatigue	-	14.38 ± 3.9	14.47 ± 4.5	n.s.
Physical fatigue	-	12.38 ± 4.4	13.10 ± 4.0	n.s.
Mental fatigue	-	11.69 ± 4.6	10.73 ± 4.4	n.s.
Reduced motivation	-	9.17 ± 3.7	9.7 ± 3.9	n.s.
Reduced activity	-	10.24 ± 3.7	10.80 ± 4.8	n.s.
Laboratory parameters				
Anti-SSA	-	19/29 (66)	28/30 (93)	p<0.0001
Anti-Ro52	-	17/29 (59)	29/30 (97)	p<0.0001
Anti-Ro60	-	15/29 (52)	29/30 (97)	p<0.0001
Anti-SSB	-	10/29 (34)	22/30 (73)	p<0.0001
IgG (g/l)	-	10.52 ± 3.61	16.38 ± 6.28	p<0.0001
C3 (g/l)	-	1.39 ± 0.34	1.25 ± 0.33	n.s.
C4 (g/l)	-	0.24 ± 0.08	0.22 ± 0.08	n.s.
CRP mg/l	-	2.00 (3.8)	2.10 (2.9)	n.s.
Medication status (%)				
Pilocarpine	-	10/29 (34)	8/30 (27)	n.s.
Hydroxychloroquine	-	19/29 (66)	16/30 (53)	n.s.
Corticosteroids	-	5/29 (17)	4/30 (13)	n.s.

Data are presented as mean ± SD, median (IQR) or as number (%) of patients according to data distribution.

Means or medians were compared using the unpaired t-test or the Man Whitney U test respectively.

HC, Healthy controls; IFNneg, IFNsignature negative; IFNpos, IFN signature positive; pSS, primary Sjögren's syndrome.

individuals from the study by Barendregt PJ *et al.*, as shown in Table 2, were controlled for a measure of depression by including it as a covariate in their analysis [1].

The 5 dimensions of fatigue and the CES-D depression score did not correlate significantly with the monocyte IFNscore in pSS patients. Whole blood MxA protein levels as assessed by the

MxA-EIA, previously described by us as a biomarker for the IFN type I signature in pSS [12], even negatively correlated with mental fatigue ($r=-0.278$; $p=0.036$).

Table 2. MFI fatigue dimension scores.

MFI dimensions	HC cohort*	pSS	
	HC (n=32)	IFNneg (n=29)	IFNpos (n=30)
General fatigue	8.16 ± 3.8	14.38 ± 3.9	14.47 ± 4.5
Physical fatigue	6.47 ± 3.2	12.38 ± 4.4	13.10 ± 4.0
Mental fatigue	6.53 ± 3.0	11.69 ± 4.6	10.73 ± 4.4
Reduced motivation	6.66 ± 2.4	9.17 ± 3.7	9.7 ± 3.9
Reduced activity	6.72 ± 3.0	10.24 ± 3.7	10.80 ± 4.8

* Healthy Control (HC) cohort was previously described by Barendregt PJ *et al.* 1998 [1].

Data are presented as mean ± SD

IDO activity correlates negatively, whereas serum tryptophan levels correlate positively with dimensions of fatigue in pSS

The systemic blood levels of TRP and KYN were assessed in sera of 49 HC, 25 IFNneg and 19 IFNpos pSS patients. The KYN/TRP-ratio – reflecting IDO enzyme activity [20] – significantly correlated with the monocyte IFN score ($r=0.371$; $p=0.018$) and the MxA-EIA ($r=0.431$; $p=0.004$) (Figure 2). Next, we correlated the KYN/TRP-ratio with aspects of disease activity in pSS, observing a positive correlation with IgG ($r=0.311$; $p=0.041$, Figure 1) and the biological component of the ESSDAI disease activity score ($r=0.376$; $p=0.012$, data not shown). Furthermore KYN serum levels positively correlated with total ESSDAI scores ($r=0.395$; $p=0.008$).

In contrast to what was hypothesized, the KYN/TRP-ratio negatively correlated with 4 of the 5 dimensions of fatigue (Figure 3), with physical fatigue ($r=-0.350$; $p=0.02$), reduced activity ($r=-0.317$; $p=0.036$), reduced motivation ($r=-0.349$; $p=0.02$) and mental fatigue ($r=-0.342$; $p=0.023$). The same 4 dimensions of fatigue – in contrast to our hypothesis – correlated positively with serum TRP levels: physical fatigue ($r=0.348$; $p=0.02$), reduced activity ($r=0.374$; $p=0.012$), reduced motivation ($r=0.425$; $p=0.004$) and mental fatigue ($r=0.446$; $p=0.002$). When applying a Bonferonni correction for multiple testing ($p<0.01$, $n=5$), the correlations between TRP with reduced motivation and mental fatigue remained significant (Figure 3). There was no significant correlation observed with general fatigue. Additionally, serum TRP levels positively correlated with CES-D depression ($r=0.337$; $p=0.025$) (Figure 2, lower panel).

We previously observed increased IDO1 and KMO, and decreased KAT mRNA expression in IFNpos pSS CD14+ monocytes, pointing towards a potential shift towards neurotoxicity in these patients [N.I Maria *et al.*, submitted; **Chapter 6**]. In this study, IDO₁ ($r=0.538$; $p=0.001$) and KMO ($r=0.416$; $p=0.12$) expression positively correlated with the KYN/TRP-ratio, whereas



KAT_I expression ($r=-0.435$; $p=0.008$) negatively correlated with the measure for IDO activity. We subsequently assessed if expression of these immunomodulatory enzymes correlated with symptoms of fatigue and depression in pSS. The sole association observed here was a negative correlation between IDO₁ mRNA expression in monocytes and reduced motivation ($r=-0.294$; $p<0.05$), which is in accordance with the negative association of IDO activity with signs of fatigue and depression. Since correlations between KMO expression and signs and symptoms of fatigue and depression were absent, this study does not lend support to the idea that skewing towards the neurotoxic arm determines these symptoms.

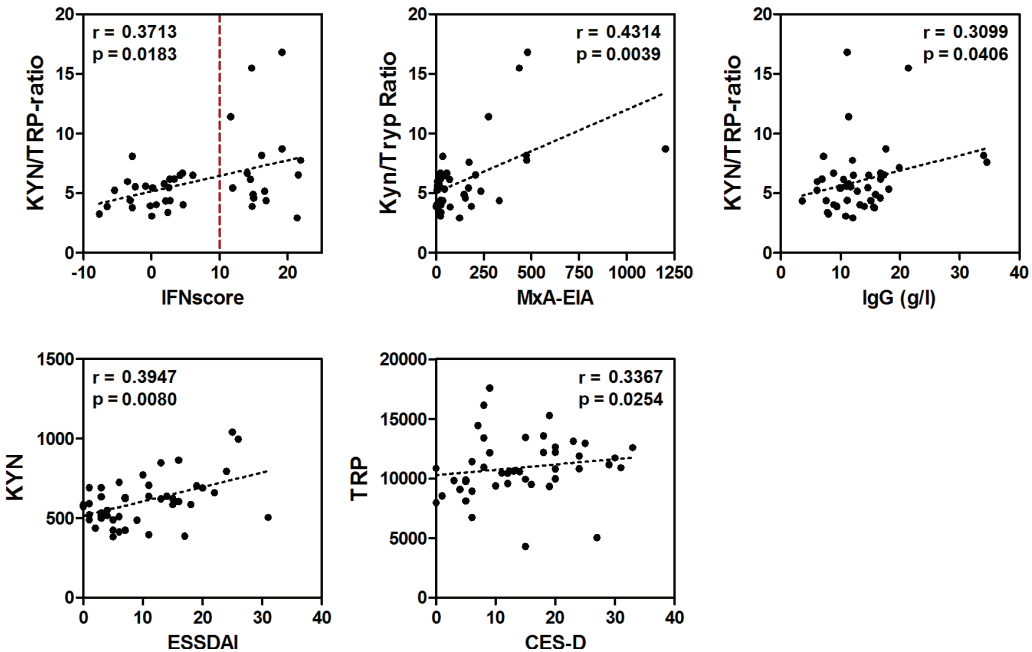


Figure 2. The Kynurenine pathway and disease activity in pSS. Correlations are depicted for the KYN/TRP-ratio reflecting IDO-enzyme activity and the monocyte IFNscore, MxA protein levels assessed by the MxA-EIA, and IgG (g/l) serum levels (upper panel). In the lower panel correlations are depicted for kynurenine (KYN) and total ESSDAI disease activity scores, and for tryptophan (TRP) and the CES-D depression scores. Correlation coefficients (r) and P values are shown. Each symbol represents an individual sample; the Spearman's rho was used for data not normally distributed.

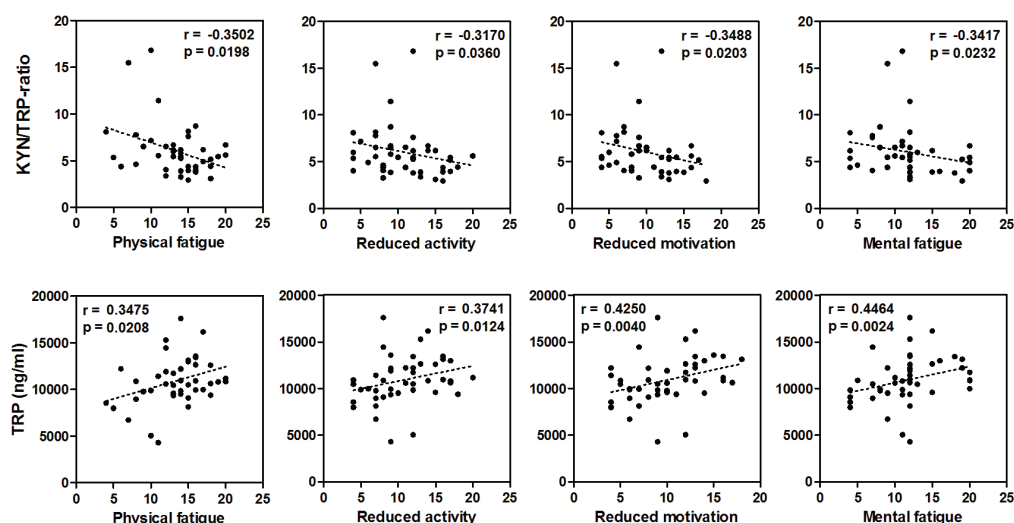


Figure 3. The KYN/TRP-ratio negatively correlates, whilst tryptophan levels positively correlate with certain dimensions of fatigue in pSS. Correlations are depicted between the KYN/TRP-ratio reflecting IDO-enzyme activity (upper panel) and the 4 dimensions of fatigue: physical fatigue, reduced activity, reduced motivation and mental fatigue. In the lower panel correlations are depicted with Tryptophan (TRP) serum levels (ng/ml) and the 4 dimensions of fatigue. Correlation coefficients (r) and P values are shown. Each symbol represents an individual sample; the Spearman's rho was used for data not normally distributed.

DISCUSSION

For this study we hypothesized that enhanced IDO activity and imbalances in the KYN pathway towards QUIN production, induced by IFNs, would be associated with increased fatigue and depressive symptoms in pSS patients. Although fatigue was high in pSS patients (in comparison to historic healthy controls), the hypothesis was refuted by finding only negative associations between IDO activity, the monocyte KMO activity, and dimensions of fatigue and depression. Furthermore, we did not find higher levels of fatigue and depression in the IFNpos as compared to the IFNneg pSS patients. On the contrary, the lower the MxA protein levels the higher the mental fatigue.

To explain these counterintuitive findings we first consider technical aspects of our study. We only assessed a relatively small sample of patients. Also ESSDAI disease activity scores were not significantly different in this small cohort between IFNpos and IFNneg pSS patients, in contrast to our previous findings, although IFNpos pSS patients of our current sample had higher presence of anti-SSA (Ro52/60) and -SSB autoantibodies as well as higher IgG levels [11]. Assessing particular fatigue symptomology in relation to the IFN signature in larger cohorts is thus warranted.



Assessing fatigue and depressive symptoms by self-assessment questionnaires is not objective, but it is the most valid assessment given the subjective nature of these variables. Despite these technical limitations, the study suggests that IFN-induced tryptophan kynurenine catabolites likely do not play a role in the fatigue and depression of pSS patients. Other physiological mechanism may be more important.

Firstly, TRP was correlated to the dimensions of fatigue and depressive symptoms. TRP is a precursor of the essential mood-regulating neurotransmitter serotonin (Figure 4) [13]. Therefore, a next step will be to further investigate the TRP-to-serotonin breakdown axis, to better understand if possible increases in TRP might indicate low serotonin production in pSS patients, thereby causing higher levels of fatigue and depression. Secondly, products of breakdown pathways other than the serotonin pathway may play a role, such as the neopterin pathway. Peripheral pro-inflammatory cytokines may also activate guanosine-triphosphate cyclohydrolase-1 (GTP-CH1), which in turn is able to mediate neopterin synthesis by macrophages. This may result in a deficit of the essential cofactor and precursor tetrahydrobiopterin (BH4), used for dopamine, norepinephrine and serotonin synthesis [4]. Thirdly, differential IDO activation and TRP metabolism has been associated with imbalanced glutamatergic neurotransmission, by affecting the glutamate N-methyl-D-aspartate receptor (NMDAr) [33]. QUIN is an endogenous NMDAr agonist, whereas KYNA has antagonistic properties on the NMDAr (see Figure 4) [5, 6]. In depression, it is proposed that increased QUIN contributes to excessive glutamatergic NMDA-activation [33]. Interestingly, in SLE, a subset of anti-DNA antibodies is known to cross-react with the NMDAr, and has been implicated in neuropsychiatric symptoms in these patients. These NMDAr-reactive autoantibodies have been proposed to alter brain-functioning following a breach in BBB integrity, subsequently causing autoantibody-mediated brain disease (reviewed in

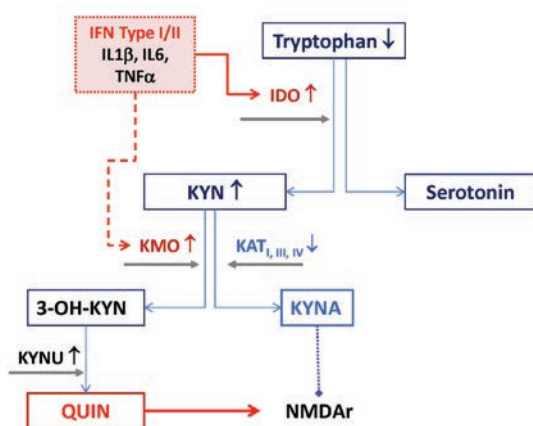


Figure 4. Tryptophan can either be degraded into kynurenine (KYN) or the essential mood-regulating neurotransmitter Serotonin. Here we find increased TRP serum levels to be associated with multiple dimensions of fatigue as well as depressive symptoms in pSS. Furthermore, differential IDO activation and TRP metabolism has been associated with imbalanced glutamatergic neurotransmission, by affecting the glutamate N-methyl-D-aspartate receptor (NMDAr). QUIN is an endogenous NMDAr agonist, whereas KYNA has antagonistic properties on the NMDAr.

[34]). The presence of NMDA-reactive autoantibodies in pSS has recently been described [35]. Further investigating the role of the NMDA-axis on brain-integrity in pSS is clearly warranted.

Although unexpected, a negative association between dimensions of fatigue and IDO activity was observed, and a positive association with TRP levels. Therefore we do not find an indication that fatigue and depressive symptoms in pSS patients are induced by an IFN-driven higher production of kyns from TRP. It is possible that alternative TRP-cat routes (to serotonin) as well as other non-IFN related mechanisms play a role in symptoms of fatigue and depression in pSS, which clearly warrant further investigation. Better understanding the role of increased TRP serum levels and symptoms of fatigue and depression in pSS might give new possibilities for specific targeting of these debilitating symptoms in pSS.

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Chapter

General Discussion

8

THE SYSTEMIC IFN SIGNATURE IN THE IMMUNOPATHOGENESIS OF PRIMARY SJÖGREN'S SYNDROME: THE INTERFERONOPATHOGENESIS

Summary and general conclusions

- I. Over half of pSS patients have systemic IFN type I overactivation, assessed as the presence of the IFN type I signature. The IFN type I signature identifies a subgroup of patients with higher clinical disease activity, higher anti-SSA (Ro52&60) and/or anti-SSB (La) autoantibody presence, higher IgG- and BAFF-levels, and lower C3 complement levels.
- II. MxA protein expression, as assessed by the MxA-EIA in whole blood, is a practical and reliable approach for the determination of systemic IFN type I activity in pSS. The MxA assay also detects systemic IFN activity in the autoimmune diseases SLE and SSc.
- III. IFNpositive pSS patients have an upregulation of TLR7 and a slight downregulation of TLR9. In contrast, IFNnegative pSS patients display a similar expression level of TLR7 as compared to healthy controls, but a significantly decreased TLR9 expression.
- IV. The RNA-sensing receptors RIG-I and MDA5 are upregulated in pDCs and monocytes of IFNpositive pSS, but – in contrast – downregulated in monocytes of IFNnegative pSS patients.
- V. IDO activity as measured by the KYN/TRP-ratio, which is driven in part by type I and type II IFNs, is enhanced in IFNpositive pSS patients and is associated with increased Tregs.
- VI. In monocytes of IFNpositive pSS the downstream KYN metabolism is abnormal (decreased KAT enzymes and increased KMO expression) possibly resulting in a shift towards potentially more pro-apoptotic and neurotoxic metabolites and a decreased immune suppression.
- VII. There is a *negative* association between multiple dimensions of fatigue in pSS patients and IDO activity, whereas TRP serum levels are *positively* associated with dimensions of fatigue in pSS.

In summary, the IFN type I signature identifies a subgroup of patients with more active disease, an imbalanced expression of TLRs and RLRs, and elevated IDO activity, which is associated with an increase in Treg cell percentages in peripheral blood.

This chapter will discuss recent developments and the implications of the above-mentioned findings for the pathogenesis of pSS, with a focus on the imbalanced RNA-sensing receptor expression. Finally, the limitations of the studies and novel perspectives are given.



THE INTERFERONOPATHOLOGY IN PSS: IN THE TOLL OF TOO MUCH INTERFERON

The presence of an interferonopathy in pSS, stems from the concept of disorders associated with a set of inborn errors of immunity causing an overactivation of IFN type I, termed ‘type I interferonopathies’ [1-4]. As described for SLE amongst others, we suggest to add the subset of IFNpos pSS patients to the list of type I interferonopathies.

Hypothetical disease model in IFNpos pSS patients

Figure 1A shows a simplified schematic representation for the interferonopathy in IFNpos pSS, based on the findings in this thesis. Here we propose that the observed RNA-sensing receptor-induced IFN production is triggered by an overwhelming abundance of ligands, particularly RNA. Inadequate damage control or apoptotic clearance contributes to the accumulation of endogenous (self-)ligands that subsequently lead to activation of PRRs [5]. Apoptotic debris is normally cleared by macrophages, which in humans do not express TLR7 or TLR9. However, delayed or defective clearance can lead to IC-formation by autoreactive antibodies forming complexes with self-apoptotic debris, which can be misdirected into pDCs (Figure 1) and B cells (not shown). Self-RNA can inappropriately activate both pDCs and B cells through TLR7, leading to IFN type I production by pDCs and autoreactive plasma cell differentiation [6-11]. pDCs and adjacent cells respond to IFN type I, hereby further inducing TLR7 mRNA expression in pDCs, monocytes and B cells [9, 10]. TLR7 upregulation and constant TLR7 triggering in pDCs induces RLR and PKR overexpression, resulting in a self-amplifying loop with a possible role for multiple RNA-sensing receptors in the sustained IFN production.

IFN type I binds to the IFNAR on monocytes, which induces expression of numerous IFN inducible genes, such as MxA, BAFF, the RLRs, PKR and also IDO. IFN-induced IDO expression in monocytes results in sustained production of immunomodulatory tryptophan metabolites and a skewed KYN/TRYP-ratio. Additionally, imbalances in the immune modulating downstream enzymes $KMO\uparrow$, and $KAT_{I, II, IV}\downarrow$, potentially cause multiple systemic effects (**Chapter 6**). A milieu with high IDO activity supports the polarization of naïve T cells into Treg cells [12-15], and might be an attempt to increase tolerance induction. Interestingly, IFNpos pSS patients have a shifted expression of the downstream KYN catabolic pathway, possibly resulting in more QUIN production and less KYNA (Figure 1). Such a shifted balance points towards a decrease in immunosuppressive and an increase in apoptosis-inducing metabolites. This complicated picture with tolerance inducing as well as immune-activating forces urges further study of these pathways.

TLR7 expression on differentiated B cells is crucial for RNA-associated autoantibody formation [16]. Interestingly RNA-associated autoantibodies are a hallmark of pSS (SSA/Ro52, SSA/Ro60 and SSB/La), particularly present in IFNpos pSS patients, thus supporting a role for defective clearance of (foreign or self) RNA in pSS pathogenesis. Such abnormalities have been reported

for pSS previously [17, 18]. If additional to an overload of RNA, an intrinsic abnormality in clearing apoptotic material is a feature confined to the IFNpos pSS subset remains to be established.

Whether specifically TLR7 seems to be at the starting point of pSS interefenopathy, or alternatively pDCs of IFNpos pSS patients are for example hyper-reactive to stimuli, remains to be elucidated.

Overall, the IFN-driven pathogenic loop in pSS, in part driven by aberrant sensing of nucleic acids, can potentially lead to functional decline or even loss of function in target tissues such as the salivary and lacrimal glands in pSS patients.

Hypothetical disease model in IFNneg pSS patients

In Figure 1B we depict the summary of our main findings in IFNnegative pSS. Here we observe decreased TLR9 expression, but most noteworthy a clear downregulated RLR expression. We assume that apoptotic clearance is functioning more appropriately in IFNneg pSS patients – or that apoptotic material might be less abundantly present in the circulation of these patients – since autoantibody production to nucleic acids is reduced. The detrimental IFN-driven amplification loop thus appears absent in these patients (see Box 1 and Figure 1B). However, low expression or absence of the PRRs discussed in this thesis could have opposite effects in the combat of foreign intruders. This subgroup will need to be better defined in the near future.

The toll of too much TLR7 in autoimmunity

TLR7/TLR9 imbalances, and particularly controlling TLR7 expression and signalling, seem to be crucial to restrict murine systemic autoimmunity [9, 19-21] (described in **Chapter 4**), indicating a role for our observations of a TLR7/9 imbalance in pathogenesis pSS. The toll of too much TLR7, leading to accelerated autoimmunity, arose from observations in murine models with a TLR7 gene duplication. The Y chromosome of this model bears a locus termed Y chromosome-linked autoimmune accelerator (Yaa). Yaa is a duplication and translocation from the X chromosome onto the Y, containing multiple genes of which TLR7. This results in a doubling of TLR7 mRNA and protein expression, as well as increased responsiveness to TLR7 ligands [22, 23]. TLR7 duplication was shown to be the specific requirement for autoimmune development in these mice, with a direct correlation between TLR7 overexpression and RNA-associated autoantibody production [19].

In **Chapter 5** we identify an upregulation of TLR7, and a slight downregulation of TLR9 in IFNpos pSS, which is in line with the previously described TLR imbalances in mouse models for systemic autoimmunity, concluding opposing effects for TLR7 and TLR9 despite similar tissue expression and, to common knowledge, similar signalling pathways [21, 24]. To date, a similar imbalance has not been reported for human SLE. A variation in copy number of TLR7 was observed in SLE patients, however, not different from the variation found in healthy individuals [25]. Furthermore, a recent study assessing TLR7 and TLR9 expression in pSS B cell subsets did



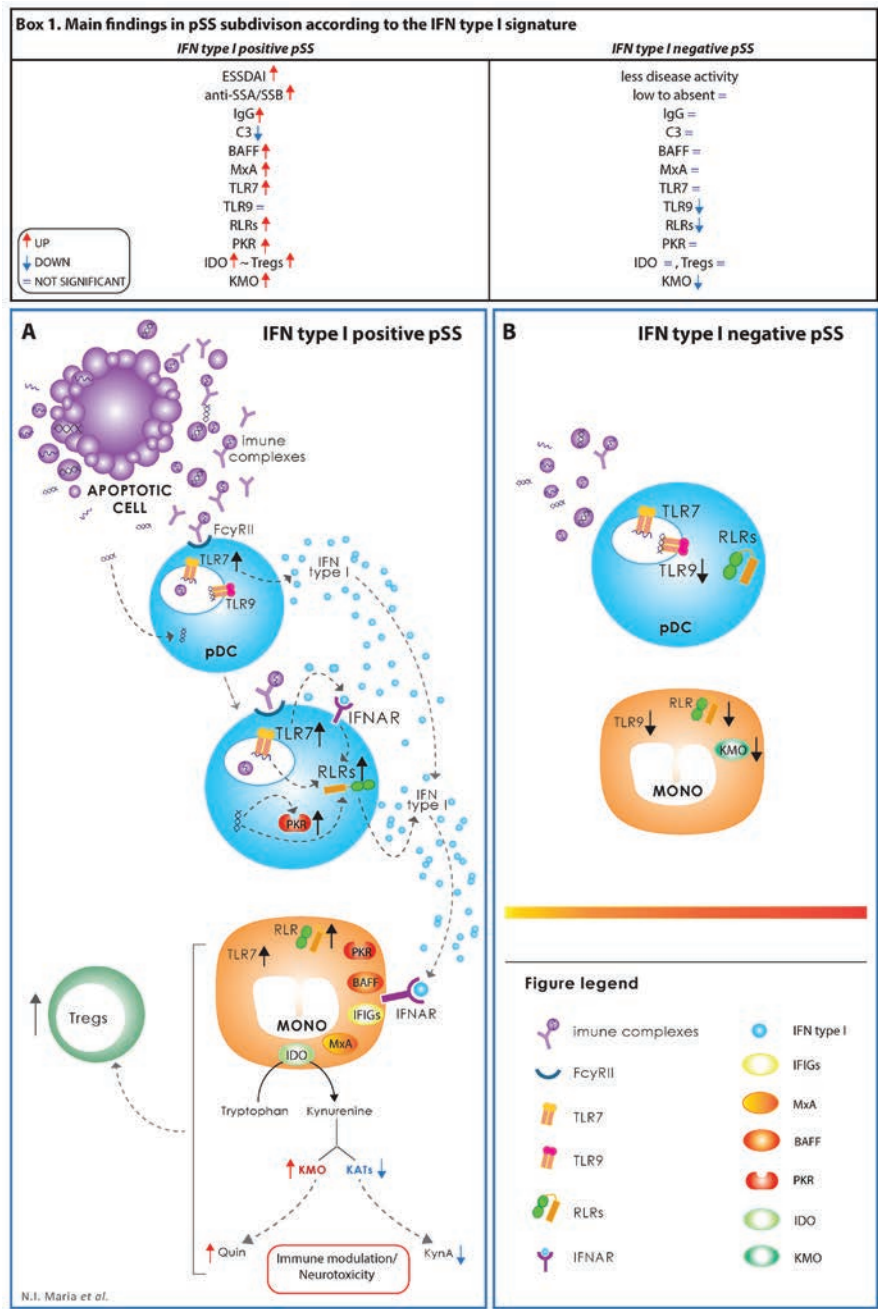


Figure 1. The toll of too much Interferon. A schematic summary of our main findings, based on subdivision according to the IFN type I signature into **A)** IFN type I positive and **B)** IFN type I negative pSS patients. Also see box 1.

not find an imbalance in TLR receptor expression. This might be explained due to the fact that patients were not divided into IFNpos and IFNneg subsets in this study, possibly resulting in a loss of subtle differences in TLR expression [26].

Subgrouping pSS patients according to distinct IFN signatures

Another point of discussion is that, in this thesis, we subdivide pSS patients into IFNpos and IFNneg pSS. In addition to the IFN type I signature, novel evidence indicates IFN activation in SS glands to be partly attributed to IFN type II activity (IFN γ) [27], describing the SS phenotype to include distinct molecular subtypes that can be stratified according to the magnitude and pattern of IFN responses [28]. Furthermore a large modular analysis in SLE recently revealed distinct type I and type II IFN signatures related to disease severity, describing 3 IFN-related modules (sub-signatures) [29]:

- *Module (M)1.2* = Mild (predominantly IFN α), comprising our IFN type I signature genes (identified in **Chapter 2** and **3**), such as the IFIGs MxA and IFIT3,
- *M3.4* = Moderate (predominantly IFN β), comprising IFIGs such as IFIH1, GBP1 and PKR (identified in **Chapter 5**) and,
- *M5.12* = Strong (also IFN β and IFN γ), comprising IFIGs such as IFI16 and ISG20.

Based on the modular approach in SLE [29] we searched for the genes annotated to the 2 latter modules (*M3.4* and *M5.12*) in our previous Affymetrix micro-array analysis, to assess the gene expression levels of these sub-signatures in IFNpos and IFNneg pSS patients (*M1.2* was clearly present in IFNpos pSS). As shown in Figure 2, the genes validated for *M3.4* and *M5.12* were all significantly upregulated in IFNpos pSS (**Chapter 5** and unpublished results).

However, Figure 2 also shows that all IFNpos pSS patients are positive for *M1.2* (mild), that fewer are positive for the *M3.4* (moderate) sub-signature and hardly any for the *M5.12* (strong) sub-signature. It is now essential to assess the relation of these distinct sub-signatures to pSS disease activity, as well as other aspects of the disease such as fatigue and depression.

Recent debate additionally focuses on a role for yet another IFN, IFN type III (IFN λ), in autoimmunity. One IFIG that can be induced by both IFN type I and IFN type III is MxA [30-33]. IFN λ was previously shown to have detrimental effects in RA patients, possibly contributing to synovial inflammation in these patients. IFN λ 1 enhanced TLR-expression, which mediated the inflammatory state [34]. Interestingly, exposure of RA-derived synovial fibroblasts to the TLR3 ligand poly(I:C), which also triggers MDA5, induced production of IFN λ 1 by these cells [34]. Needless to say that also IFN λ -driven IFIGs need to be determined to further subtype the IFN signatures and relate these to disease activity.

Can sex-differences in pSS be attributed to TLR7 upregulation?

The gene for TLR7 is located on the X chromosome, in both humans and mice. In **Chapter 1** we highlight recent evidence pointing towards a X-chromosomal link to disease-onset in



autoimmunity [35-37]. Regarding the data on Klinefelter’s syndrome and triple X syndrome the presence of an additional X chromosome could very well explain the sex-bias in SLE and pSS, independent of circulating sex hormones [38-41]. The question now arises if differences

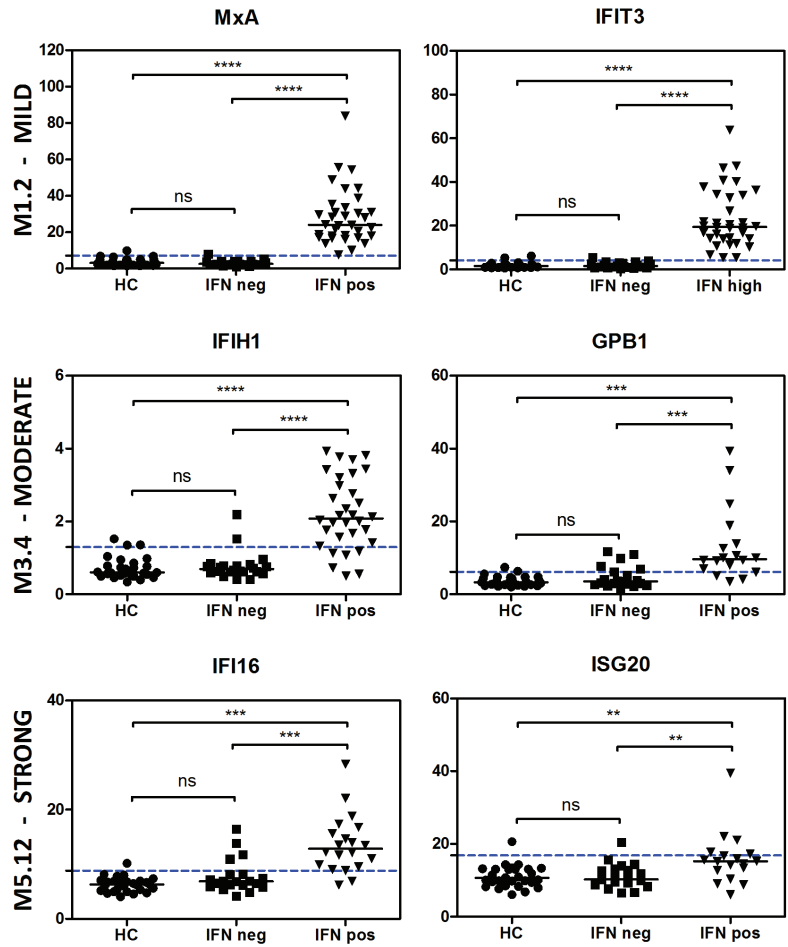


Figure 2. Distinct IFN type I and type II signatures present in pSS. Based on the previously described modular analysis in whole blood of SLE patients, describing 3 IFN modules; Module (M)1.2 = MILD, M3.4 = MODERATE and M5.12 = STRONG [10], a class comparison was performed in whole blood of pSS patients. Differentially expressed genes were considered significant using a cut-off: $p < 0.05$ and Fold change ≥ 2 . This analysis revealed certain genes in each module (not described in detail here). Here 2 genes per module are depicted, validated in HC ($n=36$), and pSS patients ($n=55$). pSS patients were stratified according to our previously described whole blood IFN type I signature (Chapter 5). For each gene, the $\text{mean}_{\text{HC}} + 1.96 \cdot \text{SD}_{\text{HC}}$ mRNA expression was calculated to assess in which of the subjects the particular gene was upregulated (blue dotted line).

in X chromosome TLR7 gene dose and/or expression contribute to the development of systemic autoimmunity. A study assessing TLR7 gene copy number differences between male and female SLE patients and HCs found no differences [25]. TLR7 therefore appears to be subjected to normal X-related inactivation, as additionally no differences in TLR7 expression were found between men and women in B cells or pDCs. Interestingly however, “female” PBMCs were shown to secrete markedly higher IFN α levels compared to “male”, when triggered with a TLR7/8 ligand [42]. This illustrates a profound sex-dependent pathway of TLR7-induced IFN α production. A sex-specific association of X-linked TLR7 was previously found with male SLE, finding an association of a functional signal nucleotide polymorphism (SNP) in the 3’UTR of TLR7 with SLE [43].

Another interesting sex-bias related finding regarding TLR7 is the observation that the inhibitory CD200-receptor (CD200R) inhibits TLR7 signalling. In female mice, absence of this CD200R signalling enhances TLR7 responses and leads to enhanced IFN type I production [44], again supporting sex differences in TLR7 responsiveness. Interestingly, aberrant CD200/CD200R expression and function has since been linked to SLE, possibly contributing to diminished binding and diminished phagocytosis of apoptotic cells in SLE [45]. If this also holds true for pSS is unknown, but could link aberrancies in clearance of apoptotic material with induction of TLR7 and IFN type I in pSS, as depicted in our model (Figure 1A). This proposed direct link between TLR7 signalling and CD200-related apoptotic clearance warrants further investigation in IFNpos pSS.

TLR7 ligation downregulates TLR9 expression: A crucial regulatory balance

In vitro studies with PBMCs and in particular pDCs revealed IFN β to specifically upregulate TLR7 and MyD88, while downregulating TLR9 mRNA [46]. These observations are in line with our own observations in HC-PBMCs as well as the CAL-1 pDC cell-line [47], where IFN type I appears to induce the TLR7-MyD88 signalling axis, whilst significantly downregulating TLR9 (unpublished observations). TLR7 and TLR9 co-expression in pDCs (and B cells) creates potential for physical interactions between the two, resulting in the tight control of TLR7 signalling by TLR9 [48]. However, studies using transfected cells with only TLR9 are providing evidence that these conclusions are not based upon an actual interaction between TLR7 and 9 [49]. TLR7 and TLR9 have also been shown to induce a different pattern of downstream IFNs [50], possibly to optimize the immune response against invading pathogens. In autoimmunity, the tilt towards TLR7 might be detrimental. Better understanding these mechanisms of action, affecting the TLR7/9 balance, will be crucial for development of improved targeted therapies in IFN-driven autoimmunity.

Do TLRs and RLRs aberrantly collaborate to amplify pSS interferonopathy in pSS?

Interestingly, co-stimulation of TLR7 and TLR9 has been shown to actually downregulate RIG-I expression in HC pDCs, in contrast to single TLR7 ligation [51]. In our IFNneg pSS patients RIG-I and MDA5 were downregulated. It can be assumed that such downregulation is the norm



to prevent overactivation of the IFN pathway when taking up apoptotic material, in order to prevent systemic autoimmunity. Complex mechanisms, including imbalances between TLR7 and TLR9, are probably the culprits behind the downregulation of RLRs in healthy non-auto-immune individuals.

In IFNpos patients RLRs are aberrantly upregulated and we assume that in particular the imbalanced TLR7 upregulation plays a role in this aberrant RLR upregulation, leading to downstream excessive activation of the IFN pathway. With other words there exists an aberrant collaboration between the two nucleic acid sensitizing pathways, the exogenous and endogenous sensing pathway. Why this is the case we do not know, but we assume that genetic and epigenetic alterations in the pathways may play a role in this aberrant response.

The PRR-IFN pathway: Genetics and epigenetics

Inappropriate or sustained activation, or defective negative regulation of certain PRR signalling pathways is associated with autoimmunity. In particular, a gain-of function mutation in IFIH1, encoding MDA5, and a loss-of-function mutation in the gene encoding the 3' repair DNA exonuclease TREX1 [52]. TREX1 degrades viral and aberrant cellular DNA, hereby activating the IFN type I producing STING-dependent pathway downstream [2]. TREX1-deficient mice are unable to degrade endogenous DNA, which results in overproduction of IFN type I and sterile inflammation, where STING deficiency limits inflammation [18]. In contrast, STING deficiency actually accelerated disease in lupus-prone MRL/lpr mice, leading to increased autoantibody production, increased expression of IFIGs and macrophage hyper-responsiveness to TLR7 and TLR9 ligands amongst others. The investigators conclude STING to be a negative regulator of TLR-mediated systemic autoimmunity, and highlight the crosstalk between TLR and cytosolic nucleic acid-sensing pathways in maintaining immune homeostasis, which has been underappreciated, to date [53].

As was previously shown for the TREX1 mutation, the gain-of-function mutation in IFIH1 was recently associated with SLE and Aicardi-Goutières syndrome (AGS), said to cause IFN-related neuro-immunological features [54, 55]. Mice bearing this IFIH1 mutation spontaneously developed lupus-like nephritis and systemic autoimmune symptoms [54]. In contrast, a loss-of-function mutation in IFIH1 is significantly associated with resistance to type 1 diabetes [56].

On the basis of these observations a cell-intrinsic mechanism can be hypothesized in which these pathways are auto-activated, even in the absence of RNA ligands, subsequently leading to constitutive signal transduction and interferonopathy. A single gain-of-function mutation in the IFIH1 gene can cause constitutive activation via MAVS, consequently resulting in chronic activation of the IFN type I pathway, through IRF7 amongst others [54]. Interestingly, deletion of MAVS but not IFNAR1 could fully ameliorate pathology in mice bearing the IFIH1 mutation, suggesting the MDA5-MAVS interaction to be the most promising therapeutic target here.

With regard to GWAS studies (also described in **Chapter 1**) these studies in pSS reveal strong associations with polymorphisms in genes involved in IFN signalling such as IRF5, a transcription factor linking TLR signalling to pro-inflammatory cytokine expression, and STAT4 [58, 60-63]. However the majority of genetic mutations described to be associated with pSS are in non-encoding regions, suspected to alter disease-susceptibility genes by altering their expression epigenetically [57-59].

Recent studies looking into effects of epigenetic imprinting in pSS are revealing interesting effects of post-translational modification. One of the first studies looking into the methylation profile of the promotor region of IRF5, did not find aberrant DNA methylation profiles for the putative regulatory regions of IRF5 [64]. Interestingly, various miRNAs have been implicated in the regulation of both TLR and RLR signalling [65]. Findings in SLE suggest miRNA-146a to be a possible negative regulator of IRF5 [66]. Additionally, miR-146a was proposed to control TLR signalling, through a negative feedback loop involving IRAK1 and TRAF6 [67]. Interestingly, miR-146a was also implicated to negatively regulating RIG-I-dependent IFN type I signalling [65, 68].

Genome-wide DNA methylation studies are currently being performed in pSS, showing various IFIGs differentially hypo-methylated in CD19⁺ B cells, with IFI44L as a top gene [Nordmark G. *et al.*, ACR 2014, Suppl. 2980]. Furthermore, active DNA de-methylation processes have been described in salivary gland epithelial cells (SGECs) in pSS [69]. Also histone hyper-acetylation of IRFs has recently been described in SLE CD14⁺ monocytes [Leung Y.T *et al.*, ACR 2014, Suppl. 870].

IDO activity, increased Tregs, but decreased immune suppressive KYNA in IFNpos pSS: a relation with mood-regulation in pSS?

Treg percentages are significantly increased in IFNpos pSS and correlate with IDO activity, indicating that IFN-induced IDO activity might be promoting Treg-differentiation in IFNpos patients (Figure 1A and **Chapter 6**). On the other hand – downstream of TRP catabolism into KYN – IFN-induced KMO expression is increased, while the potentially immunosuppressive KAT_{I, II, IV} enzymes are decreased. Interestingly, exposure of monocyte-derived macrophages to IFN β can lead to QUIN production *in vitro* [70], probably by inducing KMO overexpression in these cells. Moreover, KAT_I and KAT_{II} expression can decrease after IL-1 β treatment [71], indicating that a pro-inflammatory environment induces KMO/KAT imbalances strengthening pro-inflammation, as observed in IFNpos pSS. In IFNpos pSS interferonopathy, the presence of overactive IFN signalling thus appears to favor KYN metabolism towards the KMO branch of the pathway, down the pro-apoptotic and potentially neurotoxic QUIN arm (Figure 1A).

The controversial observation of increased Treg percentages and possibly decreased production of immunosuppressive KYNA in IFNpos pSS, remains a puzzling dichotomy. In **Chapter 7** symptoms of fatigue in pSS did neither correlated with disease activity, in line with earlier reports [74], nor with IFN positivity. pSS disease activity did however correlated with KYN



serum levels, whereas symptoms of fatigue and depression correlated with TRP serum levels, linking TRP catabolism in a somewhat unexpected way, to these debilitating symptoms in pSS. Interestingly, reducing systemic KYN levels – by inducing KAT enzyme expression and hereby the conversion of KYN to KYNA – has recently been shown to protect the brain from stress-induced changes associated with depression [72, 73], and might be an interesting new avenue to explore in IFNpos pSS.

With regard to the functionality of Tregs in IFNpos pSS, the TLR-repertoire has recently been assessed in T cells [75-77], where in particular, direct TLR-activation in Tregs was shown to inhibit their suppressive function [78-80]. Tregs might thus lose their ability to suppress after specific TLR stimulation, as has been shown previously for other co-stimulatory signals [78, 81].

LIMITATIONS

Overall our studies assessed gene expression profiles in different peripheral blood cells. We, however, did not assess genetic or epigenetic abnormalities such as mutations or differential methylation. Furthermore, protein expression was solely studied in a limited set of patients and for some of the molecules of interest. In **Chapter 5** and **6** we assess local expression of proteins of interest in a small sample set of pSS SG biopsies. Apart from these small observational studies in pSS SG tissue, all studies were executed in peripheral blood samples. Our observations in this thesis therefore solely depict systemic imbalances in pSS, largely only on the gene expression level. More in depth studies into local abnormalities with regards to our findings, as well as a detailed identification of cell types expressing proteins of interest are warranted.

The patients described in this thesis are mainly patients, visiting the outpatient clinic for routine check-ups. These patients are rarely treatment naïve, making it difficult to draw any conclusions on effect of medication. Particularly effects of medication on the IFN signature are of interest. Larger studies are necessary in order to further subdivide patients according to medication status or other clinical aspects of the disease, in order to create more homogeneous subgroups. Another limitation is that the time from onset of first symptoms, to the establishment of the diagnosis, is relatively long in our cohort.

Here we subdivide patients based on the modal distribution of the IFNscore. Although the monocyte IFN signature shows a bimodal distribution, it remains a relatively arbitrary division. Also reassessing our data, once additional type II or even type III signatures have been established is of interest. In our studies we mainly focus on the IFN type I positive pSS subtype. With regard to our findings in IFNnegative pSS patients, a more in depth study into the pathogenic pathways that characterize this later subgroup is required.

In **Chapter 6** and **7** we assess systemic IDO-activity, and expression of downstream enzymes in the KYN pathway. We do not take the effects of the liver enzyme TDO (TRP 2,3-dioxygenase) into account, which has similar effects as IDO.

PERSPECTIVES

The IFN type I signature and MxA: biomarkers for prediction and monitoring

We, as well as others, have described the presence of an IFN type I signature, assessed as upregulation of a distinct set of IFIGs [82-88]. Using the monocytic IFNscore, pSS patients can be sub-classified into IFNpositive and IFNnegative patients (**Chapter 2**). The MxA-EIA assay was found to be the best biomarker for systemic IFN activity (**Chapter 3**) and can also be used to detect IFN activation in the other IFNpositive systemic autoimmune diseases, SLE and SSc (addendum **Chapter 3**). This suggests its relevance as a classification tool for identifying the IFN type I signature in systemic autoimmune diseases regarding subsets of patients with IFN activation.

The IFN type I signature and pre-eclampsia in SLE

Recently systemic IFN type I expression has also been related to phenotypic and function abnormalities of endothelial progenitor cells and accelerated atherosclerosis development in SLE and cSLE [89]. Interestingly in pregnant SLE patients elevated levels of IFN α have been associated with pre-eclampsia even before the presentation of the clinical complications [90]. The same group recently presented data proposing MxA as a potential biomarker for pre-eclampsia in SLE [Salmon J *et al.*, ACR 2014]. The MxA assay might be an easy method for screening pregnant systemic autoimmune patients to investigate if changes in IFN activity are indeed predicting complications. These data support sub-classification in IFNpos and IFNneg subsets and monitoring of IFN type I levels in combination with extended screening for specific complications in the IFNpos patients.

The IFN type I signature in HCQ therapy prediction

To date, the antimalarial HCQ is thought to block TLR7/9-activation, however, the exact mechanism of action and its effects remain controversial (described in **Chapter 1**) [91]. In a recent clinical trial evaluating the efficacy of HCQ, pSS symptoms did not improve compared to placebo [92]. Our data in **Chapter 3** suggests that the IFN signature or alternatively the MxA assay to possibly prove useful in predicting HCQ treatment-responsiveness as well as its efficacy in pSS. To examine the potential of MxA for monitoring therapeutic efficacy, HC PBMCs were stimulated with the TLR7 ligand IQ, known to induce IFN type I production. Upregulated MxA was effectively inhibited *in vitro* by the clinically relevant concentration of 1 μ g/ml HCQ [93] (unpublished



observations). This preliminary work, although *in vitro*, gives insight into the potential for MxA as a therapeutic biomarker for prediction as well as monitoring of HCQ therapy.

The IFN type I signature in rituximab therapy prediction

A role of the IFN signature in prediction of therapeutic response to rituximab treatment has recently been reported for RA [94]. IFN positivity was suggested as a predictive biomarker for the non-response to rituximab in RA [95]. Whether this holds true for pSS remains to be established. As not all pSS patients respond equally well to rituximab, it will be interesting to see if this response can also be predicted by the IFN signature, or more practically by MxA positivity [96, 97].

The IFN type I signature for the prediction of lymphoma development in pSS

Development of lymphoma in pSS is the most severe complication of the disease, representing the ultimate outcome of the B cell overactivation associated with pSS pathogenesis [98]. Incidence of lymphoma in pSS patients is relatively high, between 5-15%, with 33% non-Hodgkin's lymphoma (NHL) [98-103]. Research has focussed on the prediction of lymphoma development in pSS [103-105]. It was demonstrated that among the pSS patients with glandular swelling, only those with 2 or more positive biomarkers present – cryoglobulinemia, C4 hypocomplementemia, anti SSB/La antibodies and leukopenia – had an increased risk of lymphoma evolution [105]. Interestingly these features are similar to our findings in IFNpositive pSS patients, described in **Chapter 2** and **3**. The direct link between IFN activity and the development of lymphoma, or increased risk of lymphoma in pSS has not been fully elucidated. One could hypothesize the effects of IFN inducing amongst others BAFF will lead to increased B cell (auto)reactivity followed by the development of B cell malignancies. Interestingly, genetic variations in BAFF, have recently been associated with the development of lymphoma in pSS [106]. The same group studied the contribution of both IFN type I and type II to lymphoma development in pSS, finding predominantly an IFN type I signature in peripheral blood and IFN type II signature in minor SGs of pSS patients [107]. Subtyping pSS patients according to their IFN signature(s), both locally and systemically, could arise as a novel predictor of lymphoma development.

Implications for TLR7-related therapy

In terms of developing new approaches for therapeutic interventions, the TLR-IFN network is a promising target and warrants more in depth investigation (Figure 3). The delicate balance between endosomal TLRs appears crucial to prevent autoimmunity, and a better understanding of these pathways and how they are balanced, will provide insight into specific targeted therapies [50, 108, 109]. Interestingly, tackling TLR7 will have effects on both pDCs and B cells simultaneously, and might prove beneficial by inhibiting multiple aspects of the disease with

one compound. Whether TLR7 blockade alone or in combination therapies will prevent and/or ameliorate pSS pathogenesis remains to be investigated [9].

With regards to systemic autoimmunity, TLR signalling has been associated with B cell receptor (BCR) signalling, where these two pathways were suggested to synergize, thereby leading to enhanced signal transduction [110, 111]. More specifically, TLR9 and BCR signal transduction pathways have been linked to B cell hyper-responsiveness in SLE [112, 113]. It has recently been speculated that in the endosomes, TLR and BCR signalling are interconnected by Bruton's tyrosine kinase (BTK) [114], and that this synergy could lead to hyper-responsiveness. This data is mainly on hyper-responses to DNA-containing antigens by TLR9. Interestingly, BTK was recently described to specifically regulate TLR9 responses in pDCs [115]. BTK contributes to MyD88-dependent TLR signalling, by directly interacting with the cytoplasmic TIR domain of most TLRs, as with the downstream adaptors MyD88, TRIF, TIRAP, and IRAK1 [114, 116-118]. Interestingly, BTK is significantly upregulated in IFNpos pSS patients (unpublished results). BTK inhibition will thus not solely target BCR signalling, B cell adhesion and migration, but will potentially also target TLR signalling. This might be an interesting dual approach in autoimmunity. Presently, preclinical trials using a small molecule BTK inhibitor Ibrutinib (PCI-32765) show promise in B cells malignancies, as BTK is crucial for survival of leukemic B cells [114, 119-123]. The effects (and side effects) of BTK inhibition in human autoimmune diseases such as SLE and pSS are however not known and if attempted in clinical trials, will probably need a careful approach. In

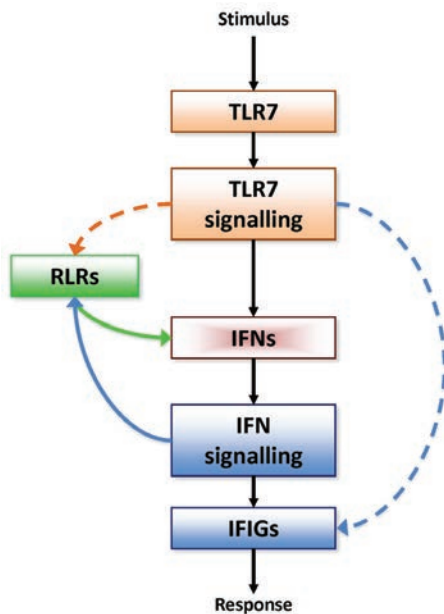


Figure 3. Potential therapeutic targets in the TLR7-IFN pathway. Adapted from Borden E. et al., 2007 [109]. Each box represents possible therapeutic targets for IFNpos pSS. In light of recent evidence however, tackling upstream of IFNs might be more effective, if indeed TLR7 signalling is IFN-independent in the induction of IFN-inducible genes (IFIGs), and TLR7 signalling might thus directly induce RLRs (dotted lines represent this proposed direct IFIG-induction by TLR7 signalling, independent of IFN).

our opinion effects of BTK inhibition first need to be better understood *in vitro*, making clinical trials premature.

With regards to tackling downstream IFN signalling, a phase 2 clinical trial in SLE with a selective JAK1 inhibitor was previously discontinued due to lack of efficacy. The JAK1 inhibitor was previously tested by us, and significantly inhibited MxA expression induced by either IFN α or IFN γ pSS serum *in vitro*. Interestingly, we recently observed an upregulation of protein tyrosine phosphatase non-receptor type 2 (PTPN2), described as the negative regulator of JAK1 [124], in whole blood of IFN type I positive pSS whereas JAK1 was significantly downregulated (unpublished results). This might explain the lack of efficacy in clinical trials. In future we suggest, particularly when using IFN-inhibiting agents, to first assess expression levels of the target in stratified pSS subgroups in order to determine its potential. Even more intriguing, however, is that there is an increasing body of evidence to suggest TLR7 to signal independent of IFN type I, inducing a large amount of IFIGs without the necessity of IFN type I subtype production and IFNAR-binding [51, 125]. Further studies addressing possible IFIG-induction by TLR7-signalling independent of IFN type I are clearly warranted.

Viral evasion mechanisms and subversion of PRR signalling: novel therapeutic approaches?

Viruses have very sophisticated ways of evading the immune system when it comes to IFN signalling. These viruses have ways of specifically inhibiting PRR-mediated recognition of viral nucleic acids by inhibiting TLRs but also RLRs and their distinct downstream signalling pathways. Viruses use specific proteases or cytoplasmic dsRNA-binding proteins amongst others, to interfere with antiviral PRR signalling. For example, HCV protein NS (non-structural) 5A inhibits TLR signalling by binding MyD88, whereas influenza A virus NS1 protein can bind to the RIG-I-IPS1 complex, hereby blocking downstream signalling (reviewed in [126]). Hence, there is a lot to be gained by using the knowledge of these viral evasion mechanisms for developing targeted therapies effectively blocking IFN signalling in IFN-driven autoimmunity. Intriguingly, TLRs and RLRs were recently shown to share downstream signalling through TRADD (TNF receptor-associated via death domain) [126-128], providing evidence for their crosstalk as well as opportunity for targeted therapy.

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2012

Addendum

SUMMARY

SAMENVATTING

LIST OF ABBREVIATIONS

ACKNOWLEDGMENTS

SUMMARY

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by accumulation of white blood cells in the salivary and lachrymal glands. Characteristic symptoms are dry eyes and mouth. Other manifestations frequently present in pSS involve amongst others the skin, joints, muscles, kidneys, and lungs, indicating that pSS is a systemic disease. pSS patients often experience disabling fatigue and a depressed mood as their most severe symptoms, reducing overall health-related quality of life. The consequences of pSS for social functioning of the patient are serious and frequently result in unemployment. To date it is however largely unknown what factors cause these disabling symptoms. The prevalence of pSS is estimated between 0.05-1%, with a nine fold predominance in females. The diagnosis of pSS can be difficult due to lack of specific markers and the heterogeneity of the symptoms. The criteria for establishing a diagnosis of pSS consist of six clinical elements, both subjective and objective. At present, no common evidence-based intervention therapy is available and treatment is mainly symptomatic. Thus, a further unravelling of the pathophysiology of pSS is essential for finding novel biomarkers and identifying new treatment targets. Interferon (IFN) type I (introduced in **Chapter 1**) has been proposed as a biomarker for systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and pSS.

IFN type I is a large family of IFNs comprising 17 different subtypes. IFN type I is produced in healthy individuals following a viral infection, finding its origin as being discovered and named for its potent ability to readily "interfere" with viruses. The research described in this thesis was aimed at unraveling the role of IFN type I activity in pSS with the following overall aims:

- I. Determine the prevalence of IFN type I activation and its relation to clinical symptoms in pSS
- II. To unravel the biological pathways underlying IFN type I activity in pSS
- III. To study downstream effects of systemic IFN type I activation in pSS

In **Chapter 2** we identified a systemic upregulation of the IFN type I system in peripheral blood monocytes of a major subgroup of pSS patients. This so-called "IFN type I signature" is assessed as the expression of multiple IFN type I inducible genes (IFIGs). The presence of the monocyte IFN type I signature was observed in over half of the pSS patients. The IFN type I signature identified a distinct subgroup of patients with increased EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) scores, presence of anti-SSA (Ro52&60) and/or -SSB (La) autoantibodies, higher immunoglobulin (Ig) G and B-cell activating factor (BAFF)-levels, and lower C3 complement levels. The abundant presence of autoantibodies leading to circulating immune complexes (ICs) is thought to be the main trigger inducing the IFN signature in pSS and SLE.

As assessment of the IFN type I signature is a laborious technique an easy assay to determine the presence of systemic IFN type I activation in pSS was developed (**Chapter 3**). MxA protein expression, as assessed by the MxA-EIA (enzyme immunoassay), proved to be a practical and

reliable biomarker for systemic IFN type I activity in pSS. The MxA assay was also found useful for the detection of systemic IFN activity in the systemic autoimmune diseases SLE and systemic sclerosis (SSc) (**Chapter 3; Addendum**).

As pSS in humans is very heterogeneous, and it is questionable whether a single pathogenic mechanism is underlying pSS, it is likely that multiple animal models are necessary to fully elucidate disease pathogenesis. Therefore, light is shed on the importance of subdividing pSS patients according to their IFN signature, and when drawing conclusions from murine models for clinical relevance in humans, models better resembling human-disease phenotypes are essential. A recommendation is made for choosing appropriate mouse models to best resemble the IFN positive and IFN negative SS-like subtypes (**Chapter 4**). On the one hand, the non-obese diabetic (NOD) mouse model could be used as a systemically IFN negative pSS model. On the other hand lupus-prone mouse models could be used as IFN positive pSS models.

Plasmacytoid dendritic cells (pDCs) have been identified as being the most potent IFN type I producing cells upon stimulation of toll-like receptor (TLR)7 and TLR9. Interestingly, in **Chapter 5** we found TLR7 upregulated in pDCs and monocytes of IFNpos pSS patients. Additionally, the downstream genes in the TLR7-IFN pathway were upregulated in IFNpos pSS. Evidence points towards a contribution of both the TLRs and the cytoplasmic RIG-I-like receptors (RLRs) RIG-I (encoded by DDX58) and MDA5 (encoded by IFIH1) to a sustained IFN-overactivation in autoimmunity. Interestingly, RIG-I and MDA5 were upregulated in IFNpos pSS, and were inducible by triggering TLR7 *in vitro*. The widespread upregulation of TLR7, RIG-I, MDA5 and their downstream signaling pathways was confined to IFNpos pSS patients. IFNneg patients had a contrasting pattern of expression with normal TLR7 and decreased TLR9, RIG-I and MDA5 expression. Taken together, we conclude an imbalanced expression pattern of the RNA-sensing receptors TLR7, RIG-I and MDA5 in pDCs and monocytes of IFNpos pSS patients. This profile could explain the pathogenic IFN production with downstream systemic effects in IFNpos pSS patients. Furthermore, we propose that the largely reduced expression of nucleic acid sensing receptors in IFNneg pSS patients explains in part their IFN negativity.

As the kynurenine (KYN) pathway can be rapidly induced by both IFN type I and type II (IFN γ), we study this pathway in **Chapter 6**. In mammals, the KYN pathway is the major metabolic pathway to catabolize tryptophan (TRP) and is tightly regulated by the immune system. Indoleamine-2,3-dioxygenase (IDO) is the rate limiting enzyme in this TRP catabolism (TRP-cat), degrading TRP to KYN. Interestingly aberrant systemic TRP-cat resulting in a shift from neuroprotective towards neurotoxic downstream metabolites, collectively called kynurenines (kyns), has been associated with mood disturbances as well as neuropsychiatric diseases. Furthermore, IDO plays a crucial role in the suppression of effector T-cell function and promotion of regulatory T-cell (Treg) differentiation. In **Chapter 6** we describe the percentage of Tregs to positively correlate with IDO and IFN type I activity in pSS. Additionally, we identify an imbalanced KYN pathway

with evidence for a shift towards potentially more pro-apoptotic and neurotoxic metabolites in IFNpos pSS patients.

The induction of fatigue in patients with virus infections treated with IFN type I, supports a role for IFN type I in fatigue in pSS. In **Chapter 7** we focused on the role of the imbalanced KYN pathway in relation to symptoms of fatigue and depression in pSS patients. We found a negative association between multiple dimensions of fatigue in pSS patients and IDO activity, whereas TRP serum levels positively associated with dimensions of fatigue in pSS. We did not find evidence that these symptoms are due to an IFN-driven imbalance. It is possible that alternative TRP-cat routes to serotonin, as well as other non-IFN related mechanisms, play a role in symptoms of fatigue and depression in pSS.

In **Chapter 8**, the overall findings are discussed, addressing the toll that pSS patients have to pay for the presence of too much IFN. The identified different pathogenic pathways probably underlie the heterogeneity of pSS, indicating that patients with pSS will most likely benefit from optimizing therapies tailored to specific subgroups of the disease. In this thesis we provide our perspective on the importance of subdividing pSS patients according to their IFNsignature(s).

SAMENVATTING

Het primaire syndroom van Sjögren (pSS) is een auto-immuunziekte die wordt gekenmerkt door droge ogen, een droge mond en een ophoping van ontstekingscellen in de speeksel- en traanklieren. Ook klachten van de huid, gewrichten, spieren, nieren en longen komen frequent voor en geven aan dat pSS een ziekte is die zich in het hele lichaam kan manifesteren, een zogenaamde systeemziekte. Moeheid wordt vaak door patiënten met pSS ervaren als meest ernstige klacht. De ziekte heeft nadelige gevolgen voor de kwaliteit van leven van de patiënt, en resulteert regelmatig in werkloosheid. Tot op heden is het echter onbekend wat de oorzaak van de ziekte is. De prevalentie van pSS wordt tussen de 0.05-1% geschat en de ziekte komt ongeveer 9 keer vaker voor bij vrouwen dan mannen. Het stellen van de diagnose pSS is moeilijk omdat specifieke kenmerken van de ziekte ontbreken en veel verschijnselen ook bij andere ziekten voorkomen. De behandeling van pSS is meestal alleen gericht op de bestrijding van de symptomen en niet op hun oorzaak. Onderzoek naar de oorzaak van de ziekte is belangrijk, omdat dit inzicht kan geven in moleculen die een rol spelen bij het ontstaan van de ziekte. Het blokkeren van deze moleculen zou in de toekomst als een specifieke behandeling voor patiënten met pSS gebruikt kunnen worden. Interferon (IFN) type I (**Hoofdstuk 1**) is een molecuul dat verhoogd is gevonden bij systemische auto-immuunziekten zoals systemische lupus erythematosus (SLE) en pSS.

IFN type I is een familie van moleculen bestaande uit 17 verschillende subtypen. IFN type I wordt normaal in het lichaam aangemaakt als er een virusinfectie is en dient om te 'interfereren' met het virus, om zo de virusinfectie te bestrijden. Na het opruimen van het virus wordt IFN type I weer afgebroken. Echter bij patiënten met pSS is een continue activatie van IFN type I gevonden. Bij een deel van de pSS patiënten blijkt IFN type I constant verhoogd aanwezig te zijn, terwijl er geen virusinfectie is en het ook niet bekend is of die er geweest is. Meerdere onderzoeksgroepen hebben aangetoond dat zowel in het bloed als in de speekselklieren er IFN activatie gevonden kan worden. Echter niet alle pSS patiënten hebben IFN type I activatie. Een belangrijke aanwijzing voor een rol van IFN type I bij het ontstaan van Sjögren is de waarneming dat bij patiënten met hepatitis of kanker, die met IFN worden behandeld, pSS kan ontstaan. Het onderzoek beschreven in dit proefschrift was gericht op het verder ontrafelen van de rol van IFN type I activatie in pSS met als doelstellingen:

- I. Het bepalen van de prevalentie van de IFN type I handtekening en de relatie van de handtekening tot klinische symptomen in pSS
- II. Het ontrafelen van de biologische processen die ten grondslag liggen aan de IFN type I handtekening in pSS
- III. Het bestuderen van de effecten van IFN activatie op het immuunsysteem van patiënten met pSS



In **Hoofdstuk 2** hebben wij bij 55-60% van de patiënten met pSS gevonden dat IFN type I verhoogd is. Deze patiënten hebben de zogenaamde monocyten IFN type I handtekening en noemen we IFN type I positief. IFN positieve (IFNpos) pSS patiënten hebben een hogere ziekteactiviteit, bepaald met de ESSDAI (EULAR Sjögren's Syndrome Disease Activity Index) score, zijn vaker positief voor bepaalde autoantistoffen, hebben lager complement en een verhoging van BAFF. BAFF is een factor die de overleving van cellen, die autoantistoffen maken, vergroot. De overmatige aanwezigheid van autoantistoffen, die tot circulerende immuuncomplexen kan leiden, wordt verondersteld de IFN activatie bij pSS en SLE te veroorzaken.

De bepaling van de IFN type I handtekening is gebaseerd op het meten van meerdere gen-expressie profielen en is zeer arbeidsintensief. In **Hoofdstuk 3** wordt de bruikbaarheid van een snelle en gemakkelijke assay voor het bepalen van IFN type I activatie in pSS beschreven. Het meten van MxA eiwit expressie met de MxA-EIA bleek een praktische en betrouwbare methode om systemische IFN type I activatie in pSS te bepalen. De MxA assay is tevens geschikt gevonden voor het bepalen van IFN type I activatie in andere systemische auto-immuunziekten zoals SLE en systemische sclerose (SSc) (**Hoofdstuk 3; Addendum**).

Aangezien pSS een erg heterogene ziekte is, en het de vraag is of één enkel mechanisme ten grondslag ligt aan pSS, zullen meerdere diersmodellen nodig zijn om de pathogenese van de ziekte op te helderen. Verder beargumenteren we het belang onderscheid te maken tussen pSS patiënten met en zonder een IFN handtekening. Dit geldt ook voor het trekken van conclusies uit studies met muis modellen. Tevens wordt een aanbeveling gedaan voor het kiezen van geschikte muis modellen voor de IFNpos en IFN negatieve (IFNneg) pSS-subtypes (**Hoofdstuk 4**). De NOD (non-obese diabetic) muis kan dienen als model voor systemisch IFNneg pSS, terwijl muizen die een lupus-achtige ziekte krijgen een model voor IFNpos pSS zouden kunnen zijn.

Plasmacytoïde dendritische cellen (pDCs) maken grote hoeveelheden IFN type I na stimulatie van 'toll-like receptor' (TLR)7 en TLR9. In **Hoofdstuk 5** vonden we een verhoogde expressie van TLR7 in zowel pDCs als monocyten van IFNpos pSS patiënten. Bovendien kwamen ook de genen die na stimulatie van de TLR-IFN route aangezet worden, verhoogd tot expressie in IFNpos pSS. Er zijn aanwijzingen voor een bijdrage van zowel de TLRs als de cytoplasmatische RIG-I-like receptoren (RLRs) RIG-I (gecodeerd door DDX58) en MDA5 (gecodeerd door IFIH1) aan de IFN-overactivatie in auto-immuniteit. De RIG-I en MDA5 receptoren kwamen net als TLR7 verhoogd tot expressie in IFNpos pSS, en konden in een celweek worden verhoogd door stimulatie van TLR7. De verhoging van TLR7, RIG-I, MDA5 was uitsluitend aanwezig in IFNpos pSS patiënten. IFNneg patiënten hadden een tegengesteld expressie patroon met normale TLR7 en verlaagde TLR9, RIG-I en MDA5 expressie. Samenvattend, de expressie patronen van RNA-herkende receptoren TLR7, RIG-I en MDA5 in pDCs en monocyten van IFNpos pSS patiënten zijn in disbalans. Dit profiel zou de abnormale IFN productie in patiënten met pSS kunnen verklaren. Verder denken wij dat de verlaagde expressie van deze receptoren in IFNneg pSS patiënten mogelijk de afwezigheid van IFN activiteit zou kunnen verklaren.

Aangezien IFN type I en type II (IFN γ) de kynurenine (KYN) biosynthese kunnen induceren, onderzochten we de KYN route in patiënten met pSS (**Hoofdstuk 6**). In zoogdieren is de KYN biosynthese de voornaamste route voor de verwerking van tryptofaan (TRP). TRP wordt door het enzym Indoleamine-2,3-dioxygenase (IDO) omgezet in KYN. Interessant is dat afwijkingen in de KYN route worden gevonden bij patiënten met stemmingsstoornissen en bij patiënten met moeheid en depressie. Tevens speelt IDO een cruciale rol in de onderdrukking van de functie van ‘effector T cellen’, terwijl het juist de tolerantie inducerende zogenaamde ‘regulatorische T cellen (Tregs)’ aanzet. Het is bekend dat IDO kan worden aangezet door IFN. In **Hoofdstuk 6** beschrijven we dat de IFN activatie in pSS patiënten gepaard gaat met een verhoging van IDO en met een verhoging in Treg cellen. Verder vinden wij in IFNpos pSS patiënten ook verhoogde expressie van enzymen die betrokken kunnen zijn bij neurotoxische effecten in het brein.

Een rol voor IFN type I bij ernstige moeheid in pSS, wordt ondersteund door het feit dat moeheid optreedt bij patiënten met hepatitis of kanker die met IFN worden behandeld. In **Hoofdstuk 7** keken we naar de rol van het afwijkende KYN patroon in pSS, in relatie tot moeheid en depressie. Hier vonden we een negatieve associatie tussen moeheid en IDO activiteit, terwijl de TRP serum spiegels juist positief associeerden met moeheid in pSS. In tegenstelling tot onze verwachting vonden we geen verband tussen de IFN type I handtekening en moeheid bij pSS.

Ten slotte worden de resultaten en implicaties van ons onderzoek besproken in **Hoofdstuk 8**. De gevonden pathogenetische routes liggen waarschijnlijk ten grondslag aan de klinische heterogeniteit van het ziektebeeld pSS. Het is aannemelijk dat patiënten met Sjögren baat zullen hebben bij therapieën die toegespitst zijn op de afwijkingen die bij de individuele patiënt aanwezig zijn. Een onderverdeling van pSS patiënten in IFN positieve en IFN negatieve patiënten is een eerste stap op weg naar een dergelijke behandeling.

LIST OF ABBREVIATIONS

Ab	antibody
Anti-SSA/B	anti-Sjögren's syndrome-associated autoantigen A/B
ACR	American College of Rheumatology
Aec	autoimmune exocrinopathy
AECG	American-European consensus group
ANA	Anti-nuclear antibodies
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BCR	B cell receptor
BDCA	blood dendritic cell antigen
BELLIS	Belimumab in Sjögren's syndrome
BH4	tetrahydrobiopterin
CARD	caspase recruitment domain
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
cGAMP	cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cGAMP synthase
CRP	C-reactive protein
DC	dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EIA	Enzyme Immuno Assay
ELISA	Enzyme-Linked Immuno Sorbent Assay
ER	Endoplasmatic reticulum
ESSDAI	EULAR Sjögren's Syndrome Disease Activity Index
EULAR	European League Against Rheumatism
FACS	fluorescence activated cell sorting
FcR	Fc receptor
FoxP3	forkhead box P3
GTPase	guanosine triphosphate hydrolysing enzyme
GTP-CH1	guanosine-triphosphate cyclohydrolase-1
GWAS	genome-wide association study
HC	healthy control
HCQ	hydroxychloroquine
HCV	hepatitis C virus

HLA	human leukocyte antigen
IC	immune complex
IDO	indoleamine 2,3-dioxygenase
IFI _{(44(L))}	IFN-induced protein
IFIG	Interferon-inducible gene
IFIT	IFN- induced protein with tetratricopeptide repeats
IFN	Interferon
IFNAR	IFN α , β -receptor
Ig	immunoglobulin
IL	interleukin
IPS-1	IFN- β promotor stimulator 1
IRAK	interleukin-1 receptor associated kinase
IRF	interferon-regulatory factor
ISG	Interferon stimulated gene
KYN	kynurenine
LT β R	lymphotoxin-beta receptor
Ly6E	lymphocyte antigen 6 complex, locus E
mAB	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
MAVS	mitochondrial antiviral signalling protein
MHC	major histocompatibility complex
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	non-obese diabetic
NS	non-structural
MyD88	myeloid differentiation primary-response gene 88
MxA	myxovirus resistance protein A
PBMCs	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cell
poly I:C	polyinosinic-polycytidylic acid
pSS	primary SS
RA	rheumatoid arthritis
RNA	ribonucleic acid
RQ-PCR	real time quantitative polymerase chain reaction
SAR	systemic acquired resistance
SGEC	salivary gland epithelial cell
SLE	systemic lupus erythematosus
SMOC	supramolecular organization center
SNP	signal nucleotide polymorphism

SS	Sjögren's syndrome
STAT	signal transducer and activator of transcription
SSc	systemic sclerosis
T1D	type 1 diabetes
TBK1	Tank-binding kinase 1
TDO	tryptophan 2,3-dioxygenase
Th	T-Helper
TIR	Toll-interleukin-1 receptor
TIRAP	Toll-interleukin-1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFAIP3	tumor necrosis factor alpha-induced protein 3
TRADD	TNF receptor-associated via death domain
Tregs	regulatory T cells
TRP	tryptophan
Yaa	Y chromosome-linked autoimmune accelerator
WT	wild type

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“For all those whom inspired me” - Naomi

En wat hebben er de afgelopen jaren toch veel mensen mij geïnspireerd, gesteund en een hele belangrijke bijdrage geleverd aan het tot stand komen van dit proefschrift. De volgende mensen wil ik graag in het bijzonder noemen:

Allereerst wil ik toch graag iedereen bedanken met het syndroom van Sjögren, die heeft meegedaan aan onze onderzoeken. Zonder jullie was dit proefschrift er nooit gekomen. In het bijzonder, dank aan de Nationale Vereniging voor Sjögren Patiënten (NVSP) voor de constante steun aan dit project, en voor de mooie en zeer geslaagde Landelijke Contact dag afgelopen oktober. Masha, Nanda en Joyce, ik wil jullie heel hartelijk danken voor de leuke tijd tijdens het internationale Sjögren congres. Bedankt voor jullie enthousiasme wat betreft dit onderzoek, en het meerdere malen uitspreken dat de NVSP trots is op mij en mijn onderzoek.

Dr. Versnel, beste Marjan, bedankt voor de mogelijkheid die je mij geboden hebt, samen met Prof. Benner, om mijn promotieonderzoek te kunnen uitvoeren op de afdeling Immunologie. Je hebt mij vooral de ruimte geboden om mezelf te ontwikkelen als onderzoeker en mijn eigen ideeën te kunnen ontplooiën. Hierdoor is vooral het vertrouwen in mijzelf als wetenschappelijk onderzoeker flink gegroeid. Je bood me ook de mogelijkheid om meerdere internationale congressen te mogen bezoeken, waar ik veel heb kunnen leren, netwerken en ons werk grootschalig heb kunnen presenteren.

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Prof. van Hagen, beste Martin, bedankt voor alle kritische input en ideeën wat betreft mijn onderzoek. Dat prikkelde altijd om nog eens goed over alles na te denken. En bedankt voor alle steun de afgelopen jaren.

Prof. Duits, beste Ashley, thank you for being here throughout my whole journey in becoming a researcher, in becoming a true scientist. You have truly been my inspiration from day 1. You know I've always wanted to become a medical doctor, just like my dad. At least since my very unexpected visit to the emergency room... switching my aspiration quickly from creative architect, to a more medical interest. You have helped me see that becoming a scientific doctor is as fulfilling, if not more so. I consider you my mentor and I hope to be able to return the favor by being an inspiration and mentor to the next generation of young, up and coming researchers to be. Masha danki pa tur kos.

Prof. Gottenberg, dear J-E, thank you for taking part in this final phase of my PhD. You and your work in the field have been a true inspiration to me. I appreciate you also voicing your

enthusiasm towards the work I have been doing during my PhD, and the discussions we have had together at conferences. As you said to me once: 'enthusiasm for research can be contagious'! Well, yours sure is! Merci beaucoup pour tout.

Dr. Lubberts, beste Erik, bedankt voor de leuke samenwerkingen de laatste jaren en de kritische blik betreffende het T cell onderzoek. Ook erg bedankt voor de deelname aan mijn leescommissie, het optreden als secretaris en de steun tijdens dit stressvolle proces. I would also like to thank my Doctoral committee members, Prof. Peter Katsikis, Prof. Errol Prens and dr. Bianca Blom for your willingness to take part in my dissertation.

Prof. van Rheeën, dear Jacco, how happy I am that I was at a seminar you gave in Leiden where I was doing my internship at the time, on your 'window model', with which you could microscopically follow breast cancer metastasis in mice using intravital live cell imaging. That was the beginning of an amazing and inspiring journey. I think this was the moment I was infected with the curious bug!! The one that infects you as a young researcher, and gives you this urge and drive to become a true scientist! I came to NY, at Einstein, where you were doing a Postdoc in the John Condeelis lab. Prof Jacco, it is people like you that inspire others to become great, and I hope to someday inspire someone in the way you have inspired me! Thank you, danki.

Dr. Brkic, Zana, my Vanilla! How I have missed you since you've left us to go to the clinic. Especially as my travel buddy! 'The choco & vanilla chronicles' are just not the same without the vanilla. I've often thanked you for being there at the beginning of my journey, introducing me to the lab and especially to the world of Interferons. I could not have done all this without you. You were my roomy, my buddy, and my council for anything and everything. I can't imagine anyone else standing there next to me at my defence! My paranimf <3

Kristy, Kwizzy, my one and only Parelnimf! Your reaction was just priceless. With disbelief you asked "your parel-what"? But thankfully there's Google, and you soon found out it was a true honor to be my pearl, mi perla. Thank you for always being there for me, any time of day, for encouraging me and believing in me. I have lived by your motto all throughout my PhD: *"Give it your all, but don't give yourself"* <3

Corine, ja die Corine! Zonder jou was dit nooit gelukt. Wat waren wij toch een "Dream team"! Na enige tijd voelden wij elkaar zo goed aan. We hoefden soms nauwelijks nog woorden te gebruiken om elkaar te begrijpen. En de beste tijden waren toch achter 'the confocal', onze mooie heart-shaped monocytes, wat een excitement! Je staat altijd startklaar als er wat gedaan moet worden... en hoe... aanpakken als de beste! In dit zelfde stukje moet ik ook even A3 bedanken. Je wist mij altijd aan het lachen te brengen als ik op jullie kamer langskwam, meestal door grapjes uit te halen met Corine! Maar daar kon ze gelukkig goed tegen. Thanks guys! I'm gonna miss you! Conny, zorg jij een beetje voor ze?

Eline, Elien, wat ben ik trots op jou zeg! En wat ben ik blij met je harde werk en steun aan mijn project. Hoe je alles zo snel oppikte, de scriptieprijs won van je studiejaar, nu werkzaam in het LUMC, en nog je vrije vrijdag heel erg lang hebt opgeofferd om mij te komen helpen met het

afronden van het project waar jij een grote bijdrage aan hebt geleverd. Inmiddels heb je bijgedragen aan alle lopende projecten! Leiden mag erg blij zijn dat ze je hebben. Nogmaals bedankt voor alles en je weet het, I'm here if you need anything. Dikke brasa.

Javad, wat ben ik blij met jou! Een hele slimme, zelfstandige en hardwerkende researcher in spé! Ook op jou ben ik erg trots! Hopelijk kunnen we nog wat moois van het cSLE project maken samen, ik wens je alvast heel veel succes en plezier straks in Johns Hopkins. We moeten elkaar maar komen opzoeken. En natuurlijk ook alle andere studenten die bijgedragen hebben aan dit proefschrift, Kim, Eline, Samara en Aster. Bedankt voor jullie inzet en enthousiasme. Ik wens jullie al het geluk en plezier toe in jullie verdere toekomst.

Lieve Daniëlle, ook zonder jou zou dit boekje er niet zijn gekomen. Wat ben ik jou toch onbeschrijfelijk dankbaar voor alles. Je positieve houding en enthousiasme waren altijd een verademing. Het feit dat je in je vrije tijd nog bezig was met het layouten, soms tot in de avonden, en dan ben ik niet de makkelijkste, maar je had altijd geduld en genoot er ook van dat je mij blij kon maken. Lieve Erna, ook jou wil ik bedanken voor alles omtrent mijn proefschrift, het nauw contact met de Pedel om alles toch nog relatief op tijd geregeld te krijgen. Sandra, jou wil ik ook bedanken voor jouw advies en hulp voor wat betreft de figuren. Jullie zijn echt top! Bedankt voor alles, thank you, danki.

Aan iedereen in de AIM, hartelijk dank voor alles de afgelopen jaren. In het bijzonder: Woutje, onze lieve Wouter, wat ben jij geweldig, altijd startklaar om iedereen te helpen. Ik wil ook jou bedanken voor alles, alle hulp met het runnen van die microarrays. Zonder jou was dat never nooit gelukt. Karin, mi Dusshii! Zo blij dat je het lekker naar je zin hebt. Brasa ku sunchi pabo mi dushi! En jouw favoriete zin 'stop di kansami' zal ik nooit vergeten. Angy mi amor, wat hebben wij het toch gezellig gehad samen! Vooral de Friday nights op het EMC, wanneer iedereen allang weekend aan het vieren was, waren wij nog aan het werk. I'm gonna miss you my darling. Harm, Thomas, Anne-Marie, jullie stonden altijd klaar als ik een keer geen bloed kon ficollen of weer eens geen raad wist met het opzetten van een nieuw experiment. Ik wil ook jullie hartelijk bedanken voor alle hulp en steun tijdens mijn promotie. Ik weet dat ik niet altijd de gezelligste collega ben geweest. Het eerste jaar ging ik nog vaak mee lunchen of even koffie drinken, maar al snel realiseerde ik hoe de tijd voorbij ging. Hierbij mijn oprechte excuses voor mijn niet-sociaal gedrag wat dat betreft.

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Prayer, Sita, how I am going to miss you... As I am writing this you are already finalizing everything here in Rotterdam in order to go back to Thailand and embark on a new chapter of your life, back home with your family and loved ones. I really hope we get to see each other again soon in the near future, either to see & explore each other's beautiful home bases or for other more memorable occasions ;). I want to thank your roomy Jeroen, dr. dushi, for keeping the fun alive during our time here! Thanks dushi.

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To all my collaborators during my PhD, thank you all for the fruitfull and pleasant collaborations. Dear dr. Waris, Matti, thank you for the wonderful visit to Turku, at the virology lab, to learn the MxA-EIA and take the skills back to Rotterdam. Thank you for the great collaborations

and the lovely sunny time in Turku. Dr. Ijpma, Arne, jou wil ik heel erg bedanken voor de hele prettige, leerzame en leuke samenwerking van de afgelopen jaren. Het was leuk hoe je interesse toonde in mijn project en ook altijd meedacht over de beste aanpak. Dear Petra, thank you for all the great collaborations, talks about science and everything else.

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For my Crypticsss!!! Best combination of science meets friends!! My lovely Frenchies, Gaetane aka Black G aka Dr. Nocturne my one and only, Laurent aka YeLau aka dr. Chiche of the Modules, Unicornec aka Divi Divi aka Divine aka dr. Divi Cornec, and SecretR aka dr. Seror...the girl from Paris South! Thank you for making research on Sjögren's that much more fun! I say cheers to many more fun cryptic adventures as well as possible masterful collaborations! And whatever you do...keep it moving ;) #Bisoussss from Blue Curaçao. TakTak!

And of course thanking people in my midst does not stop at the work front!

"A good friend knows all your stories, a best friend has lived them with you"
"If you have crazy friends you have everything"

My ladies, my twrkrz, Ki mi Lo Hasi sin bosnan? Kris, Naiche, Kimbo, Ishka, Rais, Ken, Sue en Anna... Thank you girls for all the love and support, making sure I stay sane, taking me out to dinner or drinks or dancing, or just chilling and talking about anything and everything. I could not have survived this period without you! A special shout out to my SupaFab girls... when friends become family!!! And remember, wherever we are, it is our friends that make our world <3.

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To all my basketball ladies! I'm sorry I had to stop playing... I promised to start again once I finished my PhD! Let's see... Although you might have to come visit me in NY, in order for us to shoot some hoops! Thank you all for your love and support, and giving me a place to blow off steam and stress...

"If you accept the expectation of others, especially negative ones, then you will never change the outcome" – Michael Jordan

Omaatje, wat zulde gij fier zijn geweest op uw zoeteke! Da boekske e wok vor joe nateurlik! Je was altijd leergierig en inspireerde mij ook daarin tijdens onze vakanties bij jou en opa in België. Blijf vanuit de hemel over mijn schouder meekijken <3

Tante Clorinda, I know you'll be front row up in heaven today, when I defend my thesis. How we miss you! This thesis is also for you! Hope I can make you proud <3

Mi Broedahzz, P en T, Pano en Tarsi, wat ben ik een lucky sister zeg, with brothers like you. Jullie weten het misschien niet maar ik ben super proud to have two amazing brothers like you! Wat heb ik toch veel van jullie geleerd! My ability to work together with people and my enjoyment for collaborations, for exploring and figuring things out, is in part due to the things we used to explore together when we were little. Ik hoop dat ik jullie vandaag een beetje trots kan maken! Dit proefschrift is ook voor jullie <3. Selm en Nizzy, my sisters from other misters!! I'm writing this part on the 'big beach' in Cap Ferret! Feeling the breeze and listening to the waves crashing smoothly. Thanks for giving me this little oasis to take my mind off these final stages of craziness! Thanks for all the food and wine, and all the love and support! Nizzy, a special thanks goes out to you for all the help with my beautiful illustrations, and all the help with my book itself, for staying calm when I was stressing out... Thank you my creative sistah :)

Mami en Papi, saving the best for last!!! I have already told the both of you countless times how lucky I feel to have the 2 of you as my parents. I feel blessed to have been given 2 amazing people, to raise me, teach me, inspire me, and care for me... take care of me, especially in this roller-coaster called a PhD. You guys are always there for me if I need advice, helping with anything and everything. I could fill this page with the things I want to thank you guys for, but most of all I want to thank you for always believing in me, and encouraging me to be better! I do not know where I would be without you, probably not where I am today, and the thought of having these 2 strong people in my corner makes me happy, makes me proud, makes me glad to dedicate this thesis to you, and to end this part of my thesis with thanking you. Danki mami i papi pa tur kos, pa tur e sosten i konfiansa den mi. Mi ta sigur ku sin boso mi no lo a yega kaminda mi ta awe. Danki <3

"There is no passion to be found in settling for a life that is less than the one you are capable of living" – Nelson Mandela

"First they ignore you, then laugh at you, then they fight you, then you win"
– Mahatma Gandhi



W. 2012

ABOUT THE AUTHOR

CURRICULUM VITAE

PHD PORTFOLIO

LIST OF PUBLICATIONS

CURRICULUM VITAE

Naomi Iñez Maria was born in Groningen, The Netherlands on October 10th 1985. She moved to the island of Curaçao with her family at the age of 1, where she attended primary school at George Charles Pire College. She finished her secondary education at the Radulphus College in 2003 at age 17, with a particular interest in medicine, organic chemistry and creative arts. Hereafter she moved back to the Netherlands to study Biopharmaceutical Sciences at the Leiden University. During her studies she did research internships at the Division of Toxicology, Leiden/Amsterdam Center for Drug Research, in the lab of Prof. B. van de Water – ‘Focal adhesion signalling in tumour cell migration in relation to breast cancer metastasis’, at Red Cross Blood Bank Foundation, in the lab of Prof. A.J. Duits, Curaçao – ‘Levels of endothelial progenitor cells and angiogenic factors in sickle cell disease’, and her master thesis at the Albert Einstein College of Medicine, Yeshiva University, Department of Anatomy and Structural Biology, in the lab of Prof. J. Condeelis, New York – ‘The role of N-WASP activity in invadopodia of invasive mammary carcinoma cells’. In February 2009 she received her Master’s degree in Biopharmaceutical sciences.

After her Masters she worked at the Centre for Human Drug Research as Central Nervous System observatory research manager, and thereafter as assistant-project leader in designing and monitoring clinical studies and phase I clinical trials. During this period she realized that the combination of clinical and basic science would best fit her interests and ambitions, and that she could use her creative and curious mind to explore scientific questions.

In December 2010 she started her PhD under supervision of Dr. M.A. Versnel, in the lab of Prof. H.A. Drexhage at the Department of Immunology, Erasmus MC, Rotterdam, The Netherlands. Her project focused on the immunopathology of primary Sjögren’s syndrome, in particular on the prevalence of the interferon type I signature, its associations with clinical manifestations and disease activity, as well as its potential molecular and cellular origin. During her PhD she supervised bachelor and master theses, assisted in teaching immune-histology and pathology, and presented her research at both national and international conferences in the fields of Rheumatology and Immunology. In the fall of 2014, she had the opportunity to present her work in the plenary session at the American College of Rheumatology in Boston, which was one of the highlights of her PhD. She also had the opportunity to go abroad on collaborative working visits to the lab of Dr. M. Waris, at the Department of Virology, University of Turku, in Finland, and to the lab of Prof. W-F. Ng at the Department of Rheumatology, Newcastle University, UK. Furthermore, after finishing her PhD she will receive recognition as an SMBWO immunologist.

In January 2016 she will start a PostDoc fellowship in the lab of Prof. A. Davidson at The Feinstein Institute, North Shore University Hospital, New York, continuing here research in systemic autoimmunity in both mouse and man.

“I have no special talent. I am only passionately curious” – Albert Einstein



PHD PORTFOLIO

Name PhD student	Naomi Iñez Maria
Erasmus MC Department	Immunology
Research School	Molecular Medicine (MolMed)
PhD period	December 2010 – December 2015
Promotors	Prof.dr. H.A. Drexhage, Prof.dr. P.M. van Hagen
Copromotor	Dr. M.A. Versnel

PhD Training

Courses and workshops

2005	Health Physics certificate level 5B
2007	Art. 9 certificate: Laboratory Animal handling
2008	Science Based Business (SBB) fundamentals
2009	Good Clinical Practice (GCP) Course certificate
2009	ALS (Assistant Life Support) Theoretical and Practical course
2009	Promasys training; Data Management System
2011	Molecular Medicine course
2011	Biostatistics for Clinicians (EWP22)
2011	NIBI management for PhDs & PostDocs
2011	Photoshop and Illustrator CS5 Workshop for PhDs and researchers
2011	Workshop Basic data analysis on gene expression arrays
2012	NVVI Dutch society for Immunology Lunteren “APC’s revisited”
2012	Advanced course Molecular Immunology
2012	Flow cytometry training by Becton Dickson (BD)
2013	NVVI Lunteren “The immune system on fire”
2013	Scientific Writing in English for Publication course
2013	Summer course Advanced Epidemiology NIHES <i>Netherlands Institute for health sciences</i>
	- Study Design (CC01)
	- Principles of Research in Medicine (ESP01)
	- Clinical Design Decision Analysis (ESP04)
	- Regression Analysis (ESP09)
	- The Practice of Epidemiologic Analysis (ESP65)

- 2014 Certificate Basic Teaching Qualification (BKO) “Uitvoeren van onderwijs”
- Teach the Teacher I
 - Individual guidance
- 2014 Medical Immunology course Erasmus MC
- 2014 Summer course Master Infection & Immunity: Microbiology and Virology
- 2014 Confocal microscopy course
- 2015 Gene expression data analysis using ‘R’ for RNA-Seq/Microarray

(Inter)national Scientific meetings and presentations

- 2011 Annual MolMed meeting (poster)
- 2011 11th international Sjögren’s Symposium, Athens (poster)
- 2011 Psychorheumatology Sjögren-working group, Utrecht, The Netherlands (Oral)
- 2012 American College of Rheumatology (ACR) annual meeting, Washington D.C, USA (poster)
- 2013 Annual MolMed meeting (poster)
- 2013 Working visit, Dept. Virology, University of Turku, Finland – Dr. M. Warris lab (Oral)
- 2013 National Workgroup on Systemic diseases, Amersfoort, Netherlands (Oral)
- 2013 12th international Sjögren’s Symposium, Kyoto, Japan (Oral & poster)
- 2013 NVVI annual meeting, Noordwijkerhout, the Netherlands (poster)
- 2013 Club Francophone des Cellules Dendritiques (CFCD), Paris, France (poster)
- 2014 Science Days Internal Medicine 2014, Antwerp, Belgium (Oral)
- 2014 ACR annual meeting, Boston M.A., USA (2 Orals):
- ACR Plenary Session II: Discovery 2014
 - ACR concurrent Session: Sjögren’s syndrome II
- 2014 NVVI 50th meeting, Kaatsheuvel, the Netherlands (Oral & poster)
- 2014 The Neuro-Immunology of Psychiatric Diseases, Amsterdam, The Netherlands (Oral)
- 2015 Science Days Internal Medicine, Antwerp, Belgium (poster)
- 2015 Working visit Newcastle University – W. Fai Ng lab (Oral)
- 2015 Invited presentation, Hospital for special surgery, NY – Dr.F. Barrat lab
- 2015 13th international Sjögren’s Symposium 2015, Bergen, Norway (Oral & poster)
- 2015 Landelijke contactdag NVSP: workshop for pSS patients
- 2015 ACR annual meeting, San Francisco C.A., USA (Oral & poster)
- ACR concurrent Session: Pediatric Rheumatology - Pathogenesis and Genetic

Teaching

2011-2014	Immuno-histology by digital microscopy (1 st year Medical students)
2011-2014	Immuno-pathology; Acute & chronic inflammation (2 nd year Med. students)
2012-Present	Supervising Bachelor & Master theses (research internships)

Grants and Awards

2011-Present	Travel grants from the Dutch Arthritis Research foundation (Reumafonds)
2011-Present	Travel grants from the Dutch National Foundation for Sjögren Patients (NVSP)
Oct 2013	Young Travel Award: 12 th International Symposium on Sjögren's Syndrome, University of Tsukuba, Japan
Nov 2014	Travel grant from NVVI Dutch society for immunology
Nov 2014	Travel grant from Erasmus MC Trustfonds
May 2015	Young Travel Award: 13 th International Symposium on Sjögren's Syndrome, University of Bergen, Norway; Awarded by the Scandinavian Foundation for Immunology.
Oct 2015	Nominated for the Dutch Caribbean Science Pearl award 2015
Nov 2015	Travel grant from Erasmus MC Trustfonds
Nov 2015	Travel grant from the Dutch National Foundation for Lupus Erythematosus (NVLE)

Other activities

2010-2014	Journal club at the Department of Immunology
2010-2014	Seminars and minisymposia at the Department of Immunology
2011	Writing medical ethical committee (METC) research protocols
2011	Member of the Social events committee of the Department of Immunology, EMC
2013	Collaboration with the Psychorheumatology research group of the Utrecht University on the BVS (Behandeling Vermoeidheid Sjögren) study, Maartenskliniek, Woerden, Utrecht
2014	SMBWO Immunologist exam obtained
2014-2015	Co-writing grant proposals
2014-2015	Volunteer for the IMC weekendschool Rotterdam, Department of Immunology, EMC



LIST OF PUBLICATIONS

Published:

Z. Brkic, L v Bon, M. Cossu, C.G. van Helden-Meeuwsen, M.C. Vonk, H. Knaapen, W v den Berd, V.A. Dalm, P.L v Daele, A. Severino, *N.I. Maria*, S. Guillen, W.A. Dik, L. Beretta, M.A. Versnel, T Radstake. 'The Interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis'. *In press, Ann Rheum Dis, Sept 2015; doi:10.1136/annrheumdis-2015-207392*.

N.I. Maria, P. Vogelsang, M.A. Versnel. 'The clinical relevance of animal models in Sjögren's syndrome: The Interferon signature from Mouse to Man'. *Arth Res & Ther*, 2015;17:172. DOI: 10.1186/s13075-015-0678-2.

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*"Imagination is more important than knowledge. Knowledge is limited.
Imagination encircles the world" - Albert Einstein*

*I unda ku mi ta...
Korsou lo keda mi Isla
kaminda mi Kurason
pa semper ta ankrá...*

- Naomi I. Maria