Interferon in Sjögren's syndrome and other systemic autoimmune diseases: A driver of disease pathogenesis and potential treatment target

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Interferon in Sjögren's syndrome and other systemic autoimmune diseases: A driver of disease pathogenesis and potential treatment target

Interferon bij het Syndroom van Sjögren en andere systemische auto-immuunziekten: Een sleutelrol in de pathogenese en potentieel doelwit voor behandeling

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General Introduction

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GENERAL INTRODUCTION

In this thesis we study the role of interferon (IFN) and its downstream signaling pathways on the pathogenesis of several systemic autoimmune diseases, with primary Sjögren's syndrome (pSS) being the main focus. In this chapter we introduce the immune system and describe in short how the loss of tolerance to self-proteins can lead to autoimmunity. Type I IFN activity is present in a subgroup of patients with systemic autoimmune diseases and here we describe how these IFNs are thought to contribute to the pathogenesis of systemic autoimmunity focusing on pSS. Finally, we summarize existing and new IFN targeting therapies and the latest literature on their effectivity.

THE IMMUNE SYSTEM

During life we are exposed to a wide variety of pathogens like viruses, bacteria and parasites. The immune system is a complex mechanism of biological structures and processes that helps to protect and eliminate these invaders. Furthermore, the immune system recognizes abnormal cells, thereby protecting us from cancer. Traditionally, the immune system is divided in an innate and adaptive arm.

The bodies first line of defense consist of physical and chemical barriers that prevent pathogens from entering. Examples are the skin, the enzymes in the oral cavity and the low pH of the stomach [1]. If pathogens are able to breach these barriers, the next line of defense is activated. The innate arm of the immune system consists of a variety of different cells (such as monocytes, neutrophils, macrophages and natural killer cells) and mechanisms (complement system) which can kill the invading organism. A hallmark of this type of immune response is that it is very rapid, but it lacks specificity and memory formation.

The adaptive immune response, mainly consisting of T and B cells, can recognize an almost unlimited amount of antigens and are able to form memory cells. T cells are divided in 2 major types: CD4+ T helper and CD8+ T cytotoxic cells (Tc). The CD4+ T helper (Th) subgroup can further be divided in a number of subsets including Th1, Th2, Th17, Th9, regulatory Th cells (Treg) and some recently identified novel subsets [2]. While the Tc cells are specialized in the clearing or controlling of viruses and tumors, Th cells are important for the activation and function of B cells. The primary function of B cells is to produce antibodies (immunoglobulins) [3]. These antibodies are important for the neutralization of pathogens, activation of the complement system and mediate antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis.

Innate immune responses are necessary for the activation of adaptive immunity. One of the important cells bridging innate and adaptive immunity is the dendritic cell (DC). This cell type is an antigen-presenting cell (APC) consisting of two major subtypes, conventional DC and plasmacytoid DC (pDC). DCs are innate cells and express pattern recognition receptors (PRRs), which recognize molecular patterns foreign to the host. These patterns are evolutionary highly conserved, and are called pattern-associated molecular patterns (PAMPs) [4]. An example of a PRR family are the toll-like receptors (TLRs). This family of transmembrane receptors are located both on the cell surface and in endosomal compartments. TLRs on the surface of the host cell recognize mainly components of the cell wall of pathogens, while intracellular TLRs mainly recognize (microbial) nucleic acids. Other PRRs present in the cytoplasm are the RIG-I like (RLR) and DNA sensing receptors (DSR) sensing respectively cytosolic RNA or DNA. Triggering of PRRs by PAMPs leads to a cascade of host defense responses including the production of cytokines [5]. Additionally, PRR stimulation induces maturation of DCs and facilitates the antigen presenting function by the induction of costimulatory molecules leading to activation of adaptive immunity.

AUTOIMMUNITY

The immune system is a powerful mechanism to protect us from unwanted invaders, however in order to do so it is critical to distinguish self from non-self. In patients with autoimmune diseases the immune system mistakenly reacts to self-antigens, resulting in an immune response directed against the body's own nucleic acids, proteins, cells or tissues [6]. The development of autoimmunity is multifactorial which involves amongst others a contribution of genetics and the environment. Autoimmune diseases can be classified in organ-specific or systemic. In organ-specific autoimmune diseases only one organ is affected, examples of these type of autoimmune diseases are Hashimoto's thyroiditis, type I diabetes or Addison's disease. Systemic autoimmune diseases affect multiple sites of the body like rheumatoid arthritis (RA), primary Sjögren's syndrome (pSS), Systemic Lupus Erythematosus (SLE) and Systemic Sclerosis (SSc).

In systemic autoimmune diseases autoreactive B and T cells directed against molecules found throughout the body like RNA and DNA are present [7]. During the development of B and T cells the body negatively selects cells that are targeting the body's own tissue by deleting cells that express antigen receptors with a high affinity for self-antigen. This process is called central tolerance. When autoreactive T and B cells escape this selection process there are several mechanisms in the periphery to prevent reaction to self, called peripheral tolerance. Despite these selection processes

autoreactive cells are increased in patients with autoimmune diseases resulting in loss of tolerance. In systemic autoimmunity often antinuclear autoantibodies (ANAs) can be found. These ANAs are produced by autoreactive B cells and are directed against nuclear macromolecules and their complexes like dsDNA, small nuclear ribonucleoproteins (snRNPs), histones or centromeres [8]. A large variety of different ANA specificities are described. Some are strongly associated with a particular disease, while others are expressed more heterogeneous amongst patients. ANAs can form immune complexes (ICs), which can be deposited in multiple organs triggering vascular permeability, the influx of immune cells and production of inflammatory mediators leading to tissue damage [9]. Treatment of these systemic autoimmune diseases is difficult because the autoantigens are excessively present and suppression of the immune system can disturb the delicate immune balance needed for defense against invading pathogens.

SJÖGREN'S SYNDROME AND OTHER SYSTEMIC AUTOIMMUNE DISEASES: CLINICAL FEATURES

Sjögren's syndrome (SS) is a chronic, systemic autoimmune disease characterized by infiltrations of immune cells in the salivary and lachrymal glands. These patients present with dryness of the eyes and mouth. Classical ocular symptoms are burning or itchiness and the feeling of sand in the eyes [10]. A characteristic symptom from the mouth is the need to drink when trying to swallow dry food, also called as the cracker-sign [11]. Besides these glandular problems, patients sometimes additionally suffer from extraglandular manifestations. These symptoms include fatigue, joint pain, muscle pain and Raynaud's phenomenon. In some cases the disease can also affect internal organs like the lungs or kidneys [12-14]. In the blood of SS patients often characteristic autoantibodies are found, anti-Ro/SSA present in 60-70% the SS patients and anti-La/SSB in 30-60% of the patients [15]. Anti-Ro/SSA antibodies are targeted against two cellular proteins with a molecular weight of around 52 and 60 kD, respectively called 'Ro52' and 'Ro60' [16]. Ro52 is located in the cytoplasm where it functions as an E3 ligase involved in ubiquitination [17]. Ro60 is a nuclear protein binding small, non-coding RNAs called 'Y RNAs' of which the function is largely unknown. Anti-La/SSB are targeting a 47-kD protein which shuttles between the cytoplasm and nucleus and is involved in RNA metabolism. The presence of these antibodies is often associated with earlier disease onset, glandular dysfunction and extraglandular manifestations [15]. One of the most severe complications of pSS is the development of B cell lymphoproliferative disease, which occurs in around 5-10% of the pSS cases [18-21]. Like most autoimmune

diseases SS mainly affects females and most patients are diagnosed between the age of 40 and 60. The disease is called primary SS in the absence of other autoimmune diseases. When presented in a combination with other autoimmune diseases, like RA or SLE, it is called secondary SS. Treatment of this heterogeneous disease is presently mainly symptomatic, focusing on relieving of the dryness symptoms. Deeper insight into the pathogenesis will lead to new treatment options.

Other systemic autoimmune diseases studied in this thesis are SLE and SSc. SLE is a systemic autoimmune disease which can affect both children (childhood-onset SLE) and adults (adult-onset SLE). SLE is often mistaken for other illnesses, because of the wide variation in the presentation of symptoms. Many patients suffer, similar as in pSS, of joint and muscle pain, fatigue or malaise [22]. A characteristic symptom is the butterfly rash on the face. In SLE multiple organs can be affected including skin, kidneys, central nervous system (CNS), lung and others. Patients with SLE experience flare-ups of the disease as well as periods of remission. There is no cure for the diseases and patients are often treated with immunosuppressive medication.

SSc, also formerly called scleroderma, is an autoimmune disease of the connective tissues. In this rare disease, patients suffer from thickening of the skin due to accumulation of collagen and have injuries to small arteries [23]. SSc is divided in a limited cutaneous (lcSSc) and diffuse cutaneous (dsSSc) form based on the extend of the skin involvement [24]. The localized form only affects the skin of the face, hands and feet, while the systemic form can also affect internal organs. SSc has a high mortality rate, particularly in patients with involvement of the internal organs. At the moment there is no drug available to reduce skin fibroses or organ involvement. Because of the heterogeneity in the patients, subgrouping or individual tailored therapies are important for effective treatment.

Activation of the IFN system has been observed in all of the above mentioned autoimmune diseases and appears to play a role in the pathogenesis [25-27]. Therefore we will discuss further the role of IFNs in systemic autoimmunity in this thesis.

INTERFERON

IFNs are a large group of cytokines originally defined by their anti-viral activity [28]. In humans, the IFN family is divided into three classes according to the receptor complex through which they signal: type I, type II and type III IFNs (table 1). The type I IFN class is the largest, comprising 13 subtypes of IFNα [29], IFNβ, as well as the less well described IFNε, IFNκ and IFNω. IFNγ is the sole member of type II IFN, while the most recently

Table 1. The human interferon family

Type	Subtype	Molecular weight (kDa)	Length (aa)	Receptor subunits	Downstream signaling	Primary ISG promoter	Principal source	Principal cellular targets
I	IFNα*	21.550-22.141	189**	IFNAR1, IFNAR2	JAK1, TYK2, STAT1, STAT2, IRF9	ISRE	Ubiquitous. Professionally produced by pDCs	All cells
	IFNβ	22.294	187					
	IFNε	24.414	208					
	IFNκ	25.218	207					
	IFNω	22.319	195					
II	IFNγ	19.348	166	IFNGR1, IFNGR2	JAK1, JAK2, STAT1	GAS	NK cells, NKT cells, T cells	Macrophages, T cells, B cells, APCs
III	IFNλ1 (IL-29)	21.898	200	IL10R2, IFNLR1	JAK1, TYK2, STAT1, STAT2, IRF9	ISRE	Ubiquitous	Cells of epithelial origin
	IFNλ2 (IL-28A)	22.288	200					
	IFNλ3 (IL-28B)	21.706	196					

*13 subtypes
**IFNα2 188 amino acids
IFN, interferon; ISG, interferon stimulated gene; pDCs, plasmacytoid dendritic cells; APCs, antigen presenting cells; aa, amino acids

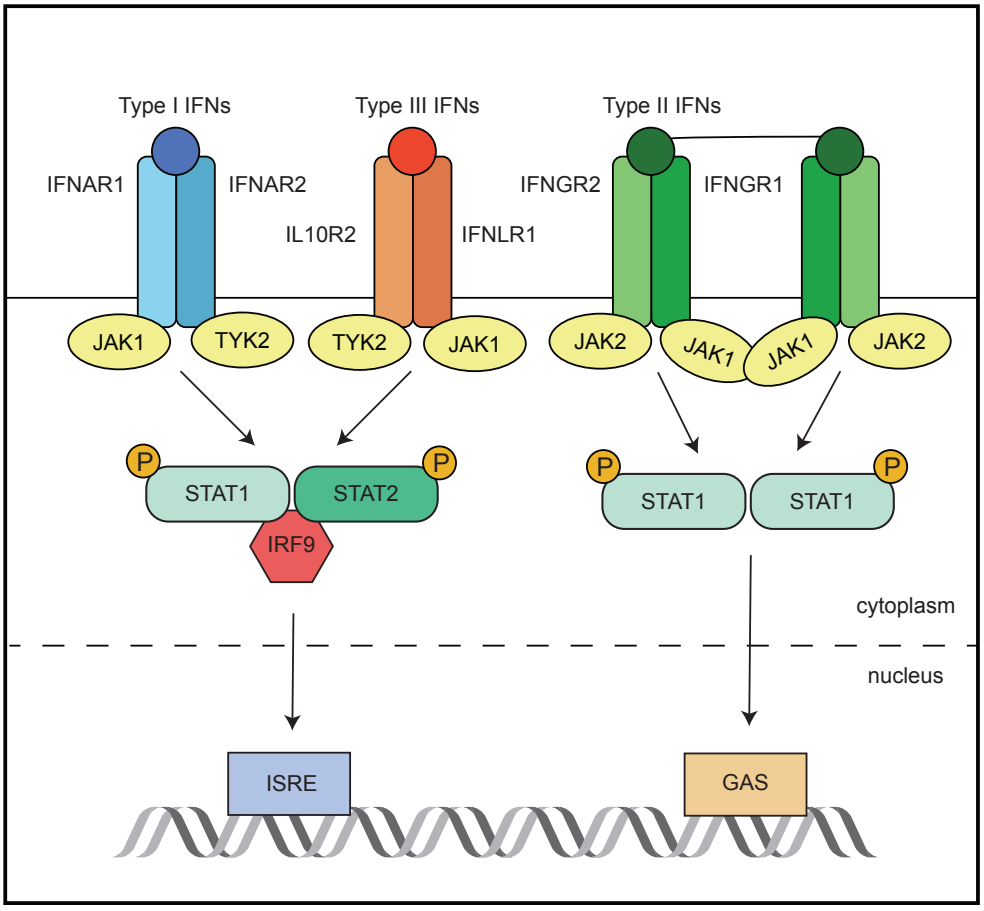

Figure 1. Receptor activation signaling pathways by type I, type II or type III interferons
Type I interferons (IFNs) (α (13 subtypes), β ω, κ, ε, δ) interact with a heterodimer of type I IFN receptor (IFNAR)1 and IFNAR2. Type III IFNs (IFNλ1, IFNλ2 and IFNλ3; also known as IL-29, IL-28A and IL-28B respectively) interact with IFNλ receptor 1 (IFNLR1; also known as IL28RA) and interleukin 10 receptor 2 (IL10R2; also known as IL10RB). These receptors are associated with two kinases from the JAK family, JAK1 and TYK2. Activation of the type I and III IFN receptor leads via JAK1 and TYK2 to recruitment and phosphorylation of signal transducers and activators of transcription (STAT)1 and 2. STAT1/2 heterodimers associate with IFN regulatory factor 9 (IRF9) to form an IFN-stimulated gene factor (ISG3) complex. ISG3 translocation to the nucleus activates IFN-stimulated response elements (ISREs). Type II IFN (IFNγ) interacts with the IFNγ receptor 1 (IFNGR1) and IFNGR2. This receptor associates with JAK1 and JAK2 and recruits and phosphorylates STAT1 homodimers. Translocation into the nucleus induces IFNγ-activated site (GAS) promoter elements.

described type III IFN class consists of IFNλ1, IFNλ2 and IFNλ3 (also known as IL-29, IL-28A and IL-28B, respectively).

Most nucleated cells have the capacity to secrete both type I and type III IFN in response to various stimuli [30]. Thus, pDCs, sometimes denoted as “professional”

producers of type I IFNs [31], can also express high levels of type III IFNs [32, 33]. Type I IFN signals via the type I IFN receptor (IFNAR) and consists of a IFNAR1 and IFNAR2 complex (figure 1). Type III IFNs signal via the type III IFN receptor consisting of a IL10R2 and IFNLR1 complex. Binding of type I and III IFNs to its respective receptor leads to downstream association with two proteins from the Janus kinase (JAK) family, JAK1 and Tyk2. Subsequently, STAT1/2 heterodimers form a complex with IFN regulatory factor 9 (IRF9) to form an IFN-stimulated gene factor (ISG3) complex. In the nucleus ISG3 binds to IFN-stimulated response elements (ISREs) leading to expression of interferon stimulated genes (ISGs). Differences in the biological effects between type I and type III IFNs are mainly due to differences in the expression of their receptors. The IFNAR is found on all nucleated cells, while response to type III IFNs appears more or less restricted to cells of epithelial origin [34].

IFNγ is mainly produced by activated T cells, natural killer (NK) cells and natural killer T (NKT) cells. Aside from its modest antiviral activity, IFNγ plays an important role in stimulating and modulating the immune responses, primarily by activating macrophages and by controlling differentiation of naïve $CD4^+$ T cells into Th1 effector cells [35]. Following binding of dimeric IFNγ to its receptor, intracellular signals are transduced via JAK1/2 and STAT1 homodimers causing induction of ISGs through binding to IFNγ-activated sites (GAS) and the initiation of gene transcription.

TYPE I INTERFERON SIGNATURE

Upregulation of type I IFNs is well studied in pSS. Due to the many different type I IFN subtypes, measurement of protein levels using Enzyme-Linked ImmunoSorbent Assay (ELISA) underestimates the type I IFN levels. Therefore, type I IFN activation is commonly assessed by measuring upregulated expression of ISGs, also called the "type I IFN signature". As there are over a thousand genes upregulated in response to type I IFNs, attempts have been made to develop a "diagnostic" consensus gene signature for clinical application [36]. Upregulation of ISGs has been found in salivary glands, peripheral blood mononuclear cells (PBMCs), isolated monocytes, pDCs and B cells of pSS patients [37-42]. However, it is still unclear how the local and systemic type I IFN signature correlate with each other. An overview of consistently identified differentially expressed ISGs is shown in **table 2**.

Although there are differences in expression of ISGs in specific tissues or cells, whole blood IFN signature analysis can now be introduced in clinical practice [43]. Currently, the easiest way to determine systemic type I IFN activation is by drawing whole blood using specific RNA stabilizing tubes [44]. These tubes can be kept at room temperature

Table 2. Differentially expressed type I IFN stimulated genes detected in various tissues and cell-types in pSS

Abbreviated name	Full name	Function
IFITM1	Interferon Induced Transmembrane Protein 1	Inhibits entry of viruses to the host cell cytoplasm
IFI27	Interferon Alpha Inducible protein 27	Mediates IFN-induced apoptosis
IFI44*	Interferon Induced protein 44	Anti-proliferative, hepatitis c-associated microtubule aggregating protein
IFI44L*	Interferon Induced Protein 44 Like	Largely unknown, but role in antiviral defense
IFIT1*	Interferon Induced Protein With Tetratricopeptide Repeats 1	Inhibits viral replication and translational initiation
IFIT2	Interferon Induced Protein With Tetratricopeptide Repeats 2	Inhibits expression of viral mRNAs lacking 2-O-methylation
IFIT3*	Interferon Induced Protein With Tetratricopeptide Repeats 3	An inhibitor of cellular as well as viral processes, cell migration, proliferation, signaling, and viral replication
IRF7	Interferon Regulatory Factor 7	Key transcriptional regulator of type I IFN-dependent immune responses
IFI16	Interferon Gamma Inducible Protein 16	Modulates p53 function and inhibits cell growth via Ras/Raf pathway
OAS1	2'-5'-Oligoadenylate Synthetase 1	Activates latent RNase L, resulting in viral RNA degradation and the inhibition of viral replication
Mx-1/MxA*	Myxovirus-resistence protein 1	GTPase, prevents replication process of several RNA and DNA viruses

*genes used for whole blood type I IFN signature [49].

for up to 5 days after sampling, which facilitates transportation. After RNA isolation the ISGs are quantified by qPCR. For the calculation of the type I IFN score the mean expression level and standard deviation (SD) of a set of ISGs in a healthy control group is used to standardize expression levels of each of these genes per patient. The standardized expression levels are subsequently summarized for each patient to generate a type I IFN score. Type I IFN positivity is defined as two or three standard deviations above the mean in the HC group [45, 46]. In pSS, 50-80% of the patients have a positive type I IFN signature [47, 48].

A FEED-FORWARD LOOP INVOLVING AUTOANTIBODIES AND INTERFERONS

All nucleated cells have the capability to produce type I IFN in response to viral infection through stimulation of TLRs and cytosolic sensors of nucleic acids. Intriguingly, the same receptors that help clear infections also contribute to sustained type I IFN production in systemic autoimmune diseases. Key cytosolic sensors of RNA include retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) RIG-I and MDA5 (figure 2). In pSS these sensors were recently found to be up-regulated in type I IFN positive patients compared to IFN negative patients, thus pinpointing a novel mechanism which may contribute to the increased IFN production [42]. Both RLRs and DSRs signal via TBK1/IKKε. Additionally we showed an upregulation of TBK1 in pSS, indicating a potential contribution of DSRs to the chronic upregulation of type I IFN [49, 50]. The endosomal receptors TLR7 and TLR9 respond to single stranded RNA and double stranded DNA, respectively. Following ligation, signaling via the myeloid differentiation primary response 88 (MyD88) pathway leads to activation of IFN regulatory factor (IRF) 7, a central regulator of type I IFN transcription. Importantly, the professional type I IFN

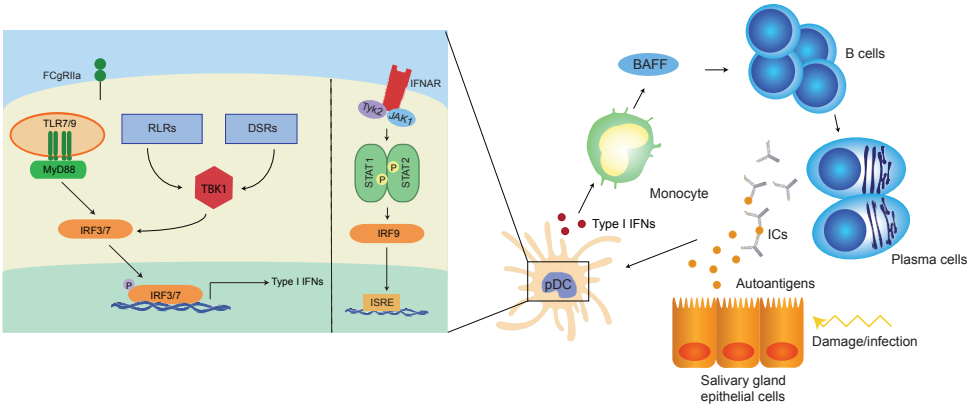

Figure 2. Schematic overview of the pathogenesis of systemic type I interferon (IFN) activation in pSS
An unknown trigger results in damage to salivary gland epithelial cells and leads to accumulation of apoptotic debris including RNA and DNA. Free or complexed self-RNA or self-DNA induces triggering of the Toll-like receptors (TLR), RIG-I like receptors (RLR) or DNA sensing receptors (DSR) in plasmacytoid dendritic cells (pDCs). Upon triggering of these receptors, MyD88 or TBK1 are activated, followed by translocation of IRF3/7 from the cytosol to the nucleus resulting in production of type I IFNs. Binding of type I IFN to the receptor for type I IFN (IFNAR), results in phosphorylation of STATs and complex formation with IRF9. This complex translocated into the nucleus and interferon response elements (ISRE) are subsequently activated, followed by expression of interferon-induced genes (ISGs). Among the ISGs produced is B-cell activating factor (BAFF), which leads to expansion of B cells and differentiation into autoantibody producing plasma cells. The produced autoantibodies bind to autoantigens forming more immune complexes (ICs) which can again trigger TLR7/9 on pDCs.

producing cells, pDCs, selectively express TLR7 and TLR9 and thus rapidly respond with type I IFN production in response to nucleic acids [31]. Differential mRNA expression of TLR7 and TLR9 in various cell subsets has been reported in pSS patients compared to controls, with increased expression of TLR7 in pDCs and monocytes from IFN positive patients [42], and in naïve B cells [51]. Decreased expression of TLR9 has been reported in pDCs from IFN negative patients and in monocytes from both IFN negative and IFN positive patients compared to controls [42]. However, the expression of TLR9 seems to be increased in minor salivary glands from pSS patients compared to sicca controls [52].

In addition to upregulated RNA and DNA sensors, patients with pSS also appear to have abundant ligands to activate these signaling pathways. It has been suggested that the sustained activation of the type I IFN system relates to the endogenous stimulation of TLRs by immune complexes (ICs) formed by autoantibodies and nucleic acids. Sera from pSS patients in combination with apoptotic or necrotic cells induces IFNα production in PBMC from healthy donors [53]. The capacity of pSS sera to induce IFNα production is likely dependent on ICs formed by autoantibodies targeting RNA-binding proteins such as the Ro/SSA and La/SSB autoantigens. The IFNα production is abrogated by RNAse, but not DNAse treatment, indicating the importance of RNA for endogenous stimulation of IFNα production. Further experiments demonstrated that the IFNα production was inhibited by blocking FcγRIIa, a receptor expressed on pDCs that preferentially binds ICs [53]. Thus, a mechanism for endogenous type I IFN production in which nucleic acids gain access to endosomal TLRs through FcγRIIa-mediated endocytosis of ICs leading to activation of pDCs and production of type I IFN was proposed. Further evidence for a role of IFN in the pathogenesis of pSS comes from genetic studies where polymorphisms in both IFN inducing and response pathways are associated with increased risk of pSS [54-60].

Aberrant type I IFN activation drives autoantibody production, in part by direct activation of autoreactive B cells [61]. IFNα can induce expression of the autoantigen Ro52 [62], and the cytotoxic effect of type I IFNs on some cells contributes to the accumulation of cellular debris. Moreover, pDCs have the ability to induce plasma cell differentiation and immunoglobulin production through type I IFN and IL-6 [63]. Additionally, type I IFNs induce the expression of B-cell activating factor (BAFF) via IRF1 and IRF2 [64]. Serum levels of BAFF correlate with higher levels of anti-Ro/SSA autoantibodies [65, 66], and a clear correlation between presence of a type I IFN score and higher serum levels of BAFF and BAFF mRNA expression in monocytes of pSS patients has been reported [26].

Recently, increasing attention has been drawn to the importance of the CD40-CD40L (CD154) axis in pSS. This co-stimulatory pathway is central in the interaction between B cells and T cells, leading to B cell activation with immunoglobulin class-switching and cytokine production. Several antagonistic drugs targeting this pathway have been

developed [67]. Interestingly, type I IFNs have been shown to be important co-factors for CD40L-mediated cytokine production of immature monocyte-derived dendritic cells [68]. Additionally, activated platelets expressing CD40L were shown to augment IFNα secretion from pDCs stimulated with ICs through a CD40L-CD40 interaction, a mechanism of possible importance in pSS [69].

Negative regulation of IFN production and signaling occurs at several levels e.g. by inhibition of pattern recognition receptors (e.g. TLRs) and the IFNAR as well as their downstream effector molecules such as transcription factors (e.g. IRFs) resulting in repressed transcription of ISGs. Important systems for regulating IFN responses include post-transcriptional modifications such as phosphorylation/dephosphorylation and ubiquitination, epigenetic modifications (histone modifications) and the more recently recognized regulation by non-coding RNAs such as microRNAs and lncRNAs [70]. It has become apparent that many of the negative regulators of IFN production and signaling are in fact ISGs themselves, but aberrances in the negative regulation is less studied in pSS. One such pathway relates to the E3 ubiquitin ligase TRIM21, Ro52, a major autoantigen in pSS [17, 71, 72]. TRIM21 is induced by IFN and mainly acts by ubiquitination of several IRFs downstream of TLRs, including IRF3, IRF5 and IRF7 [73, 74] and thus acts as a negative feedback loop for IFN signaling. Further underlining the role of TRIM21 in regulation of inflammatory responses is the observation that TRIM21 deficient mice develop an autoimmune-like condition with uncontrolled inflammation, kidney disease, hyper-gammaglobinaemia, and anti-nuclear autoantibodies in response to minor tissue injury caused by metallic ear-tagging [74]. Notably, anti-Ro52 autoantibodies from patient with pSS targeting the RING domain of TRIM21 were shown to inhibit its E3 ligase activity by sterically blocking interaction with E2 ubiquitin-conjugating enzyme [75]. However, the relevance of this finding *in vivo* remains uncertain as it has not been convincingly described how autoantibodies may reach their intracellular antigens.

Altogether, a feed-forward loop is sustained in patients with pSS in that type I IFNs induce B cell activation and production of autoantibodies, which in turn will lead to the formation of ICs that promote escalation of type I IFN production. Further adding to the vicious cycle is the increased apoptosis induced by IFN and increased expression of the autoantigen Ro52.

INNATE IMMUNE CELLS ARE TYPE I INTERFERON PRODUCERS AND IMPORTANT RESPONDERS

Several innate immune cell subsets are believed to contribute to type I IFN production and the pathogenic process in pSS. pDCs are a rare blood cell population (0.2-0.8% of peripheral blood cells), but the most potent producers of type I IFN [76]. Upon triggering of pDCs by any of the three different pathways/routes described above, type I IFN expression is induced. The increased presence of type I IFN producing pDCs in pSS salivary glands underlines their role in the feed-forward pathogenic IFN loop [38]. Classical DCs upregulate MHC class I and II as well as costimulatory molecules upon type I IFN stimulation. These mature DCs are excellent professional antigen presenting cells that can induce differentiation of naïve CD4+ T cells [77]. Activated DCs also produce BAFF and thereby stimulate humoral responses [78]. Interestingly, the peripheral blood CD14-CD16+ monocyte subset of DC precursors is increased in pSS compared to controls [37]. Furthermore, immature DCs are decreased in the blood of pSS patients, while mature DCs accumulate in the salivary glands [79, 80] in which the glandular epithelium has been shown to secrete autoantigens (Ro/SSA, La/SSB and Sm) in exosomes [81]. The accumulated DCs may present these autoantigens to autoantigen-specific T cells and thus perpetuate immune cell infiltration in the salivary gland.

Also monocytes are important responders and amplifiers of the pathogenic type I IFN expression. This is likely due to the high expression of IFNAR on their surface and the production of cytokines such as BAFF that influence proliferation, differentiation and survival of autoreactive B cells [43, 82, 83]. In line with this role of monocytes in pSS is the observation of a positive correlation between elevated serum levels of BAFF with higher levels of autoantibodies and several other disease parameters [84, 85]. The importance of BAFF production in the pathogenesis is supported by the development of pSS-like disease in BAFF transgenic mice [86].

In the salivary gland tissue of pSS patients, the presence of macrophages correlates with high infiltration of inflammatory cells and with development of lymphoma [80, 87-89]. Specifically, the presence of IL-18 producing macrophages correlated with higher focus scores, gland swelling and decreased C4 serum levels [80], and a contribution to the development of lymphoma by these macrophages was therefore proposed.

Type I IFN enhances NK cell maturation, and several studies in pSS have addressed the role of this cytotoxic subset of lymphocytes in pSS [90]. Reduction of $CD56^{bright}$NK cell numbers in association with reduced NK cell activity and expression of activating receptors has been detected in peripheral blood of patients with pSS [91, 92], and enrichment of NKp44+ NK cells was demonstrated in the salivary glands [93]. However,

the literature on NK cell numbers in pSS shows conflicting data as another study reported low numbers of infiltrating NK cells [88]. Genetic links to NK cells have also been reported in that polymorphisms of NKp30, a NK specific activating receptor which regulates cross-talk between NK cells and DCs, associated with pSS and patients were shown to have higher levels of NKp30 and increased IFN-γ production compared to controls [93].

RELATIONSHIP BETWEEN INTERFERON ACTIVITY AND CLINICAL FEATURES OF SJÖGREN'S SYNDROME

Systemic upregulation of type I IFN in pSS is associated with the presence of anti-Ro/SSA and anti-La/SSB autoantibodies, higher serum IgG, lower complement C3 levels and lower lymphocyte and neutrophil counts [47, 48]. Patients with upregulation of type I in combination with type II IFN were also shown to have lower Schirmer's test scores [48]. Additionally, several studies show increased EULAR Sjögren's syndrome disease activity index (ESSDAI) scores in patients who are type I IFN positive [47, 94, 95]. Local upregulation of type I IFN in the salivary glands of pSS patients was also associated with the presence of anti-Ro/SSA autoantibodies and higher IgG levels. Furthermore, local upregulation of type I IFN was associated with higher focus scores and ocular surface staining score, and lower unstimulated whole salivary flow rate and Schirmer's test score [96]. Determination of the type I IFN signature can therefore be of additional value for monitoring of the disease.

Lymphoma development occurs in a small percent of the pSS patient [18-21]. A link between type I IFN and lymphomas has been suggested as type I IFN induces BAFF in monocytes, which in turn stimulates B cell proliferation. A high IFNγ, low IFNα mRNA ratio in salivary gland tissue in pSS patients has also been associated with lymphomagenesis [97], while IFN type I or type II scores overall could not discriminate between non-lymphoma and lymphoma patients.

Inflammation is often associated with fatigue [98]. Pro-inflammatory cytokines, like type I IFNs, are important players in the inflammatory response and are therefore thought to play a role in the development of fatigue. Animal studies have indicated that pro-inflammatory cytokines induce a set of physiological and behavioural changes in mice, interpreted as a strategy to fight infections, and denoted "sickness behaviour" [99]. Fatigue is one of the components of "sickness behaviour". Therefore fatigue in humans might be considered part of a biologically triggered coping strategy. Interestingly, patients receiving IFNα treatment for viral hepatitis or melanoma often develop severe

fatigue, suggesting a link between IFNs and fatigue [100-103]. In addition, patients with chronic fatigue syndrome, a condition in which patients suffer from persistent debilitating fatigue, often have elevated levels of IFNα. However, we and others have shown that there is no link between upregulation of type I IFN and fatigue in pSS [21, 48, 104-106].

INTERFERON TARGETING THERAPEUTICS

Multiple targets to interfere within IFN-related pathways have been identified. A schematic overview of IFN targeting therapeutics is shown in **figure 3**. A treatment affecting this pathway for which there is longstanding clinical experience is hydroxychloroquine (HCQ), which among other things inhibits TLR7/9 activation. HCQ was shown to directly bind nucleic acids, thereby blocking their TLR7/9-binding epitopes [107]. HCQ is commonly used to treat arthralgia, arthritis, fatigue and cutaneous manifestations in pSS [108]. Although it is successfully used in the treatment of SLE [109], its efficacy in pSS remains questionable. In pSS, HCQ treatment leads to a decrease of hypergammaglobulinemia and reduced erythrocyte sedimentation rate in several studies, but with little or no effect on improvement of dryness, pain or fatigue [110-116].

To specifically target type I IFN activation, the first strategies focused on blocking IFNα using monoclonal antibodies (mAb) (sifalimumab and rontalizumab) or using a therapeutic vaccine that elicits anti-IFN antibodies (IFNα-kinoid). Treatment with rontalizumab led to improvement in SLE patients with low type I IFN scores, but not in patients with high IFN scores [117]. Sifalimumab showed moderately positive results in SLE, however, treatment did not completely abolish the type I IFN signature [118, 119]. IFNα-kinoid induced polyclonal anti-IFNα activity and led to a decrease of IFN scores in SLE patients [120]. However, none of these therapies block other type I IFNs like β, ω, κ or ε, which could explain the lack of response in some patients. Anifrolumab, a mAb targeting the IFNAR blocks the activity of all type I IFN subtypes. This mAb has demonstrated more encouraging results in SLE and was particularly effective in patients with high IFN signature scores [121], but has not yet been tested in pSS.

As mentioned above, increased levels of BAFF are observed in pSS, likely enhancing pathogenic B cell activation. Belimumab is a mAb targeting BAFF, which has been shown to improve ESSDAI and ESSPRI scores in pSS, although there was no effect on fatigue, pain, salivary flow, Schirmer's test or focus scores [122, 123]. Interestingly, particularly patients with upregulation of type I IFN and subsequently higher BAFF levels were good responders to belimumab [124]. This observation again indicates the usefulness of the

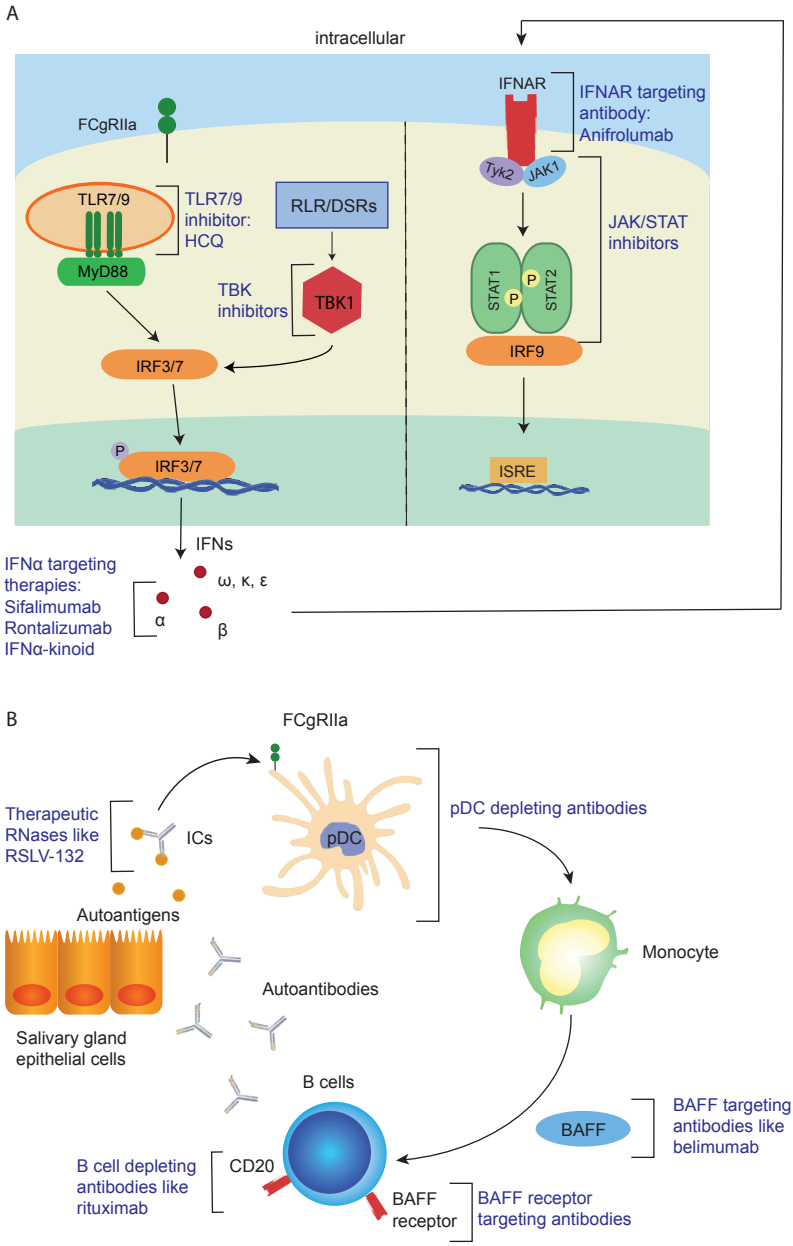

Figure 3. Schematic overview of therapeutics targeting the type I interferon (IFN) pathway
Therapeutics in different stages of development target intracellular molecules including toll-like receptors (TLRs), tank-binding kinase 1 (TBK1), Janus kinase/signal transducers, and activators of transcription (JAK/STAT). Furthermore, therapies have been developed targeting IFNα directly or its receptor (IFNAR), or are targeted against plasmacytoid dendritic cells (pDCs), B-cell activating factor (BAFF) or its receptor, and B cells. Also, therapeutic RNases targeting immune complexes (ICs) are under development.

IFN signature to identify the patients who are most likely to benefit from a particular treatment. Besides belimumab, atacicept and briobacept are two other BAFF-blockers with potential to be used in pSS. Atacicept is a non-selective BAFF blocker mimicking the transmembrane activator and calcium modulator cyclophilin ligand interactor (TACI) receptor, thus binding and inactivating both BAFF and proliferation-inducing ligand (APRIL). In SLE, atacicept reduced immunoglobulin levels and mature B cell numbers. Although some patients showed improvement of SELENA-SLEDAI scores, the results were insufficient to draw conclusions on the efficacy [125]. Briobacept is also a BAFF inhibitor, which showed a decrease in anti-dsDNA antibodies and improvement of glomerular changes in a lupus mouse model [126]. So far, no data from clinical trials using briobacept have been published.

Depletion of B cells using rituximab, an antibody against CD20, has shown variable results in pSS. Several open-label studies demonstrated that the treatment was well tolerated and induced rapid but transient B cell depletion in the blood and salivary glands in correlation with improvement of ESSDAI scores [127-129]. Two small randomized controlled trials showed improvement of fatigue scores (VAS and MFI), unstimulated salivary flow and several laboratory parameters like B cell numbers and rheumatoid factor levels [130, 131]. More recently however, a large randomized controlled trial failed to demonstrate improvement of unstimulated salivary flow and Schirmer's test [132]. VAS fatigue levels, dryness and disease activity scores significantly improved early in the trial but not after 24 weeks of treatment. The latest and largest clinical trial with rituximab in pSS did not reveal improvement of VAS fatigue, ESSDAI or ESSPRI scores [133]. Only unstimulated salivary flow improved after 36 and 48 weeks of treatment. A relevant observation is that upon rituximab treatment the B cell depletion was associated with increased BAFF levels, indicating a feedback mechanism [134]. Several case reports have shown that sequential treatment of rituximab and belimumab was beneficial in specific cases of SLE and pSS [135-137]. Larger studies are needed to draw any firm conclusion on the effects of sequential rituximab and belimumab therapy.

Most of the current treatments target molecules or cells upregulated in response to IFNs. A different strategy could be to prevent the actual production type I IFN. Prevention of the triggering of pDC, as the main type I IFN producers, would be the most obvious approach. RNases (like RSLV-132), that degrade RNA-containing immune complexes, or treatment with endoglycosidase, an IgG glycan-hydrolyzing bacterial enzyme from Streptococcus pyogenes are interesting novel developments [138, 139]. Other therapeutic approaches focus on targeting molecules in the IFN pathway like TLRs, MyD88, IRAKs, and PI3K [25, 140, 141]. Furthermore, inhibition of the JAK/STAT pathway, though not specific for IFN inhibition, has also been shown to reduce induction of ISGs. There are several inhibitors available to block the JAK/STAT signaling pathway

including baricitinib, tofacitinib, ruxolitinib, filgotinib and several others. All these small molecule kinase inhibitors are in different phases of clinical trials for a variety of autoimmune diseases, including monogenic interferonopathies [142, 143]. Another therapeutic option is to target the pDCs directly. BDCA2 and CD123, both present on the surface of pDCs, have been used as targets for depletion of pDCs. In vitro studies using blood from SLE patients showed that both pDC depleting mAbs reduced type I IFN production after immune complex or TLR stimulation [144, 145].

A monoclonal antibody targeting CD40 and disrupting the costimulatory interaction mediated by CD40-CD40L interaction has shown promising results in a phase II trial presented at the ACR 2017 [146]. Although a full publication is not yet available, the investigators reported decreased serum levels of CXCL13, improvement in ESSDAI as well as ESSPRI and physician's and patient's global assessments.

Many of the new therapeutic options are small molecules. There are some advantages of small molecules over biologicals, the first one being that small molecules can often be administered orally, whereas biologicals require injection or infusions [147]. Small molecules are often less specific than biologicals, however, this could also be an advantage as some off-target effects might be beneficial. Protein-like biologicals usually have longer half-life than small molecules. Such biologicals require less frequent administration, which could be an advantage. However, in case fast elimination is required, a shorter half-life might be more beneficial. Additionally, small molecules are often less complex and less expensive to manufacture and could therefore be far less expensive than biologicals, although currently similarly priced. Lastly, small molecules often have longer shelf-lives and do not require refrigeration for storage, which could be an advantage in more isolated areas.

SCOPE OF THIS THESIS

Type I IFNs are elevated in a subgroup of patients with systemic autoimmune diseases. However, there are still questions remaining about the mechanisms leading to the production of these IFNs. In this thesis we dive further into the immunopathogenesis of IFNs in pSS and other systemic autoimmune diseases. Additionally, we study the link between IFNs and fatigue and explore other biological pathways underlying this symptom, which is frequently present in patients with systemic autoimmune diseases.

In **chapter 2** we describe the presence of multiple IFN related modular signatures, thereby identifying a subgroup of pSS patients with besides type I IFN activation, also additional IFN type II activation. Patients with IFN type I plus IFN type II activation showed higher IgG levels and erythrocyte sedimentation rate and a reduced level

of lymphocytes compared to patients with only IFN type I activation or without IFN activation. Furthermore, pSS patients with IFN type I plus IFN type II activation showed increased eye dryness reflected in Schirmer's test score compared to patients without IFN activation. We found no difference in fatigue in patients with or without IFN activation.

In **chapter 3** and **4** we focus on the immunopathogenesis of IFNs in systemic autoimmune diseases. In *chapter 3* we assess the presence of IFN type I score in a cohort of cSLE patients and show elevated expression levels of TLR7, RLRs and DSRs in the IFN type I positive subset. Both RLRs and DSRs signal via TBK1 to induce IFN type I gene expression. Blocking of this signaling pathway with a TBK1/IKKε inhibitor resulted in reduced IFN stimulated gene expression. This indicates a role for cytosolic nucleic acid binding receptors in the production of type I IFN in systemic autoimmunity. In *chapter 4* we extend this study by showing the upregulation of TBK1 and its downstream signaling molecules IRF3 and IRF7 in IFNpos systemic autoimmunity. We stimulated PBMCs with a TLR7 ligand to mimic the IFN activation observed in IFNpos systemic autoimmunity. Here, addition of a TBK1/IKKε inhibitor reduced IFN gene expression and production back to baseline levels. Also in cells of patients with systemic autoimmunity the addition of a TBK1/IKKε inhibitor significantly reduced IFN related gene expression, indicating TBK1 as a potential treatment target in patients with IFNpos autoimmunity.

A large number of pSS patients use HCQ for the treatment of pSS, however data regarding the efficacy of HCQ is limited. In **chapter 5** we describe the effect of HCQ, a TLR7/9 inhibitor and the most frequently prescribed drug for pSS, on IFN related gene expression. In this study we studied samples the previously published JOQUER trial. We showed that treatment for 24 weeks with HCQ significantly downregulated type I IFN scores, RLR and DSR expression. However, after subgrouping of patients positive or negative for IFN activation at baseline no differences in HCQ response were observed.

Chapter 6 discusses the latest literature on the role of IFNs in the pathogenesis of pSS and the difficulties studying IFN-induced gene expression. Furthermore, we discuss the possible role of the IFN signature in the clinic and describe treatment options targeting IFNs.

In **chapter 7** we focus on fatigue, which is a frequent extraglandular manifestation in pSS. Although there appears to be a relation between inflammation, pro-inflammatory cytokines and fatigue, there are no indications that IFN positive pSS patients are more fatigued than IFN negative patients. We described this in *chapter 2* and this observation was confirmed by others. In order to study the biological pathways underlying fatigue in pSS we used a novel proteomics technique. In *chapter 7* we describe the search for biomarkers for fatigue in pSS using SOMAscan technology. This resulted in a proteomic signature for fatigue.

Finally, in **chapter 8** we summarize and discuss the findings of this thesis, focusing on detection methods for IFNs, immunopathogenesis of IFNs in systemic autoimmunity and fatigue research in pSS. Additionally, we discuss some aspects for future research.

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Chapter 2

Systemic interferon type I and type II signatures in primary Sjögren's syndrome reveal differences in biological disease activity

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ABSTRACT

Objective Assess the relationships between systemic interferon type I (IFN-I) and II (IFN-II) activity and disease manifestations in primary Sjögren's syndrome (pSS).

Methods RT-PCR of multiple IFN-induced genes followed by principal component analysis of whole blood RNA of 50 pSS patients was used to identify indicator genes of systemic IFN-I and IFN-II activities. Systemic IFN activities were analyzed in two independent European cohorts (n=86 and 55, respectively) and their relationships with clinical features analysed.

Results Three groups could be stratified according to systemic IFN activity: IFN inactive (19-47%), IFN-I (53-81%) and IFN-I+II (35-55%). No patient has isolated IFN-II activation. IgG levels were highest in patients with IFN-I+II, followed by IFN-I and IFN inactive patients. The prevalence of anti-SSA and anti-SSB was higher among those with IFN activation. There was no difference in total-EULAR SS Disease Activity Index (ESSDAI) or ClinESSDAI between the 3 subject groups. For individual ESSDAI domains, only the biological domain scores differed between the 3 groups (higher among the IFN active groups). For patient reported outcomes, there were no differences in EULAR Sjögren's syndrome patient reported index (ESSPRI), fatigue or dryness between groups, but pain scores were lower in the IFN active groups. Systemic IFN-I but not IFN-I+II activity appeared to be relatively stable over time.

Conclusions Systemic IFN activation is associated with higher activity only in the ESSDAI biological domain but not in other domains or the total score. Our data raise the possibility that the ESSDAI biological domain score may be a more sensitive endpoint for trials targeting either IFN pathways.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is characterized by lymphocytic infiltrations in salivary and lachrymal glands. This is accompanied by sicca symptoms and frequently also extraglandular manifestations [1-3]. Treatment is mainly symptomatic and biologicals so far have shown limited efficacy.

Interferons (IFNs) play a pivotal role in the pathogenesis of pSS. The presence of IFN-induced gene expression in the salivary glands, peripheral blood mononuclear cells (PBMCs), isolated monocytes and B cells of pSS patients has been demonstrated [4-8]. This so-called 'IFN type I signature' was associated with higher disease activity and higher levels of autoantibodies [9]. Recent findings also show activation of interferon type II (IFNγ)-induced gene expression in salivary glands of pSS patients [10, 11]. To our knowledge detailed analysis of modular IFN type I and II activation patterns in a large cohort pSS patients has not been performed.

Type I and type II IFN bind to different receptors, but induce partially overlapping gene expression patterns. Therefore it is difficult to determine which types of IFNs triggers the IFN-induced gene expression pattern observed in pSS. However, understanding the relative contribution of IFN type I and type II may deepen our knowledge in pSS pathogenesis and promote a stratified approach to therapeutic development.

Systemic type I IFN activation has been extensively characterized in systemic lupus erythematosus (SLE). In clinical trials blocking of IFN type I had limited efficacy, possibly due to unopposed type II IFN activation [12-14]. In SLE, Chiche *et al.* have reported three strongly upregulated IFN-annotated modules (M1.2, M3.4 and M5.12) from peripheral blood transcriptomic data. Each of these modules has a distinct activation threshold [15]. The M1.2 transcriptional module was induced by IFNα, while both M1.2 and M3.4 transcripts were upregulated by IFNβ. M5.12 was poorly induced by IFNα and IFNβ alone. Transcripts belonging to M3.4 and M5.12 were only fully induced by a combination of type I and type II IFNs and displayed a more dynamic pattern when studied over time in SLE. Interestingly, M5.12 was mainly upregulated in SLE patients with high disease activity and correlated with renal flares. These data indicate that detailed modular analysis for pSS can contribute to the discovery of better biomarkers and development of stratified therapeutic intervention.

Fatigue is a major complaint in pSS patients [16-20] and is associated with a poor quality of life [21]. Patients receiving IFNα treatment for viral hepatitis can develop severe fatigue [22] and in rare cases also develop pSS-like symptoms [23-25]. Here we investigate a possible correlation between IFN activation and fatigue.

In this study, we performed a detailed analysis, using the IFN annotated modules described for SLE, in two large clinically well-characterized pSS cohorts - the United

Kingdom Primary Sjögren's Syndrome Registry (UKPSSR) and the Rotterdam (The Netherlands) cohort. Furthermore, we assessed the relationships between these IFN modules and fatigue as well as other clinical features.

METHODS

Patient recruitment

PSS patients and healthy controls (HC) from the UK cohort were from the UKPSSR collected in 30 centres [26]. PSS patients and HC from the Rotterdam cohort were recruited at the Erasmus Medical Centre, Rotterdam, the Netherlands. All pSS patients fulfil the 2002 American-European Consensus Group classification criteria [27]. Disease activity was assessed using EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) and Clinical ESSDAI (ClinESSDAI) [28, 29]. HC did not suffer from autoimmune disease or use corticosteroid. Characteristics of patients are summarized in supplementary table S1. Written informed consents were obtained from all participants in the study, in compliance with the Declaration of Helsinki. Medical Ethical Review Committee of the Erasmus MC and North West Research Ethics Committee approved this study.

Blood collection, measurement of laboratory parameters and real-time PCR

Blood was collected in clotting tubes for serum preparation and in PAXgene RNA tubes for whole blood RNA analysis. RNA isolation, cDNA preparation and real-time PCR were performed according to manufacturer's protocol. See supplementary methods for extended protocols.

Calculation of IFN score for each module

To identify correlated groups of genes and reduce data complexity, the expression of IFN-inducible genes (from M1.2, M3.4 and M5.12) were added to a principle component analysis. Kaiser-Meyer-Olkin measure of sampling adequacy were respectively 0.882; 0.907; 0.888 for M1.2, M3.4 and M5.12. In order to assess the amount of variance explained by each factor, eigenvalues extracted.

The IFN score for each module was defined by the relative expression of 5 indicator genes. For M1.2 these genes were IFI44, IFI44L, IFIT1, IFIT3, MXA; for M3.4 ZBP1, EIFAK2, IFIH1, PARP9, GBP4; and for M5.12 PSMB9, NCOA7, TAP1, ISG20 and SP140. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN scores per subject represent the sum of these standardized scores, calculated as previously

described [33, 34]. Patients were divided in groups being positive or negative for M1.2, M3.4 or M5.12, using a threshold of $mean_{HC} + 2 \times SD_{HC}$.

Assessment of fatigue and depressive symptoms

In the UK cohort, fatigue was assessed using the profile of fatigue and discomfort-Sicca symptoms inventory (PROFAD-SSI), visual analogue scale (VAS) for fatigue and the EULAR Sjögren's syndrome patient reported index (ESSPRI) [28, 35, 36]. In the Rotterdam cohort fatigue was assessed using the Dutch version of the multidimensional fatigue inventory (MFI) [37]. Depressive symptoms were assessed using the hospital anxiety and depression scale (HADS) for the UK cohort and the Dutch-validated Center for Epidemiologic Studies Depression (CES-D) for the Rotterdam cohort [38, 39].

Statistics

Independent T-test was used to compare means and the Mann-Whitney U test was used to compare medians. Categorical data were compared using Fisher's exact test and correlations were assessed using Spearman's rho (r_s). Multiple group comparisons were analyzed using One-Way ANOVA or Kruskal-Wallis. For extended statistics see supplementary methods.

RESULTS

Presence of IFN annotated modules in whole blood of pSS patients

To select 5 indicator genes for each of the previously described IFN annotated modules (M1.2, M3.4, M5.12) [15], 11-16 genes were selected using micro-array data of differentially expressed genes in monocytes of pSS patients (supplementary table S2, unpublished results) [4]. Expression levels of these genes were assessed in 50 pSS patients and 38 HCs (Rotterdam cohort) using RT-PCR and added into a principle component analysis to identify correlated groups of genes in order to reduce data complexity.

Five indicator genes for each module were selected and subsequently determined in a cohort of 86 pSS patients (UK cohort), followed by a replication cohort of 55 pSS patients (Rotterdam cohort). A flow chart summarizing this selection procedure is shown in supplementary figure S1. All IFN annotated modular scores were expressed significantly higher in pSS patients than in HC (supplementary figure S2). Furthermore, the three IFN modules strongly correlated with each other as depicted for the UK and Rotterdam cohorts combined ($P<0.001$) (figure 1A).

To study the modular expression over time, the modular IFN scores of 15 pSS patients of the Rotterdam cohort were determined at two different time points. The average period between two time points was 1.8 ± 0.8 years. There were no significant differences in M1.2 and M3.4 score between the two time points. In the M5.12 module there was a significant difference in score between the two time points (figure 1B).

Of the M1.2 positive patients, 90-96% were also positive for M3.4 and 66-67% were also positive for M5.12 when both cohorts were combined. Only three patients were positive for M3.4, while negative for M1.2 and M5.12. There were no patients positive for M5.12 and negative for M1.2.

In the UK cohort; 81, 78, 55, 53 and 19 percent of the patients were positive for M1.2, M3.4, M5.12, all the modules or none of the modules respectively (figure 1C). In the

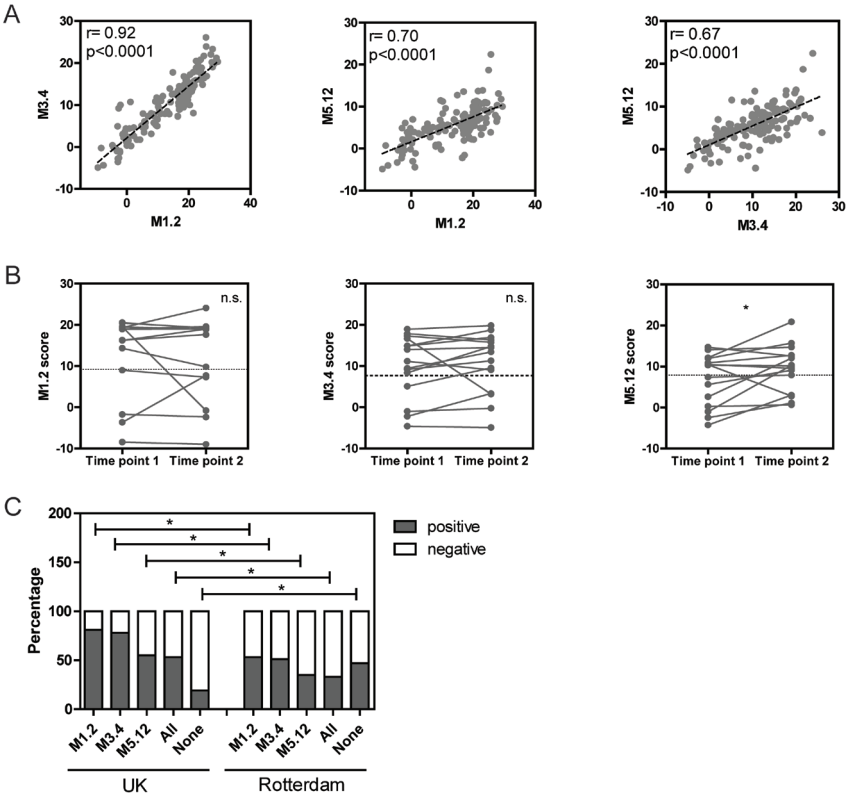

Figure 1. Presence of IFN annotated modules in pSS patients from UK and Rotterdam cohort
A, Correlation between modular scores of the UK and Rotterdam cohorts combined (n=141) **B,** Modular scores over time in pSS patients (UK and Rotterdam cohorts combined) (n=15) **C,** Comparison positivity for modules M1.2, M3.4, M5.12, all modules or none of the modules between the UK cohort and the Rotterdam cohort. Dotted lines indicate positivity threshold for each score. Independent T-test was used to compare means of normally distributed samples and Wilcoxon signed-rank test to compare dependent medians. Categorical data were compared using Fisher's exact test. For correlations, Spearman's rho correlation test was used.

Rotterdam cohort this was respectively; 53, 51, 35, 33 and 47. The percentage of patients positive for each module was lower in the Rotterdam cohort. Compared to the UK cohort, patients in the Rotterdam cohort used more hydroxychloroquine (HCQ) (supplementary table S1). However, there were no differences in IFN scores between patients treated or untreated with HCQ in both cohorts (supplementary figure S3A-F).

Systemic upregulation of IFN-inducible genes is associated with higher prevalence of autoantibodies

While M1.2 and M3.4 modular gene expression largely overlapped in pSS patients, there was a subgroup that was additionally positive for M5.12. Therefore pSS patients were subgrouped in patients without (negative for modular IFN activation), IFN type I (positive for M1.2 only) or IFN type I+II (positive for M1.2 + M5.12) inducible gene expression. These three subgroups were subsequently investigated for associations with clinical data and functional tests. Functional tests were only available for the UK cohort. Patients with systemic IFN activation (I or I+II) were more often positive for anti-SSA, anti-SSB and had higher IgG levels compared to patients without systemic IFN activation in both cohorts (UK cohort: table 1, figure 2A,D and E; Rotterdam cohort: supplementary table S3, supplementary figure S4A-E). Furthermore, patients with IFN type I+II-inducible gene expression showed significantly higher IgG and ESR levels and lower lymphocyte counts and hemoglobin levels compared to patients with only IFN type I-inducible gene expression (UK cohort: table 1, figure 2A, B and C). Schirmer's test scores were significantly lower in IFN type I+II positive patients compared to patients without IFN activation (UK cohort: table 1, figure 2F).

Systemic upregulation of IFN-inducible genes is associated with higher biological disease parameters but not clinical ESSDAI

To investigate differences in disease activity between patients without, with IFN type I and with IFN type I+II-inducible gene expression, the ESSDAI and its sub-domains were compared between the different subgroups. The frequency of pSS patients positive for the biological domain was higher in patients with IFN activation compared to patients without IFN activation (UK cohort: table 2; Rotterdam cohort: supplementary table S4). In fact, activity in the biological domain is largely confined to the IFN active groups. The frequency of pSS patients positive for the hematologic domain was higher in patients with IFN type I+II-inducible gene expression compared to patients without IFN-inducible gene expression or with only IFN type I-inducible gene expression in the UK cohort. There were no differences in total-ESSDAI or ClinESSDAI scores between the different subgroups.

Table 1. Comparison laboratory parameters in the UK cohort after stratification on IFN activation

	pSS			
	Neg (n=16)	**IFN I (n=22)**	**IFN I+II (n=47)**	
Laboratory parameters				
Anti-SSA[c]	11/16 (69)	20/22 (91)	44/47 (94)	p=0.026
Anti-SSB[c]	5/15 (33)	15/21 (71)	32/46 (70)	p=0.007
IgG (g/l)[b]	10.9 (9.1-13.4)	14.8 (12.4-17.9)	18.6 (14.1-26.2)	p<0.001
IgA (g/l)[a]	2.3 ± 1.1	2.8 ± 1.0	3.4 ± 1.8	P=0.077
IgM (g/l)[b]	1.1 (0.9-1.7)	1.1 (0.8-1.5)	1.3 (1.0-1.7)	P=0.389
C3 (g/l)[a]	1.3 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	P=0.403
C4 (g/l)[a]	0.3 ± 0.04	0.2 ± 0.06	0.2 ± 0.1	P=0.179
Hb (g/dl)[a]	13.2 ± 1.0	13.4 ± 1.1	12.4 ± 1.1	p=0.001
WCC (*10E9)[b]	6.5 (4.4-8.2)	5.7 (4.2-6.8)	5.7 (4.7-6.8)	P=0.217
Lymphocytes (*10E9)[a]	2.0 ± 0.7	1.8 ± 0.5	1.6 ± 0.5	p=0.007
Neutrophils (*10E9)[b]	3.8 (2.3-5.1)	3.2 (2.3-4.4)	3.1 (2.7-4.3)	P=0.607
Plt (*10E9)[a]	305.8 ± 70.5	276.5 ± 60.6	264. ± 66.1	P=0.087
CRP mg/l[b]	3 (2.0-5.0)	5 (2.9-5.0)	5 (2.6-5.0)	P=0.567
ESR (mm/hr)[b]	14 (5.0-16.0)	19 (10.5-26.0)	31.5 (15.3-50.0)	p<0.001
Functional tests				
Schirmer's test (mean of both eyes)[b]	9.6 (4.0-22.5)	4.8 (1.0-13.6)	3.5 (0.5-8.0)	P=0.028
Unstimulated saliva flow (ml/5 min)[b]	0.7 (0.1-2.9)	0.4 (0.0-1.1)	0.2 (0.0-1.0)	P=0.274

Data are presented as mean ± SD[a], median (IQR)[b] or as number (%)[c] of patients according to data distribution. Means or medians were compared using the One-Way ANOVA or Kruskal-Wallis. Categorical data were compared using Fisher's exact test. pSS, primary Sjögren's syndrome; Neg, IFN negative; IFN I, IFN type I; IFN I+II, IFN type I and II; Ig, immunoglobulin; C, complement; Hb, hemoglobin; Plt, platelets; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

Systemic upregulation of IFNs is not associated with fatigue or depression

To investigate if there was a difference in patient-reported symptoms between patients without, with IFN type I and with IFN type I+II-inducible gene expression, validated questionnaires for fatigue, depression and anxiety were analyzed. Patients without IFN activation and those with IFN type I-inducible gene expression had higher pain scores, compared to patients with IFN type I+II-inducible gene expression (table 3). There were no differences in fatigue, depression or anxiety between the pSS sub-groups.

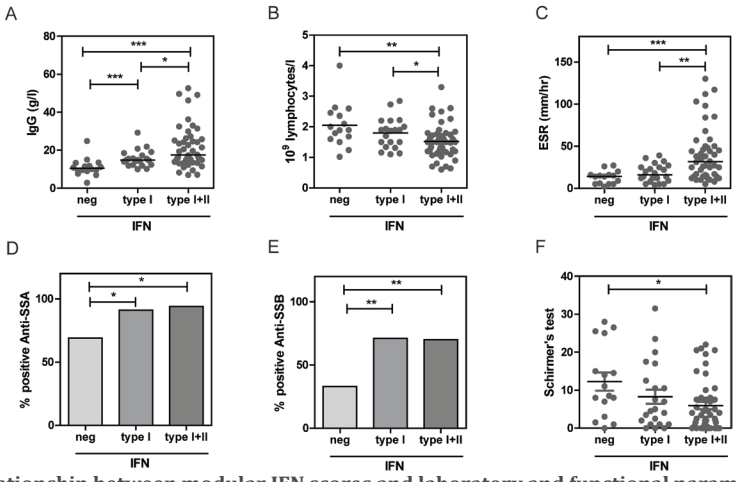

Figure 2. Relationship between modular IFN scores and laboratory and functional parameters
IgG levels **(A)**, lymphocyte counts **(B)** and erythrocyte sedimentation rate **(C)** % positive for anti-SSA **(D)** % positive for anti-SSB **(E)** Schirmer's test **(F)** in IFN neg (n=16), M1.2 positive (IFN type I-inducible) (n=22) or M5.12 positive (IFN type I+II-inducible) (n=47) pSS patients. Kruskal-Wallis (A, C and F), One-way ANOVA (B) and Fisher's exact test (D and E) were used to compare multiple groups. * Represents P value of <0.05, ** represents P value of <0.005, *** represents P value of <0.0005.

DISCUSSION

In this study we show the presence of systemic upregulation of IFN type I and IFN type I+II signatures in two large clinically well-characterized European pSS cohorts, using five indicator genes of the previously described IFN annotated modules. IFN type I (M1.2), induced mainly by IFNα, was the most prevalent in both cohorts. IFN type I+II (M1.2 + M5.12), induced by IFNα, IFNβ and IFNγ was present in approximately 66% of the patients positive for IFN type I. Compared to patients without or with only IFN type I-inducible gene expression, pSS patients with IFN type I+II-inducible gene expression were more often positive for the biological domain of the ESSDAI and had higher levels of IgG, higher ESR and lower lymphocyte counts in the UK cohort. In the Rotterdam cohort IgG levels of patients with IFN type I+II were also higher compared to HCs and there was a trend towards lower lymphocyte counts. There were no differences in patient-reported fatigue or depression between patients with and without systemic IFN activation.

We have previously shown that systemic IFN activation in peripheral blood monocytes in a subset of pSS patients [9]. This type I IFN signature correlated with higher anti-SSA/anti-SSB autoantibody frequencies and hypergammaglobulinemia. Comparison of these genes with the modules we tested in this study, revealed that the IFN type I signature genes we used were all of the M1.2 module and thus type I induced. Indeed all patients positive for M1.2 were previously found to have a positive monocytic IFN signature [9].

Table 2. Comparison of the ESSDAI and its sub-domains in the UK cohort after stratification on IFN activation

	pSS			
	Neg (n=16)	**IFN I (n=22)**	**IFN I+II (n=47)**	
ESSDAI[a]	3 (0.5-5.0)	2.5 (0.0-5.0)	4 (0.0-8.0)	P=0.472
ClinESSDAI[a]	4 (0.5-6.0)	2 (0.0-4.5)	4 (0.0-9.0)	P=0.929
ESSDAI domain[b]				
Constitutional	4/16 (25)	4/22 (18)	10/47 (21)	p=0.879
Lymphadenopathy	0/16 (0)	2/22 (9)	3/47 (6)	p=0.489
Glandular	6/16 (38)	2/22 (9)	8/47 (17)	p=0.167
Articular	7/16 (44)	6/22 (27)	17/47 (36)	p=0.664
Cutaneous	1/16 (6)	0/22 (0)	2/47 (4)	p=0.660
Pulmonary	1/16 (6)	1/22 (5)	7/47 (15)	p=0.574
Renal	0/16 (0)	0/22 (0)	3/47 (6)	p=0.867
Muscular	0/16 (0)	0/22 (0)	0/47 (0)	-.
PNS	0/16 (0)	0/22 (0)	2/47 (4)	p=0.437
CNS	0/16 (0)	0/22 (0)	0/47 (0)	-
Hematological	0/16 (0)	0/22 (0)	7/47 (15)	p=0.046
Biological	0/16 (0)	8/22 (36)	23/47 (49)	p=0.002

Data are presented as median (IQR)[a] or as number (%)[b] of patients according to data distribution. Medians were compared using Kruskal-Wallis. Categorical data were compared using Fisher's exact test. pSS, primary Sjögren's syndrome; Neg, IFN negative; IFN I, IFN type I; IFN I+II, IFN type I and II; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index

Until now no detailed studies on the presence of a systemic IFN type II signature in pSS have been performed. A recent study in pSS has reported the presence of systemic type II IFN-induced gene expression, although using different genes from this study [40]3. Similar percentages of type I and type I+II positive patients were reported. However, 6.8% of the patients were exclusively positive for type II IFNs; in contrast, we did not find patients only positive for M5.12. This difference could be explained by the selection of GBP1 as a gene mainly induced by IFN type II. According to the modular analysis, where our study was based upon, this gene belongs to the M3.4 module and therefore can also be induced by IFNβ.

The distribution of the modular IFN expression we detect in pSS is very similar to that earlier described for SLE. In pSS as well as SLE, M1.2 is the most prevalent module followed by M3.4 and M5.12 [15]. Additionally, similar to SLE, in pSS patients M5.12 was never upregulated without concomitant upregulation of M1.2 and M3.4. In SLE 87% of the patients showed upregulation of at least one of the modules. In our study

Table 3. Comparison of fatigue, depression, symptom profile and disease damage index after stratification on IFN activation

	pSS			
	Neg	**IFN I**	**IFN I+II**	
UK cohort	n=16	n=22	n=47	
SSDDI	7.3 (5.0-7.3)	7.3 (4.0-9.0)	7.0 (3.0-8.7)	p=0.791
Fatigue VAS	85.0 (75.5-93.5)	77.0 (20.5-87.8)	76 (15.0-84.0)	p=0.149
PROFAD-Physical	5.5 (4.5-6.0)	5.0 (2.6-6.0)	4.8 (1.8-5.5)	p=0.122
PROFAD-Mental	4.0 (2.5-5.8)	4.5 (1.0-5.4)	3.5 (0.5-5.0)	p=0.531
HADS anxiety	6.0 (4.5-7.0)	7.5 (5.0-11.0)	10.0 (5.0-12.0)	p=0.192
HADS depression	7.0 (3.0-11.0)	8.0 (2.5-10.0)	5.0 (1.0-10.0)	p=0.322
Total ESSPRI	7.0 (6.2-8.7)	6.7 (4.1-7.6)	5.8 (2.7-7.3)	p=0.047
ESSPRI sub-domains				
Pain	7.0 (5.0-9.0)	6.0 (2.3-8.0)	3.5 (1.0-7.0)	p=0.003
Fatigue	8.0 (7.0-9.0)	7.5 (3.5-9.0)	7.0 (2.0-8.0)	p=0.159
Dryness	7.0 (5.5-8.0)	7.0 (4.0-8.5)	6.0 (3.0-8.0)	p=0.938
Mental fatigue	7.0 (5.0-8.5)	5 (1.0-8.0)	3.0 (1.0-7.0)	p=0.058
Rotterdam cohort				
MFI sub-domains	n=25	n=11	n=19	
General fatigue	15.0 (12.0-17.8)	16.0 (13.0-18.0)	14 (2.75)	p=0.793
Physical fatigue	14.0 (12.0-16.0)	14.0 (10.0-15.0)	13.5 (9.0-20.0)	p=0.305
Mental fatigue	12.0 (8.0-15.0)	11.0 (5.0-12.0)	10.0 (8.0-15.0)	p=0.322
Reduced motivation	11.0 (8.0-14.0)	9.0 (5.0-13.0)	9.0 (5.9-11.0)	p=0.529
Reduced activity	11.0 (7.0-13.0)	11.0 (8.0-16.0)	11.0 (7.0-13.0)	p=0.941
CES-D	17.5 (8.0-23.5)	12.0 (8.0-20.0)	13.5 (10.0-20.0)	p=0.760

Data are presented as median (IQR). Medians were compared using Kruskal-Wallis. SSDDI, Sjögren's Syndrome Disease Damage Index; PROFAD, Profile of Fatigue and Discomfort; HADS, Hospital Anxiety and Depression; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index; CES-D, Center for Epidemiologic Studies Depression; MFI, Multiple Fatigue Inventory

81% in the UK cohort was positive for at least one of the modules and in the Rotterdam cohort 53%. Longitudinal data indicated in both diseases M5.12 as being the module most susceptible to change over time, although our data are based upon a small sample number. A difference between SLE and pSS is that the M3.4 module largely overlaps with the M1.2 in pSS, while in SLE patients this was not observed [15]. The IFN modules correlated with auto-antibodies, anti-dsDNA titers in SLE and anti-SSA/anti-SSB in pSS. M5.12 in SLE correlated with SELENA-SLEDAI scores, flares and the cutaneous domain, and in pSS this module weakly correlated with the pulmonary and renal domain of the

ESSDAI (data not shown) in the UK cohort, but not the total-ESSDAI scores. A reason why we did not detect significant differences in total-ESSDAI or most ESSDAI domain scores in pSS could be because extraglandular manifestations in pSS are less frequent than in SLE. Alternatively, IFN activity may be linked only to some but not all extraglandular manifestations.

Anti-inflammatory drugs can affect IFN signatures [41]. In this study the frequency of patients positive for the modular IFN scores were lower in the Rotterdam cohort compared to the UK cohort. One possible explanation for this could be that patients in the Rotterdam cohort were treated more often with HCQ than patients in the UK cohort. We have shown before that patients treated with HCQ have lower IFN type I scores [41]. In addition, HCQ has been shown to impair IFNα production by pDCs [42]. In this study we also stratified patients based on HCQ use. Although there appeared to be a trend toward lower IFN type I (M1.2) scores among those taking HCQ, there were no significant differences detected in any of the modular scores. However, this is a cross-sectional study with no data on pre-treatment IFN scores. Moreover, because of the contribution of other IFNs in pSS the overall effect of HCQ may not result in a significant difference in IFN score. Consistently, in SLE it was shown that HCQ treatment only lowered expression of MxA, with other interferon inducible genes such as OAS1 and IFI27 being unaffected [43].

Interestingly, there were no significant differences in total-ESSDAI or ClinESSDAI scores between patients without or with type I or type I+II IFN activation, except for the biological domain. This could have significant implication on selection of primary endpoints in clinical trials evaluating novel therapies. For instance, therapies targeting type I or II IFN may improve ESSDAI biological domain score, but have no impact on total-ESSDAI or ClinESSDAI scores. It is also of interest that systemic IFN activity is not associated with disease activity in other ESSDAI organ domains. One possible explanation is that the sample size in this study did not have the power to detect such differences. Another intriguing possibility is that mechanisms other than systemic IFN activation might be responsible for the clinical manifestations in these other organ domains.

Recently, salivary gland analysis of pSS patients revealed a predominant type II activation pattern [11, 40]. Comparing these data with our results on systemic IFN activation we conclude that systemically the IFN type I expression dominated over IFN type II expression. This indicates that local and systemic IFN activation patterns within the same patient may differ. Future study of IFN activation patterns in paired samples from peripheral blood and salivary gland tissue of the same patient would be of interest and might help to define the role for systemic IFN activation as a biomarker for pSS.

Blocking systemic IFNα activation in SLE showed a reduction of SELENA-SLEDAI scores in a small subset of patients [13, 14, 44]. Interestingly, post-hoc analysis revealed

a possible effect in patients with low baseline IFN activity. This might be due to a contribution of IFN type II or IFNβ to the pathogenic process. A recent study targeting the IFNAR in SLE patients with moderate-to-severe disease showed encouraging clinical effectivity in patients with a high IFN signature at baseline, while patients with low IFN signatures did not respond different compared to the placebo group [45]. Our findings here in pSS and earlier finding in SLE show distinct activation patterns (IFNα, IFNβ and/or IFN type II) which all lead to upregulation of IFN-inducible genes. Stratification of patients based on their IFN activation pattern will identify subgroups that are most likely to benefit from a specific targeted treatment. For instance, patients positive for M1.2 and M3.4 could benefit from blocking the receptor for type I interferons (IFNAR), while patient additionally positive for M5.12 blocking the IFN type II pathway (as well as IFNAR blockade) might be necessary.

In contrast to our hypothesis, patients with IFN activation were not more fatigued than those without IFN signatures. This might be caused by the relatively low patient number or the subjective nature of fatigue. However, our data are in line with our previous study showing no correlation between IFN type I score and VAS fatigue score [46]. Additionally, we showed for the UK cohort lower levels of pro-inflammatory cytokines, amongst which IFN type II, in high fatigued pSS patients [30]. These data indicate that fatigue is not directly related to activation of IFN induced gene expression.

This study has several limitations. First is the study of gene expression levels in peripheral blood cells, instead of in a specific cell type. However, we previously investigated the IFN type I signature in monocytes of pSS patients and identified the same set of signature genes as here in whole blood cells. Also all patients positive for the monocytic IFN signature were also positive for M1.2 when whole blood cells were collected simultaneously. Another limitation is that the Rotterdam cohort is collected in an academic reference center and therefore may have a disproportionally higher percentage of atypical pSS patients while the UK cohort is a national biobank with 30 recruitment centers. This may also explain the differences in the prevalence of renal complications between the two cohorts.

Taken together, this study describes the prevalence of systemic (IFN type I and/or type II) activation in pSS. Stratification according to this activation pattern revealed differences in disease features. These data raise the possibility that the biological-ESSDAI rather than total-ESSDAI score may be a more sensitive endpoint for trials targeting either type I or type II IFN pathways.

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SUPPLEMENTARY FILES

Blood collection, preparation

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

Measurement of complement, immunoglobulin levels and auto-antibodies

For the UK cohort, anti-SSA and anti-SSB antibodies were measured in the National Health Service approved pathology laboratory of the recruiting hospital. For the Rotterdam cohort, C3, C4, IgG, IgA, IgM were measured as described previously [9, 30]. Anti-SSA and anti-SSB were determined by EliA (Thermo Scientific), confirmed with ANA profile immunoblot (EuroImmun) and re-confirmed where needed by QUANTA Lite ELISA-kit (INOVA).

Real-time quantitative PCR

Total RNA was isolated from PAXgene tubes and reverse-transcribed to cDNA. For calculation of relative expression, samples were normalized to expression of the household gene Abl [31]. Relative expression values were determined from normalized CT values using 2^-ΔΔCT method (User Bulletin, Applied Biosystems) [32].

Statistics

Normally distributed data were expressed as means with standard deviations and the independent T-test was used to compare means. When data was not normally distributed, values were expressed as medians with interquartile ranges (IQRs) and comparisons were made using the non-parametric Mann-Whitney U test. To compare categorical data, Fisher's exact test was used. Correlations were assessed using Spearman's rho (rs). Multiple group comparisons were analyzed using the One-Way ANOVA test or Kruskal-Wallis. Values of $p<0.05$ were considered statistically significant. Analysis was performed using IBM SPSS 21.0 (SPSS, Chicago, IL, USA). Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA) was used for graphs.

TABLES

Supplementary table S1. Demographics, characteristics and medication use by participants

		pSS	
	HC (n=88)	**UK cohort (n=86)**	**Rotterdam cohort (n=55)**
Demographics			
Female, n (%)	76/88 (86)	86/86 (100)	51/53 (93)
Age, mean (SD), years	52.7 (14.9)	55.7 (11.7)	60.0 (12.6)
Patient characteristics			
Disease duration, mean (SD), years	-	6.14 (5.3)	14.0 (8.5)
Medication status, n (%)			
Hydroxychloroquine	-	32/86 (37)	33/55 (60)
Corticosteroids	-	12/86 (14)	9/55 (16)

pSS: primary Sjögren's syndrome; HC: healthy control.

Supplementary table S2. Determined genes for each of the different modules

M1.2	**M3.4**	**M5.12**
Serping	AIM2	DHRS9
IFI44	CCL8	ECGF1 = TYMP
IFI44L	DDX58	EVT7
IFIT1	DHX58	IFI16
IFIT3	EIF2AK2	ISG20
Ly6e	GBP1	RNF213
MXA	GBP4	NCOA7
XAF1	IDO	NT5C3
ISG15	IFIH1	PSMB9
RSAD2	IFIT2	REC8
CXCL10	IFITM1	SAMD9
	IRF7	SP140
	PARP9	TAP1
	STAT1	
	UBE2L6	
	ZBP1	

Supplementary table S3. Comparison laboratory parameters in the Rotterdam cohort after stratification of pSS patients based on IFN activation

	pSS			
	Neg (n=25)	**IFN I (n=11)**	**IFN I + II (n=19)**	
Anti-SSA, n (%)	13/25 (52)	10/11 (91)	19/19 (100)	P=0.001
Anti-Ro52, n (%)	13/25 (52)	9/11 (82)	16/19 (84)	P=0.046
Anti-Ro60, n (%)	11/25 (44)	10/11 (91)	19/19 (100)	p<0.001
Anti-SSB, n (%)	8/25 (32)	5/11 (45)	16/19 (84)	P=0.003
IgG, g/l, median (IQR)	10.6 (7.2-11.7)	14.0 (9.8-16.6)	17.3 (11.4-22.9)	p<0.001
IgA, g/l, median (IQR)	1.9 (1.7-2.6)	3.0 (2.6-3.6)	3.0 (2.1-3.3)	P=0.045
IgM, g/l, median (IQR)	0.8 (0.5-1.2)	0.9 (0.8-1.4)	0.8 (0.6-1.0)	P=0.768
Vit D, median (IQR)	127.4 (71.0-160.7)	157.2 (105.1-177.7)	106.5 (69.6-111.1)	P=0.224
C3, g/l, mean (SD)	1.23 (0.24)	1.19 (0.33)	1.09 (0.42)	P=0.302
C4, g/l, median (IQR)	0.2 (0.2-0.3)	0.2 (0.1-0.2)	0.2 (0.2-0.3)	P=0.069
Hb, mmol/l, mean (SD)	8.54 (0.61)	8.12 (0.57)	7.9 (0.67)	P=0.231
Thrombocytes, *10E9, median (IQR)	270.0 (212.0-293.0)	259.5 (228.3-295.0)	206.0 (199.0-251.0)	P=0.209
Lymphocytes, *10E9, mean (SD)	1.87 (0.56)	1.75 (0.6)	1.26 (0.65)	P=0.030
Neutrophils, *10E9, median (IQR)	3.9 (2.8-5.6)	2.6 (2.2-5.5)	3.2 (2.8-3.8)	P=0.014
CRP, mg/l, median (IQR)	3.4 (0.9-6.1)	2.3 (0.6-5.5)	1.9 (1.0-7.8)	P=0.569

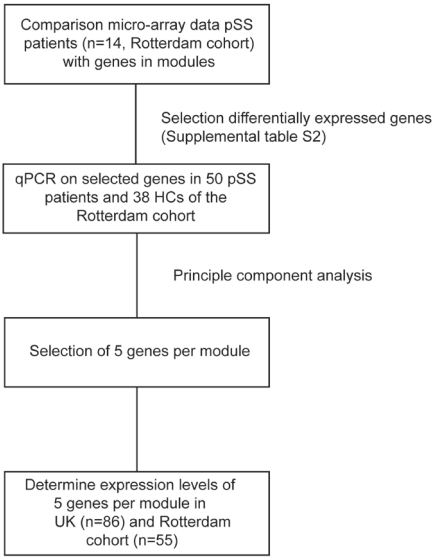
Means or medians were compared using the One-Way ANOVA and Kruskal-Wallis. Categorical data were compared using Fisher's exact test. pSS: primary Sjögren's syndrome; Neg: IFN negative; IFN I: IFN type I; IFN I+II: IFN type I and II; Ig: immunoglobulin; C: complement; Hb: hemoglobin; Vit D: Vitamin D.

Supplementary table S4. Comparison of the ESSDAI and its sub-domains in the Rotterdam cohort after stratification of pSS patients based on IFN activation

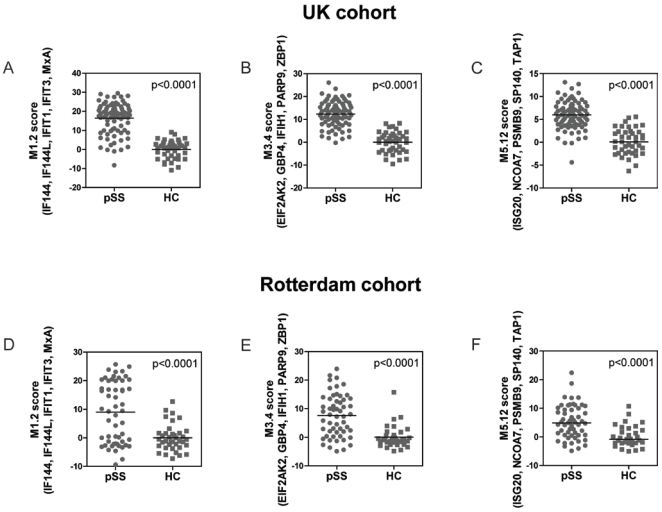
	pSS			
	Neg (n=25)	**IFN I (n=11)**	**IFN I+II (n=19)**	
ESSDAI, median (IQR)	5.0 (3.0-12.0)	6.0 (4.0-12.0)	5.0 (3.0-14.3)	P=0.252
ClinESSDAI, median (IQR)	7.0 (3.0-20.0)	6.0 (0.0-13.0)	7.0 (3.0-27.0)	P=0.574
ESSDAI domain, n (%)				
Constitutional	10/25 (40)	1/11 (9)	7/19 (37)	
Lymphadenopathy	2/25 (8)	0/11 (0)	1/19 (5)	P=0.050
Glandular	2/25 (8)	2/11 (18)	1/19 (5)	P=0.773
Articular	17/25 (68)	3/11 (27)	8/19 (42)	P=0.718
Cutaneous	4/25 (16)	3/11 (27)	9/19 (47)	P=0.119
Pulmonary	7/25 (28)	3/11 (27)	6/19 (32)	P=0.075
Renal	2/25 (8)	0/11 (0)	3/19 (16)	P=0.913
Muscular	1/25 (4)	1/11 (9)	0/19 (0)	P=0.338
PNS	2/25 (8)	1/11 (9)	2/19 (11)	P=0.262
CNS	0/25 (0)	0/11 (0)	2/19 (11)	P=0.959
Hematological	1/25 (4)	1/11 (9)	3/19 (16)	P=0.140
Biological	3/25 (12)	3/11 (27)	12/19 (63)	P=0.403

Medians were compared using Kruskal-Wallis. Categorical data were compared using Fisher's exact test. pSS: primary Sjögren's syndrome; Neg: IFN negative; IFN I: IFN type I; IFN I+II: IFN type I and II; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index.

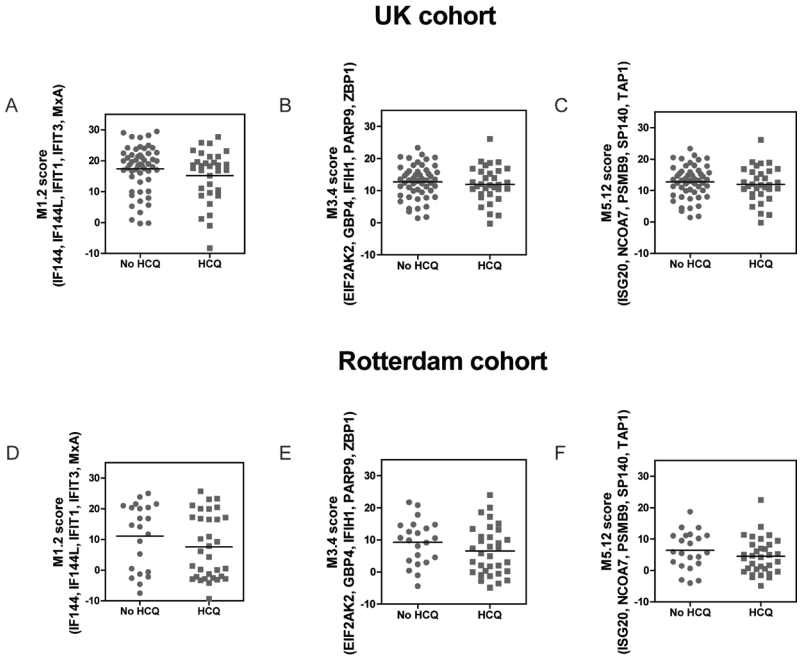
FIGURES



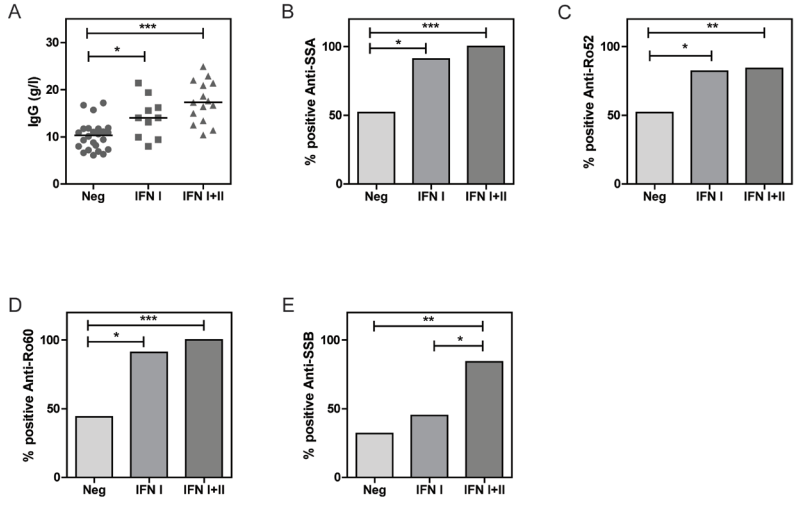
Supplementary figure S1. Flow chart of gene selection
Micro-array data of 14 pSS patients from the Rotterdam cohort were compared to genes in the modules. Differentially expressed genes for each module were selected and gene expression was determined in 50 pSS patients and 38 HCs of the Rotterdam cohort by RT-qPCR. Expression data was added into a principle component analysis. For each module 5 indicator genes were selected. Expression of these 5 indicator genes were determined in a pSS cohort of the UK (n=86) and Rotterdam (n=55).



Supplementary figure S2. Modular IFN scores in whole blood of pSS patients
Comparison modular scores (M1.2, M3.4 and M5.12) between pSS patients and HCs. UK cohort patients (n= 86) and HCs (n= 44) **(A-C)** and Rotterdam cohort patients (n=55) and HCs (n=38) **(D-F)**. Independent T-test was used to compare means of normally distributed samples.



Supplementary figure S3. Effect HCQ on modular IFN scores
M1.2, M3.4 and M5.12 IFN scores stratified on HCQ usage in the UK cohort (n= 86) **(A-C)** and Rotterdam cohort (n=55) **(D-F)**. Independent T-test was used to compare means of normally distributed samples.



Supplementary figure S4. Relationship between modular IFN scores and laboratory parameters in the Rotterdam cohort
IgG levels **(A)**, %-positive for Anti-SSA **(B)** % positive for Anti-RO52 **(C)** %-positive for anti-RO60 **(D)** %-positive for anti-SSB **(E)** in IFN neg (n=25), IFN type I (n=11) or IFN type I+II positive (n=19) pSS patients in the Rotterdam cohort. Kruskal-Wallis **(A)** and Fisher's exact test **(B to E)** were used to compare multiple groups. *P<0.05, **P<0.005, ***P<0.0005.

Chapter 3

Type I IFN signature in childhood-onset systemic lupus erythematosus: a conspiraty of DNA- and RNA-sensing receptors?

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ABSTRACT

Background Childhood-onset SLE (cSLE) is an incurable multi-systemic autoimmune disease. Interferon Type I (IFN-I) plays a pivotal role in the pathogenesis of SLE. The objective of this study was to assess the prevalence of the IFN-I signature and the contribution of cytosolic nucleic acid receptors to IFN-I activation in a cohort of primarily white cSLE patients.

Methods The IFN-I score (positive or negative), as a measure for IFN-I activation, was assessed using real-time quantitative PCR (RQ-PCR) expression values of IFN-I signature genes (IFI44, IFI44L, IFIT1, Ly6e, MxA, IFITM1) in CD14+ monocytes of cSLE patients and healthy controls (HC). Innate immune receptor expression was determined by RQ-PCR and flow cytometry. To clarify the contribution of RNA-binding RIG-like receptors (RLRs) and DNA-binding receptors (DBRs) to IFN-I activation, peripheral blood mononuclear cells (PBMCs) from patients were treated with BX795, a TANK-binding kinase 1 (TBK1) inhibitor blocking RLRs and DBRs pathways.

Results The IFN-I signature was positive in 57% of cSLE patients and 15% of the HCs. Upregulated gene expression of TLR7, RLRs (IFIH1, DDX58, DDX60, DHX58) and DBRs (ZBP-1, IFI16) was observed in CD14+ monocytes of the IFN-I-positive cSLE patients. Additionally, RIG-I and ZBP-1 protein expression was upregulated in these cells. Spontaneous IFN-I stimulated gene (ISG) expression in PBMCs of cSLE patients was inhibited by a TBK1-blocker.

Conclusions IFN-I activation, assessed as ISG expression, in cSLE is associated with increased expression of TLR7, RNA- and DNA-binding receptors, and these receptors contribute to IFN-I activation via TBK1 signaling. TBK1-blockers may therefore be a promising treatment target for SLE.

BACKGROUND

Childhood-onset Systemic Lupus Erythematosus (cSLE) is a lifelong multi-systemic autoimmune disease that shares disease pathogenesis with adult-onset SLE but in most studies is characterized by a more severe disease course and poorer prognosis [1-3]. Interferon type I (IFN-I) plays a central role in the pathogenesis of SLE [4-7]. Surprisingly, trials blocking exogenous IFN-I or its receptor show so far limited effectivity, possibly due to our lack of knowledge of the pathways leading to IFN activation [8].

About half of the adult-onset SLE patients exhibit increased activation of IFN-I signaling or so-called positive IFN-I signature [4, 5, 9]. This IFN-I signature is usually assessed by measuring IFN-I stimulated gene expression. In a USA cohort of primarily non-white cSLE patients with high disease activity approximately 80-90% IFN-I activation has been reported [6, 10]. To our knowledge the prevalence of the IFN signature has not been studied in other cSLE cohorts.

The endosomal Toll-like receptors (TLRs) 7 and 9 induce IFN expression in response to internalized RNA- and DNA-containing immune complexes. Loss of the regulation of TLR7 and TLR9, both binding exogenous self-nucleic acids, has been linked to SLE-disease pathogenesis in mouse models as well as in humans [11, 12]. In addition to the TLRs, induction of IFN-I expression can also be initiated by two cytosolic nucleic-sensing receptor families, known as i) the RIG-like Receptors (RLRs) sensing RNA and ii) the DNA-binding Receptors (DBRs) (figure 1). In Sjögren's syndrome we recently observed upregulation of RLRs that may contribute to IFN-I positivity in this disease [13]. The DBRs, like IFI16 and ZBP-1/DAI, bind intracellular dsDNA [14, 15] and as a result initiate production of IFN-I. Interestingly, mutations in the RLRs, DBRs and their downstream signaling molecules lead to systemic IFN-I activation in diseases grouped as 'type I interferonopathies' [16-18]. Patients with these diseases show similarities to the SLE disease-phenotype, pointing towards a central role of these molecules in IFN-activation and potentially in the pathogenesis of SLE [16-18].

The objective of this study was to determine the prevalence of the IFN-I signature in a cohort of primarily white cSLE patients and address the potential contribution of cytosolic nucleic acid receptors to IFN activation.

METHODS

Patients and controls

Twenty-three cSLE patients fulfilling at least 4 of the American College of Rheumatology Criteria were recruited at the outpatient clinic of the department of

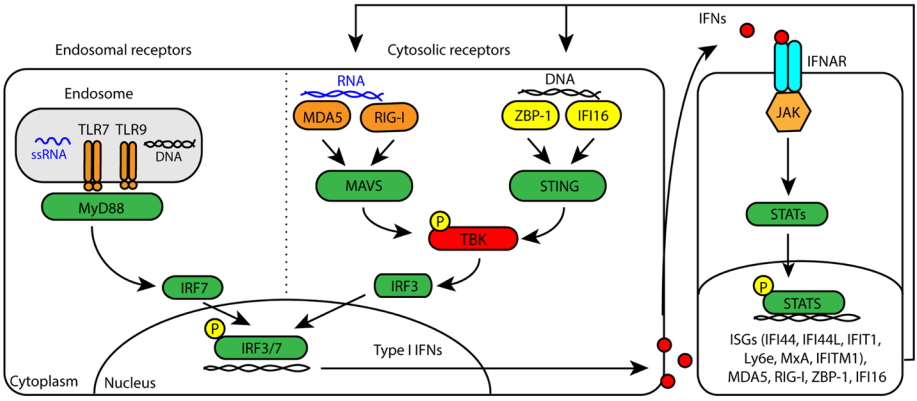

Figure 1. Simplified scheme of the induction of interferon (IFN) type I production by three signaling pathways
1) Endosomal receptors toll-like receptor (TLR)7 and TLR9; 2) RNA-binding cytosolic receptors MDA5 and RIG-I; 3) DNA-binding receptors ZBP1 and IFI16. These pathways contribute to the activation of Interferon regulatory factors (IRFs), which induce the expression of type I IFNs. Binding of IFN to cells which express the interferon alfa receptor (IFNAR) activates a cascade which leads to the expression of various IFN stimulated genes (ISGs), known as the IFN type I signature.

pediatric rheumatology of the Sophia Children's Hospital, Erasmus Medical Centre. Thirteen healthy controls (HC), specifically checked for not having (viral) infections and without having family members with autoimmune diseases, were included. Patient characteristics are summarized in table 1. The Medical Ethical Review Board of the Erasmus Medical Centre approved the study and written informed consent was obtained from all participants and their parents or legal guardians.

Blood collection and isolation of monocytes and plasmacytoid dendritic cells

Blood samples were collected in sodium-heparin tubes (Greiner Bio-One, Germany) followed by isolation of peripheral blood mononuclear cells (PBMCs) as described before [19]. 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). For membrane staining, cells were incubated for 15 min in the dark with: anti-CD14 (APC/Cy7; Becton Dickinson Biosciences, San Diego, USA), anti-BDCA-4 (PE; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD123 (PE-Cy7; eBioscience, San Diego, USA), anti-CD3 (PerCP-Cy5; Becton Dickinson Biosciences) and anti-CD19 (APC; Becton Dickinson Biosciences). Cells were sorted using a FACSAria III cell sorter (BD Bioscience) and analyzed using FlowJo Sofware (TreeStar Inc., Ashland, USA).

Table 1. Patient and control characteristics

		cSLE		
	HC (n=13)	**IFNpos (n=13)**	**IFNneg (n=10)**	
Demographics				
Ethnicity				
White	13/13 (100%)	9/13 (69%)	7/10 (70%)	Ns[c]
Non-white	0/13 (0%)	4/13 (31%)	3/10 (30%)	Ns[c]
Gender				
Male (%)	3/13 (23%)	2/13 (15%)	2/10 (20%)	Ns[c]
Female (%)	10/13 (77%)	11/13 (85%)	8/10 (80%)	Ns[c]
Median age (years)	22 (15±25)	15.8 (4.8±18.2)	15.1 (9.3±17.5)	Ns[a]
Disease duration (years)	-	0.85 (0±3.4)	1.5 (0±4.7)	Ns[b]
SELENA-SLEDAI	-	4 (0±20)	3 (0±13)	Ns[b]
Laboratory parameters				
ANA	-	12/13 (92%)	9/10 (90%)	Ns[c]
Anti-ds-DNA	-	4/13 (31%)	2/10 (20%)	Ns[c]
Anti-Ro52/Ro60	-	6/13 (46%)	0/10 (0%)	p=0.019[c]
Anti-La	-	2/13 (15%)	0/10 (0%)	Ns[c]
Anti-RNP	-	5/13 (31%)	0/10 (0%)	p=0.046[c]
C3 (g/l)	-	0.89 (0.3±1.27)	1.1 (0.77±1.72)	p =0.014[b]
C4 (g/l)	-	0.16 (0.02±0.2)	0.19 (0.1±0.37)	p=0.049[b]
IgG (g/l)	-	10.3 (7.1±27.6)	9.6 (8.4±28)	Ns[b]
Medication (%)				
Hydroxychloroquine	-	10/13 (77%)	10/10 (100%)	Ns[c]
Mycofenolaatmofetil	-	3/13 (23%)	6/10 (60%)	Ns[c]
Prednison	-	6/13 (46%)	5/10 (50%)	Ns[c]
Other medication	-	5/13 (38%)	5/10 (50%)	*Ns*[c]

Data are presented as median (IQR) or as number (%) of patients according to data distribution. ANA, antinuclear antibody; Anti-RNP, antibodies to ribonucleoprotein; C, complement; cSLE, childhood-onset systemic lupus erythematosus; Ig, immunoglobulin; IFNpos= IFN signature positive; IFNneg= IFN signature negative; HC, healthy control; Non-white ethnicity = Hindu and Suriname; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.
[a]Groups compared using the Kruskal-Wallis (three groups),
[b]Groups compared using the Mann-Whitney U test (two groups),
[c]Groups compared using the Fisher's exact test (categorical data).

RQ-PCR

RNAeasy columns (Qiagen, Hilden, Germany) were used to isolate total RNA followed by reverse-transcription to cDNA using a High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, USA). RQ-PCR analysis was performed by a 7900HT Fast Real-Time PCR System using predesigned primer sets (Applied Biosystems). Data were normalized to the expression of the household gene ABL to calculate the relative expression. ABL was previously described as a reliable household gene for myeloid cells [20]. ABL did not show differences in expression upon stratification of samples according to the IFN-stimulated gene expression scores (unpublished results). Fold change values were determined from normalized CT values using 2^-ΔΔCT method (User Bulletin, Applied Biosystems).

Monocyte IFN-I signature and MxA protein assessment

Principle component analysis showed a subset of 6 genes (IFI44, IFI44L, IFIT1, Ly6e, MxA, IFITM1) to explain more than 95% of the total variance of 11 IFN-I-inducible genes tested. Since the expression of the 6 IFN-I-inducible genes were not normally distributed, log transformations of expression values were performed and IFN scores were calculated as described previously [19]. The mean and standard deviation (SD) of each IFN-inducible gene in the HC group were used to standardize expression levels of each gene for each study subject. Patients with cSLE were stratified into patients positive for the IFN-I signature (IFNpos; IFN score ≥10) and patients negative for the signature (IFNneg; IFN score <10). Flow cytometric analysis of MxA on CD14+ monocytes and the MxA-EIA was performed as previously described [21].

Flow cytometric analysis of RLRs and DBR

Membrane staining was performed as described above with additionally an AnnexinV-BV421 staining (Milteny Biotec). Subsequently, cells were fixed and permeabilized by a permeabilization bufferset (eBioscience) with 1% paraformaldehyde, 0.5% saponin and stained with either rabbit anti-Mx1 (ProteinTech, Chicago, USA), rabbit anti-MDA5 (Abcam, Cambridge, UK), rabbit anti-DDX58 (Abcam), rabbit anti-IFI16 (Abcam) and rabbit anti-ZBP1 (Thermofischer, Rockford, USA)) and incubated in the dark for 45 min on ice. As a secondary antibody, chicken anti-rabbit-AF488 (Invitrogen, Carlsbad, USA), was used. Unstained cells and isotype-matched controls (Becton Dickinson Biosciences) were used to assess antibody specificity. Analysis was performed as previously described [21].

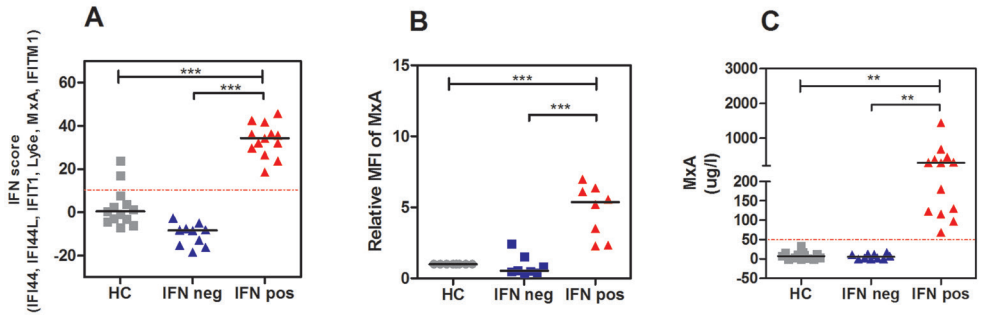

Figure 2.
A) Prevalence of the interferon (IFN) type I signature in childhood-onset systemic lupus erythematosus (cSLE). Dotted line indicates the cut-off value of 10 for discrimination between IFN-negative (IFNneg) and IFN-positive (IFNpos) subjects. **B)** Relative MxA expression was calculated as (MxA-specific staining patient (MFI)-isotype control patient (MFI))/(MxA-specific staining healthy control (HC) (MFI)-isotype control HC (MFI)). MxA is shown for HCs, IFNneg and IFNpos cSLE patients. **C)** MxA levels (ug/l) determined by MxA-enzyme immunoassays (EIA) in whole-blood lysates of HCs and cSLE patients. Dotted line indicates the cut-off value of 50 for discrimination between IFNneg and IFNpos subjects. Every symbol represents one subject; horizontal lines describe the medians; Groups were compared with One-way ANOVA (three groups). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

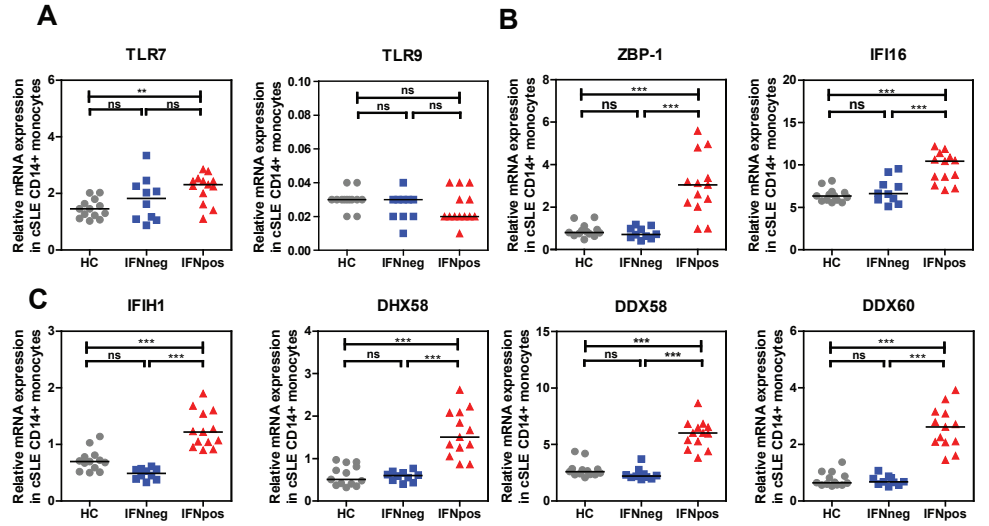

Figure 3.
Upregulation of toll-like receptor (TLR)7 and cytosolic RNA- and DNA-binding receptors in interferon (IFN) type I positive childhood-onset systemic lupus erythematosus (cSLE). Relative mRNA gene expression of **(A)** TLR7, TLR9, **(C)** DDX58, DDX60, IFIH1, DHX58, **(B)** ZBP-1 and IFI16 in CD14+ monocytes of cSLE patients (n=23) and healthy controls (HCs) (n=13). Each symbol represents an individual sample; horizontal lines represent medians. To compare the three groups One-way ANOVA was used. Ns, not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

In vitro stimulation bioassays

PBMCs were seeded at a concentration of 2×10E6/250 µL, and starved during 1 hour at 37° in RPMI with 0.5% fetal calf serum, 0.05% P/S. Cells were subsequently stimulated for 5 hours with 0.5 µg/mL Imiquimod (R837, IQ; InvivoGen, San Diego, USA), in the presence or absence of specific inhibitors for TBK1/IKKε (BX795, 1 µM, InvivoGen), TLR7 (IRS661, 2 µM, TIB-Molbiol, Berlin, Germany) and TLR7+TLR9 (ALX-746-255, 5 µM, Enzo Life Sciences, Lausen, Switzerland).

Statistical analysis

The non-parametric Mann-Whitney U (two groups) and Kruskal-Wallis (three groups) tests were used to analyze comparisons between medians. Paired T-test was used to compare means of paired data. Fisher's exact test was used to compare categorical data. Spearman's rho (rs) coefficient were calculated for correlations. Values of $p<0.05$ were considered statistically significant. Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA) was used to design the graphs and IMB SPSS 20.0 (SPSS, Chicago, IL, USA) was used for the statistical analysis.

RESULTS

Prevalence of the IFN-I signature in cSLE

The IFN-I score was calculated for each subject by summing up the standardized expression levels of the 6 IFN-I-inducible genes. Since there was a bimodal distribution of IFN inducible genes in cSLE patients, we set the threshold at an IFN-I score of 10. Using this threshold, 57% (13/23) of the patients with cSLE and 15% of the HCs (2/13) was

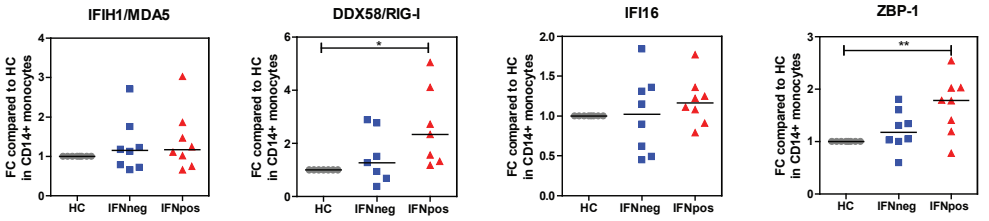

Figure 4.
Upregulated protein expression of RIG-I and ZBP-1 in interferon (IFN) positive CD14+ monocytes of childhood-onset systemic lupus erythematosus (cSLE) patients. Flow cytometric analysis of MDA5, RIG-I, IFI16 and ZBP-1 in CD14+ monocytes of IFN-positive (IFNpos) cSLE patients (n=8), IFN-negative (IFNneg) cSLE patients (n=8) and healthy controls (HCs) (n=8). Each symbol represents an individual sample. To compare the three groups Kruskal-Wallis was used. Data represented in fold change (FC) compared to HC. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

IFN-I positive (figure 2A). Previously we found that MxA protein expression using flow cytometry on CD14^{+} monocytes and a whole blood enzyme immunoassay are applicable biomarkers for systemic IFN-I activation in Sjögren's syndrome [21]. Both assays were tested simultaneously on the same PBMC samples. Results from these assays confirmed the results obtained by IFN-induced gene expression analysis (figure 2B, C).

Increased expression of TLR7, RLR and DBR in CD14+ monocytes of cSLE

Upon ligand binding the TLRs, RLRs and DBRs all initiate IFN-I production (figure 1). The gene expression of TLR7, 9, four RLRs and two DBRs was assessed in CD14+ monocytes of cSLE patients stratified in IFNpos and IFNneg. TLR7 expression was significantly upregulated in IFNpos patients compared to HCs (figure 3A). There were no significant differences in TLR7 expression between IFNneg and IFNpos patients or between IFNneg patients and HCs. In addition, TLR9 expression did not differ between the groups.

The expression levels of the RLRs IFIH1, DHX58, DDX58 and DDX60 and the DBRs ZBP-1 and IFI16 were significantly upregulated in IFNpos patients compared to IFNneg patients and HCs (figure 3B, C). There was no significant difference between IFNneg patients and the HCs in RLR or DBR expression levels. Furthermore, expression levels of the RLRs and DBRs showed a positive correlation with IFN scores (supplementary figure S1).

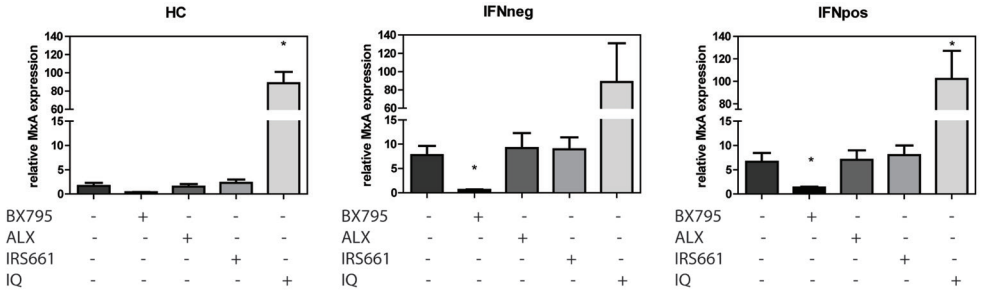

Figure 5.
TBK1/IKKε inhibits IFN type I activation in childhood-onset systemic lupus erythematosus (cSLE) peripheral blood mononuclear cells (PBMCs). Relative MxA gene expression after 5-hr culture of PBMCs of healthy controls (HCs), IFN-negative (IFNneg) or IFN-positive (IFNpos) patients with cSLE stimulated with imiquimod (IQ) (1 μg/ml)) or incubated with TBK1/IKKε inhibitor (BX795)(1 μM), toll-like receptor (TLR)7 and 9 inhibitor (ALX-746-255) (2 μM) or TLR7 inhibitor (IRS661) (5 μM). Cells without addition of stimuli or inhibitors are cultured in starvation medium and used as control for baseline IFN activation level. Gene expression data are presented as means ± SEM of 4 independent experiments (n=5 per group). Means were compared to starvation medium using the paired t-test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Increased RIG-I and ZBP-1 protein levels in cSLE

To study protein expression of RLRs and DBRs we performed a flow cytometric analysis of MDA5, RIG-I, IFI16 and ZBP-1 expression in CD14+ monocytes of IFNpos, IFNneg cSLE patients and HCs. The gating strategy and a representative figure are depicted in supplementary figure 2. RIG-I and ZPB-1 protein expression was significantly upregulated in CD14+ monocytes of IFNpos cSLE patients compared to HC (figure 4). There were no significant differences found in MDA5 and IFI16 protein levels in CD14+ monocytes of patients and HCs. pDCs are known to upregulate RLRs and DBRs upon IFN-I activation. In pDCs of IFNpos cSLE patients the expression of ZBP1 and IFI16 was significantly upregulated (supplementary figure 3).

TBK1/IKKε inhibitor blocks IFN-I activation in cSLE PBMCs

To study the contribution of the RLR and DBR pathways to IFN-I activation in cSLE we blocked these pathways using a TBK1/IKKε inhibitor (BX795). A titration of BX795 is shown in supplementary figure 4. TLRs were blocked with inhibitors for TLR7 (IRS661) [13] and TLR7+9 (ALX-746-255). As a positive control for the effectivity of the blockers HC PBMCs were stimulated with the TLR7-agonist imiquimod (IQ) to induce IFN-I positivity followed by incubation with these inhibitors (supplementary figure 5). PBMCs of IFNpos and IFNneg cSLE patients without any additional stimulation exhibited an increased spontaneous IFN-stimulated gene expression compared to HCs as determined by MxA expression (figure 5). Incubation with the TBK1/IKKε inhibitor completely reduced the spontaneous IFN-I stimulated gene expression in cSLE cells. Inhibition of TLR7 or 7+9 had no effect on the intrinsic spontaneous IFN activation in PBMCs of IFNneg and IFNpos with cSLE (figure 5).

DISCUSSION

This study shows increased expression of TLR7 and the cytosolic receptors of the RLR and DBR families in monocytes of IFN-I positive cSLE patients. Blocking of the RLR and DBR signaling pathway downregulated IFN-I stimulated gene expression indicating a contribution of these receptors to systemic IFN-I activation in SLE.

In our cohort of cSLE patients 57% had a positive IFN-I signature. This is in line with our earlier observations in a cohort of adult-onset SLE patients [9]. Previous studies reported 80-90% positivity in cSLE patients, these were primarily non-white patients with relative high disease activity [6, 10]. In contrast, our cohort consists of mainly white cSLE patients with low disease activity which may account for at least part of the difference in prevalence as the presence of IFN-I signature is related to disease

activity [19]. As in the other cSLE cohorts, most cSLE patients in our cohort used anti-inflammatory medication. The presence of an IFN-I signature in patients receiving medication indicates that current treatments are not or only partly able to downregulate IFN-I stimulated gene expression.

Upon stratification in IFNpos and IFNneg cSLE patients, we found an upregulation of TLR7 in IFN positive cSLE. This supports a role for TLR7 in the induction of IFN-I activation in SLE as has been demonstrated in animal models [22, 23]. Interestingly, a Mexican cohort of cSLE cases showed that the gene dosage of TLR7 is an important risk factor for cSLE susceptibility [24]. In our ex vivo experiments TLR7 or TLR7+9 inhibitors did not decrease IFN-I activation in cSLE patients. This is likely due to the short culture period of 5 hours that does not allow formation of nucleic-acids containing immune complexes, that are required for TLR7/9 driven IFN induction. Therefore, the exact role of TLRs in comparison with cytosolic receptors remains to be established.

The expression of cytosolic receptors belonging to the RLRs and DBRs, was upregulated in IFNpos cSLE patients compared to HCs and IFNneg patients. Accumulating evidence indicates an important role for aberrancies in these receptors and their downstream signaling molecules in monogenic diseases with clinical similarities to SLE [16, 17]. Interestingly, a recent study showed a correlation between IFN-I activation and the expression of an endogenous virus-like genomic repeat element L1 in kidney tissue of lupus nephritis patients. As such a L1 element activates RLRs this supports a role of this receptor family in SLE [25].

The potential contribution of RLRs and DBRs to IFN-I activation was also supported by our ex vivo experiments showing a clearly decreased IFN-I stimulated gene expression in all cSLE patients upon ex vivo blocking of TBK1. TBK1 is at the crossroad downstream of the RLR and DBR signaling pathways. Interestingly, TBK1 upregulation in SLE PBMCs has been observed [26] and inhibition of TBK1 activity suppressed IFN-I induced autoimmunity in a mouse model for SLE [27]. Blockade of TBK1/IKKε with BX795 was also found to inhibit IFN-I stimulated gene expression in PBMCs of a patient with a gain-of-function mutation in STING, that resulted in over-secretion of IFN-I [28]. In our ex vivo experiments, PBMCs of IFNneg patients also showed a higher spontaneous intrinsic IFN-I stimulated gene expression, that could be decreased by TBK1 inhibition with BX795. This is probably due to stimulation of the IFN-I inducing pathways by the presence of more dead cells and cell material, which we always observe in SLE samples compared to controls, despite the same isolation procedure. This is in line with data on a higher vulnerability of SLE cells in the literature [29].

To date, inhibiting IFN activation by blocking IFN-I receptor (IFNAR) by biologicals so far showed encouraging results but the treatment was only effective in a subset of the patients [30]. More upstream interference using TBK1 inhibitors to prevent the

induction of IFN expression might be a better approach. With TBK1 as an upstream signaling hub inducing IFN-I expression and more than 20 patented TBK1 inhibitors already developed, a novel treatment target for clinical applications might enter the field. Compared to most other biologicals, small-molecule TBK1 inhibitors have two advantages: 1) the inhibitors can be taken orally and 2) they are expected to have less side effects due to the high specificity [27].

This study has limitations. All patients are receiving treatment which could have affected the IFN-I activation and due to lack of reliable assays to detect systemic IFN-I activation in serum the IFN-induced gene expression is used.

Several studies in PBMCs of adult SLE patients describe a difference between IFNα or IFNβ induced genes [7, 31]. We did not make this distinction in our study as we used monocytes and these IFN subtype specific induced genes can differ per cell type. Additionally the treatment presently tested in clinical trials is focused on blocking the IFNAR, which is binding both IFNα and IFNβ.

Furthermore, we studied mRNA and protein expression from monocytes but the patient's pDCs are only studied by flowcytometry. However, monocytes are considered important responders to RLR and DBR triggering and the ex vivo cultures of PBMCs of the patient are simulating the in vivo situation. The TBK1 inhibitor used also inhibits IKKε. Therefore a role of IKKε in the IFN-I activation in SLE cannot be excluded.

CONCLUSIONS

Overall, the IFN-I signature was present in 57% of patients with cSLE and associated with increased expression of TLR7 and cytosolic nucleic acid binding receptors. These RLRs and DBRs contributed to the spontaneous ex vivo IFN-I stimulated gene expression via TBK1 signaling. Inhibitors of TBK1 are therefore a promising treatment target for SLE.

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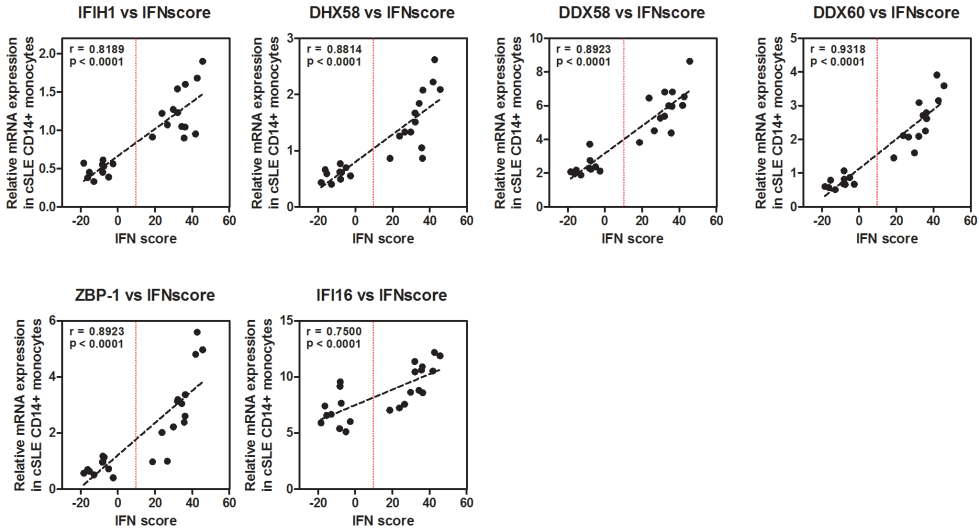
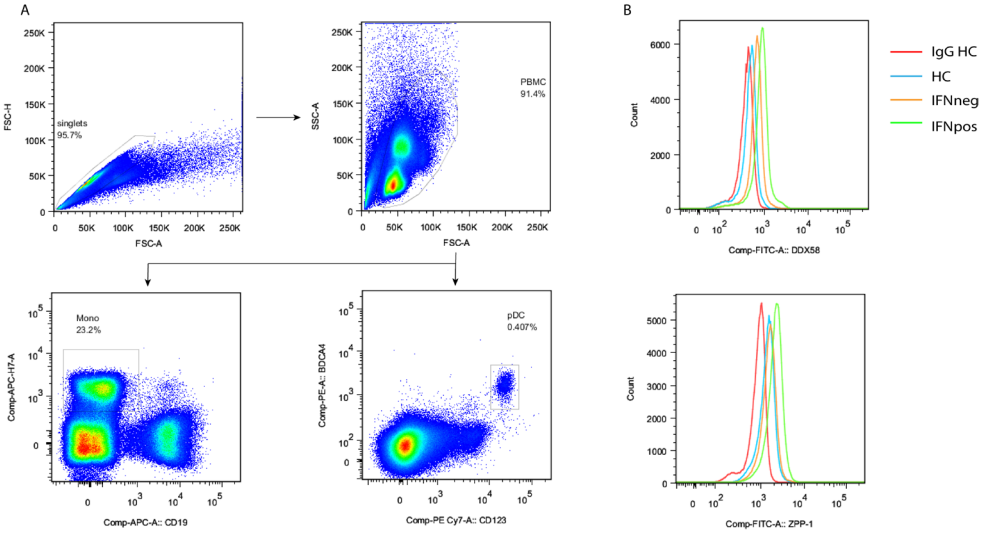
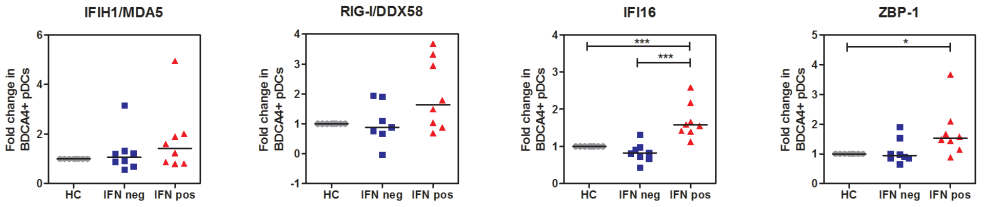
SUPPLEMENTARY FILES



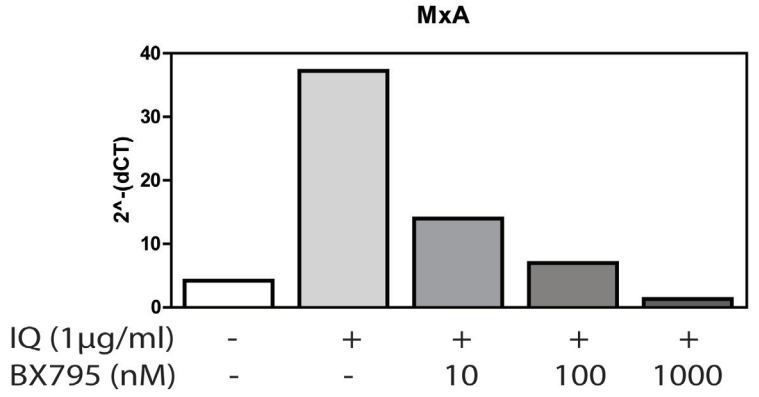
Supplementary figure S1. Correlations of RLR and DBR expression levels with IFN scores
Correlation between interferon (IFN) type I score and mRNA expression of 6 IFN inducible genes (IFIH1, DDX58, DDX60, DHX58, ZBP-1, IFI16) in CD14+ monocytes in patients with cSLE (n=23). The correlation coefficients (r) and p values are shown. For correlations Spearman's ρ correlation test was used and to compare means the Mann–Whitney U test was used.



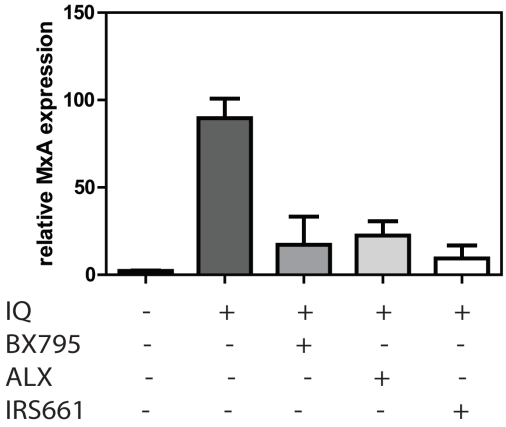
Supplementary figure S2. Gating strategy and representative histogram
A) Gating strategy for CD14+ monocytes and CD123/BDCA4+ pDCs. PBMCS were gated after removal of doublets and dead cells. Within the PBMC fraction, CD14+ monocytes were gated and CD123/BDCA4+ pDCs **B)** representative histogram of protein expression levels (top: DDX58/RIG-I, bottom: ZBP-1).



Supplementary figure S3. RLR and DBR protein expression in pDC of cSLE patients
Flowcytometric analysis of MDA5, RIG-I, IFI16 and ZBP-1 in pDCs of IFNpos cSLE patients (n=8), IFNneg cSLE patients (n=8) and healthy controls (n=8). Each symbol represents an individual sample. To compare the three groups One-way ANOVA was used. Data represented in fold change compared to HC. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Supplementary figure S4. Titration curve of BX795
Titration of BX795 on HC PBMCs. Relative MxA gene expression after 5 hr culturing of PBMCs of HCs with imiquimod (IQ) (1 µg/ml)) and increasing amounts of TBK1/IKKε inhibitor (BX795)(10, 100 and 1000 nM). Gene expression data are presented as means ± SEM.



Supplementary figure S5. Effectivity of inhibitors for TBK1, TLR7 and TLR7+9 to downregulate Imiquimod induces MxA expression

Relative MxA gene expression after 5 hr culturing of PBMCs of HCs with imiquimod (IQ) (1 μg/ml)) and/or incubated with TBK1/IKKε inhibitor (BX795)(1 μM), TLR 7 and 9 inhibitor (ALX-746-255) (2 μM) or TLR 7 inhibitor (IRS661))(5 μM). Cells without addition of stimuli or inhibitors are cultured in starvation medium and used as control for baseline IFN activation level. Gene expression data are presented as means ± SEM of 2 independent experiments.

Chapter 4

TBK1: a key regulator and potential treatment target for interferon positive Sjögren's syndrome, systemic lupus erythematosus and systemic sclerosis

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ABSTRACT

Objective Upregulation of type I interferons (IFN-I) is a hallmark of systemic autoimmune diseases like primary Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). Expression of IFN-I is induced by three different receptor families: Toll-like receptors (TLRs), RIG-like receptors (RLRs) and DNA-sensing receptors (DSRs). TANK-binding kinase (TBK1), is an important signaling hub downstream of RLRs and DSRs. TBK1 activates IRF3 and IRF7, leading to IFN-I production and subsequent induction of interferon-stimulated genes (ISGs). The objective of this study was to explore the potential of BX795, an inhibitor of TBK1, to downregulate IFN-I activation in pSS, SLE and SSc.

Methods TBK1, IRF3, IRF7 and STAT1 were determined by qPCR in PAXgene samples and phosphorylated-TBK1 (pTBK1) was analyzed by flowcytometry in plasmacytoid dendritic cells (pDCs) from IFN-I positive (IFNpos) patients. Peripheral blood mononuclear cells (PBMCs) of pSS, SLE and SSc patients and TLR7 stimulated PBMCs of healthy controls (HCs) were cultured with the TBK1 inhibitor BX795, followed by analysis of ISGs.

Results Increased gene expression of TBK1, IRF3, IRF7 and STAT1 in whole blood and pTBK1 in pDCs was observed in IFNpos pSS, SLE and SSc patients compared to HCs. Upon treatment with BX795, PBMCs from IFNpos pSS, SLE, SSc and TLR7-stimulated HCs downregulated the expression of the ISGs MxA, IFI44, IFI44L, IFIT1 and IFIT3.

Conclusions TBK1 inhibition reduced expression of ISGs in PBMCs from IFNpos patients with systemic autoimmune diseases indicating TBK1 as a potential treatment target.

INTRODUCTION

In systemic autoimmune diseases like primary Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) upregulation of type I interferons (IFN-I) is a hallmark [1-3] and potential treatment target. Systemic upregulation of IFN-I is present in 50-90% of the patients with pSS, SLE and SSc as determined by various methods in different cohorts of patients [1-5]. Plasmacytoid dendritic cells (pDCs) produce IFN-I in response to RNA- and DNA-containing immune complexes (ICs) activating the endosomal toll-like receptors (TLR) 7 and 9. IFN-I expression can also be induced by RIG-like receptors (RLRs) and DNA-sensing receptors (DSRs) upon activation by cytosolic nucleic acids (RNA/DNA). A dysregulated expression of the RLRs RIG-I and MDA5 in IFN-I positive (IFNpos) pSS patients was previously described by us [6]. In lupus nephritis and glands of pSS patients, the expression of endogenous nucleic acids encoded by a virus-like element correlated with IFN-I activation, indicating a contribution of RLRs and DSRs to IFN-I activation [7]. Gain of function mutations in the nucleic acid-sensing routes in interferonopathies like Aicardi-Goutières also support a role for nucleic acids-sensing receptors in systemic IFN-I activation [8, 9].

Tumor necrosis factor (TNF) receptor-associated factor NF-κB activator (TANK)-binding kinase 1 (TBK1) is a kinase downstream of the RLRs and DSRs. TBK1 is a non-canonical IκB kinase (IKK) which requires, just like its closely related structural homologue IKKε, phosphorylation at Ser^{172} to become activated. Activated TBK1 and IKKε phosphorylate interferon regulator factor (IRF) 3 and 7 followed by translocation to the nucleus and subsequent induction of transcription and production of IFN-I. IFN-I can then bind to the receptor of IFN-I (IFNAR), which is present on immune cells, and via the JAK-STAT pathway lead to induction of interferon stimulated genes (ISGs) [10, 11]. Interestingly, among those ISGs are RLRs and DSRs indicating a close interplay between the various IFN-I inducing pathways (figure 1A). Additionally, IKKε has been implicated to be involved in inducing STAT1 phosphorylation downstream of the IFNAR [12].

Currently, trials targeting the IFNAR in SLE show encouraging results and support the pathogenic role of IFN-I [13]. Blocking more upstream the actual transcription of IFN-I by inhibition of TBK1, as a signaling hub irrespective of the route of activation, might potentially be a better treatment target. Interestingly BX795, a molecule which inhibits TBK1 and IKKε, has recently been shown to inhibit IFN-I production and signaling in human PBMCs with a mutation-induced interferonopathy [8]. Here we hypothesize that in IFNpos autoimmune diseases like pSS, SLE and SSc, phosphorylation of TBK1 (pTBK1) is upregulated due to activation of RLRs and/or DSRs. Inhibition of TBK1 activity could downregulate IFN type I production.

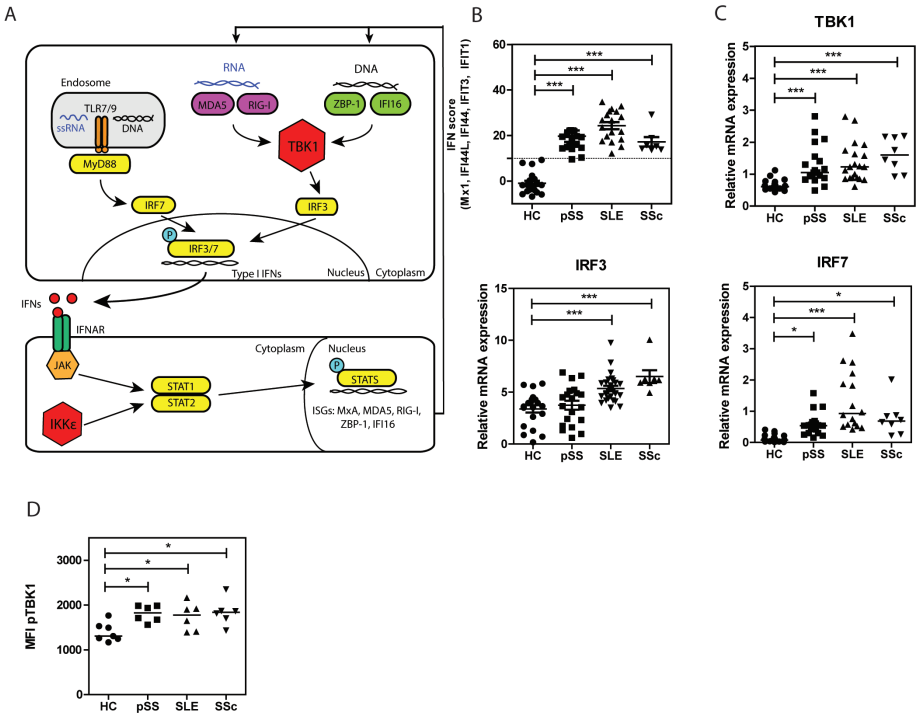

Figure 1. Systemic activation of TBK1 in type I IFN positive autoimmunity
(A) Simplified scheme of the type I IFN inducing pathways and the signaling hub TBK1/ IKKε (in red), which can be targeted by the inhibitor BX795. **(B)** IFN scores of type I IFN signature positive pSS, SLE, SSc patients and healthy control (HC) tested in this study. Line indicates cut-off value between IFN positive and negative. **(C)** Gene expression of TBK1, IRF3 and IRF7 was determined in type I IFN signature positive pSS (n=20), SLE (n=20), SSc (n=8) patients and healthy controls (n=20). **(D)** Protein expression of phosphorylated-TBK1 (pTBK1) in blood-derived plasmacytoid dendritic cells of pSS (n=6), SLE (n=6), SSc (n=6) patients and healthy controls (n=7). Expression of pTBK1 was calculated as 'pTBK1-specific staining (MFI)'-'isotype control (MFI)'. For three or more group comparisons Kruskal-Wallis was used. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

PATIENTS AND METHODS

Patients and controls

Healthy controls (HCs) and patients with a positive diagnosis for pSS according to 2002 American-European Consensus Group classification criteria; for SLE according to the ACR revised criteria for SLE and for SSc according to the ACR/EULAR 2013 classification criteria for SSc were recruited at the Erasmus Medical Centre, Rotterdam, the Netherlands [14-16]. HCs did not suffer from autoimmune diseases nor used corticosteroids. Characteristics of patients are summarized in supplementary table S1. The study was approved by the Rotterdam Medical Ethical Review Committee and written informed consent was obtained from all subjects.

Blood collection

Blood samples were collected in PAXgene RNA tubes (PreAnalytix, Switzerland) for whole blood RNA analysis and sodium-heparin tubes (Greiner Bio-One, Germany) for isolation of peripheral blood mononuclear cells (PBMCs).

RQ-PCR

RNAeasy columns (Qiagen, Hilden, Germany) were used to isolate total RNA from PBMCs followed by reverse-transcription to cDNA using a High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Total RNA from PAXgene RNA tubes was isolated according to manufacturer's protocol. RQ-PCR analysis was performed by a Quantstudio™ 5 Real-Time PCR System using predesigned primer sets (Applied Biosystems). Data were normalized to the expression of the household gene Abl to calculate the relative expression. Fold change values were determined from normalized CT values using 2^-ΔΔCT method (User Bulletin, Applied Biosystems).

Calculation of IFN-I score

The IFN-I score was defined by the relative expression of 5 genes: IFI44, IFI44L, IFIT1, IFIT3 and MxA. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN-I scores per subject represent the sum of these standardized scores, calculated as previously described [1, 17, 18]. Patients were divided in groups being positive or negative for systemic IFN-I activation, using a threshold of $mean_{HC}$ + 2 x SD_{HC}.

Flow cytometric analysis of pTBK1

PBMCs were thawed, centrifuged 5 min (1500 rpm, 4°C) and resuspended in PBS. For membrane staining cells were incubated for 20 min in the dark with anti-BDCA-4 (PE; Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD123 (PE-Cy7; eBioscience, San Diego, USA). Subsequently, cells were fixed and permeabilized by a permeabilization bufferset (eBioscience). After this, cells were stained with rabbit anti-pTBK1/NAK (Ser172) (D52C2) (Cell Signaling Technology, Danvers, USA), rabbit anti-TBK1/NAK (Ab109734) (Abcam, Cambridge, UK) or rabbit anti-Mx1 (ProteinTech, Chicago, USA), and incubated in the dark for 45 min on ice. As a secondary antibody, chicken anti-rabbit-AF488 (Invitrogen, Carlsbad, USA), was used. Unstained cells and isotype-matched controls (Becton Dickinson Biosciences) were used to assess antibody specificity. Cells were measured on a FACSCanto II (BD Bioscience) and analyzed using FlowJo Sofware (TreeStar Inc., Ashland, USA).

Bioassays

PBMCs were seeded at a density of 2×10E6/250 µL, and starved for 1 hour at 37°C in RPMI-1640 medium with 0.5% fetal calf serum and 0.05% penicillin/streptomycin. Cells were stimulated for the indicated period with 0.5 µg/mL Imiquimod (R837, IQ; InvivoGen, San Diego, USA), in the presence or absence of the TBK1/IKKε inhibitor BX795 (1 µM, InvivoGen). At the end of the culture period the viability was analyzed by trypan blue staining.

IFN-I Reporter assay

IFN-I was measured by bioassay using HEK-Blue IFN-α/β cells (InvivoGen) according to manufacturer's protocol.

Statistical Analysis

The non-parametric Mann-Whitney U (two groups) and Kruskal-Wallis (more than two groups) tests were used to analyze comparisons between medians. Values of $p<0.05$ were considered statistically significant. Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA) was used to design the graphs and IBM SPSS 24.0 (SPSS, Chicago, IL, USA) was used for the statistical analysis.

RESULTS

Phosphorylated TBK1 is upregulated in IFN-I positive pSS, SLE and SSc

To investigate pTBK1 and the signaling pathway of the cytosolic RLRs and DSRs in IFNpos autoimmune diseases we selected pSS, SLE and SSc patients with systemic upregulation of IFN-I. IFN-I positivity was defined by the relative expression of 5 ISGs and depicted as an IFN score (figure 1B) [1, 2]. To study a possible role of the cytosolic RLR and DSR in IFN-I induction, the expression of the downstream signaling molecules TBK1 and IRF3 was assessed in PBMCs of IFNpos patients. In addition IRF7, downstream of the TLR7,9 IFN-inducing route and the IFN-stimulated gene STAT1, downstream of the IFNAR, were analyzed (figure 1C and supplementary figure S1A). Upregulation of TBK1, IRF7 and STAT1 gene expression was observed in IFNpos pSS, SLE and SSc patients and IRF3 gene expression was upregulated in SLE and SSc compared to HCs. The observed upregulation of TBK1, IRF7 and STAT1 in IFNpos pSS are confirming our previous observations [6].

To focus on pDCs, as main source of IFN-I, BDCA4+CD123+ cells were stained with an antibody recognizing the phosphorylated form of TBK1 (Ser^{172}) (for gating strategy see supplementary figure S1B). pDCs of IFNpos pSS, SLE and SSc patients showed an

upregulation of pTBK1 compared with HCs indicating activation of this signaling route (figure 1D). In addition, total TBK1 was also determined in BDCA4+CD123+ cells from IFNpos SLE patients and HCs. MxA, a protein upregulated in IFNpos autoimmunity, was measured as a positive control. There was no difference in total TBK1 between IFNpos SLE patients and HCs, while MxA protein expression was significantly higher expressed in IFNpos SLE BDCA4+CD123+ cells compared to HCs (supplementary figure S1C, D). These results suggest that increased phosphorylation of TBK1 plays a role in the observed IFN-I upregulation, and this is not because of differences in total TBK1 levels.

BX795 downregulates IFN-I activation in TLR7-stimulated PBMCs

BX795 is a relatively specific inhibitor for TBK1, which also inhibits the closely related kinase IKKε, which is amongst others involved in the signaling downstream of the IFNAR [8]. To assess the effectivity of BX795 to downregulate IFN activation, HC-PBMCs were stimulated with the TLR7-agonist imiquimod (IQ), which induces rapid IFN-I production and upregulation of ISGs including several RLRs. A titration of BX795 on TLR7-stimulated PBMCs is shown in supplementary figure S2. In HC-PBMCs stimulated with IQ, BX795 downregulated IFN-I production and mRNA levels of the ISGs MxA, IFI44, IFI44L, IFIT1 and IFIT3 to the unstimulated level (figure 2A, B). Restimulation of PBMCs with IQ showed that the PBMCs were still viable and able to produce IFN-I after 24 hours (data not shown).

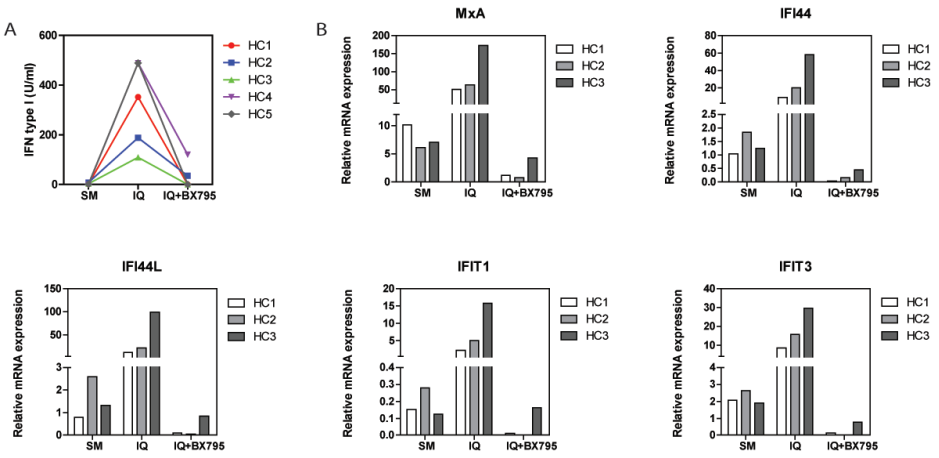

Figure 2. Type I IFN inhibition by BX795 in healthy control peripheral blood mononuclear cells after Toll-like receptor 7 triggering with imiquimod
(A) Type I IFN protein production as determined by HEK-Blue IFN-α/β reporter system in the culture supernatant at baseline (SM) and after 24 hours imiquimod (0.5 μg/mL) stimulation without and with BX795 (1 μM). **(B)** Relative mRNA gene expression of the Interferon Stimulated Genes MxA, IFI44, IFI44L, IFIT1 and IFIT3 at baseline and after 5 hours imiquimod (0.5 μg/mL) stimulation without and with BX795 (1 μM).

BX795 downregulates IFN-I activation in PBMCs of patients with systemic autoimmune diseases

To assess the effect of BX795 on IFN-I activation in pSS, SLE and SSc PBMCs we incubated unstimulated PBMCs of IFNpos patients with BX795. PBMCs, particularly of SLE patients and to a lesser extent of pSS and SSc patients, exhibited an increased IFN-I activation under non-stimulating conditions as determined by expression of the ISG MxA compared to HC-PBMCs (figure 3A). BX795 treatment significantly reduced the spontaneous IFN-I activation of pSS, SLE and SSc PBMCs. Also the expression of the ISGs IFI44, IFI44L, IFIT1 and IFIT3 was downregulated by BX795 treatment in pSS (figure 3B), SLE and SSc PBMCs (supplementary figure S3A, B).

DISCUSSION

Systemic autoimmune diseases like pSS, SLE and SSc are diseases with an unmet need for evidence-based therapy targeting pathogenic factors. We describe for the first time that a TBK1/IKKε inhibitor downregulates IFN-I activation in PBMCs of patients with pSS, SLE and SSc.

In addition to the TLR7,9 pathway also RLRs and DSRs can induce IFN-I transcription. The partly overlapping downstream signaling pathways after activation

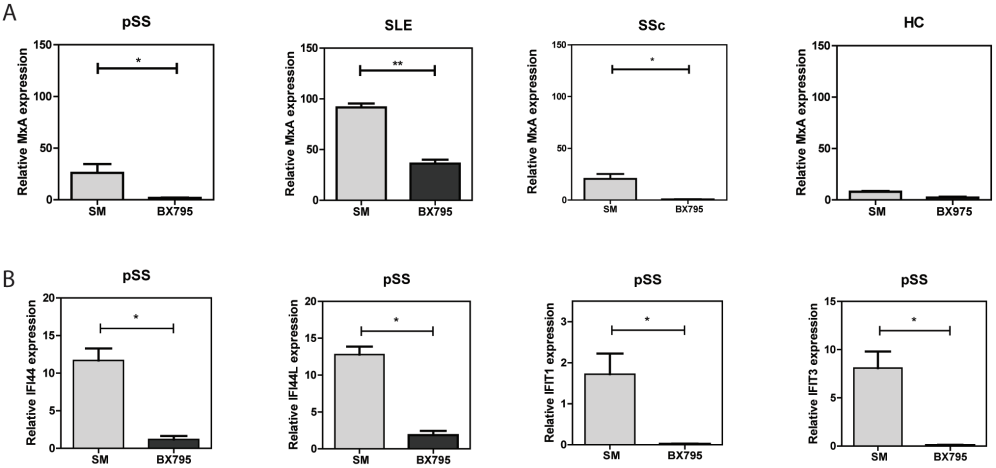

Figure 3. BX795 treatment inhibits spontaneous type I IFN activation in peripheral blood mononuclear cells of patients with systemic autoimmunity
(A) Effect of BX795 (1 μM) after 5 hours incubation on the mRNA expression of the Interferon Stimulated Gene MxA by peripheral blood mononuclear cells of pSS (n=6), SLE (n=3), SSc patients (n=3) and healthy controls (n=4). **(B)** and on the Interferon Stimulated Genes IFI44, IFI44L, IFIT1, IFIT3 in pSS patients (n=6). Mann-Whitney U was used for two group comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

of RLRs and DSRs offer the opportunity to inhibit common signaling hubs irrespective of the activating route. TBK1 is such a hub and the presence of already more than 35 patented pharmacological inhibitors, amongst which are several small molecule inhibitors, indicating inhibition of TBK1 as a novel treatment option for IFNpos systemic autoimmunity [19]. An advantage of TBK1 inhibitors is that they are already used to treat cancer and inflammatory diseases and their high stability and low costs compared to biologicals [19].

Interestingly, upregulation of TBK1 mRNA has been found in leukocytes from SLE patients and SLE lymphoblast cell lines. Treatment of these cells with a TBK1 inhibitor showed reduced expression of the ISGs CXCL10 and RSAD2 [20]. These data and the observed hyperphosphorylation of TBK1 in isolated monocytes from a few SLE patients [21] point towards a role of TBK1 as signaling hub in SLE. Here we describe the upregulation of pTBK1 in pDCs of IFNpos patients with pSS, SLE and SSc supporting a role the RLRs and DSRs. This observation is in line with our previous data showing upregulation of RLRs and TBK1 in pSS pDCs and PBMCs of (childhood-onset) SLE patients [6, 22].

TBK1 inhibition has recently been described effective in reducing IFN-I activation in PBMCs of four patients with an autoinflammatory syndrome characterized by a gain-of-function mutation in the gene encoding stimulator-of-interferon-genes (STING) [8]. This interesting observation using the same inhibitor as here, shows inhibition of the phosphorylation of IRF3 downstream of TBK1 and reduced activity in an IFNβ-reporter assay. Like in our study, BX795 inhibited ISG mRNA expression. Additional support for a role of TBK1 in the pathogenesis of *TREX*-induced interferonopathies is provided by the decreased IFN activation in a human cell line with a *TREX* mutation upon treatment with a TBK1 inhibitor [23]. These data together support further exploration of the potential of TBK1 inhibitors as treatment for IFNpos systemic autoimmune diseases.

A limitation of this study is that BX795 inhibits not only TBK1 but also IKKε, which is in addition to being downstream of TLR3 and 4 also downstream of the IFNAR. We show that pDCs, which lack TLR3 and 4, have upregulated pTBK1 supporting a contribution of the RLR/DSR pathway to the observed IFN activation. In addition, microarrays of SLE leukocytes show elevated TBK1 expression but not of IKK genes [20]. However, a possible contribution of IKKε downstream of the IFNAR to our observations should be considered and might even be advantageous as IKKε inhibition by BX795 will reduce ISG induction via the IFNAR.

In conclusion, this report describes for the first time, the effect of the TBK1/IKKε inhibitor BX795 on IFN- I activation in blood cells of patients with three different systemic autoimmune diseases. TBK1 might therefore be a promising target for therapeutic intervention in patients with IFNpos autoimmunity.

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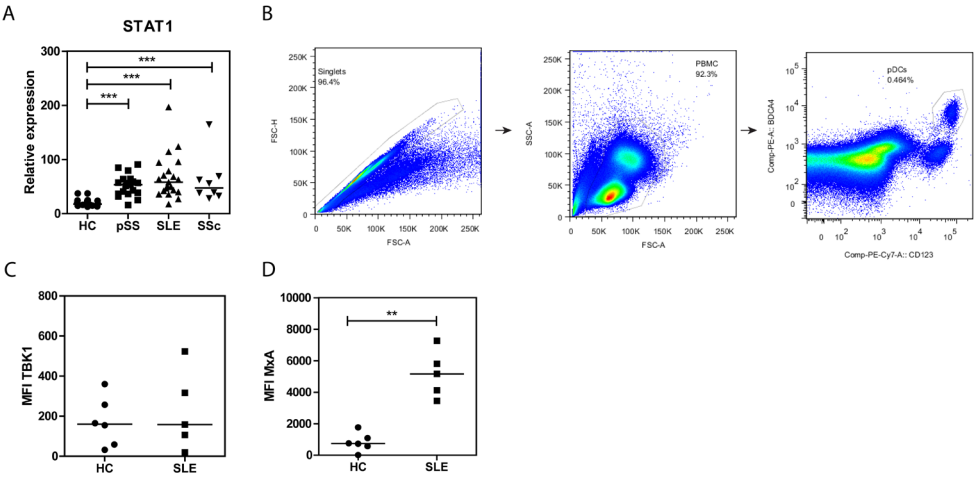
SUPPLEMENTARY FILES

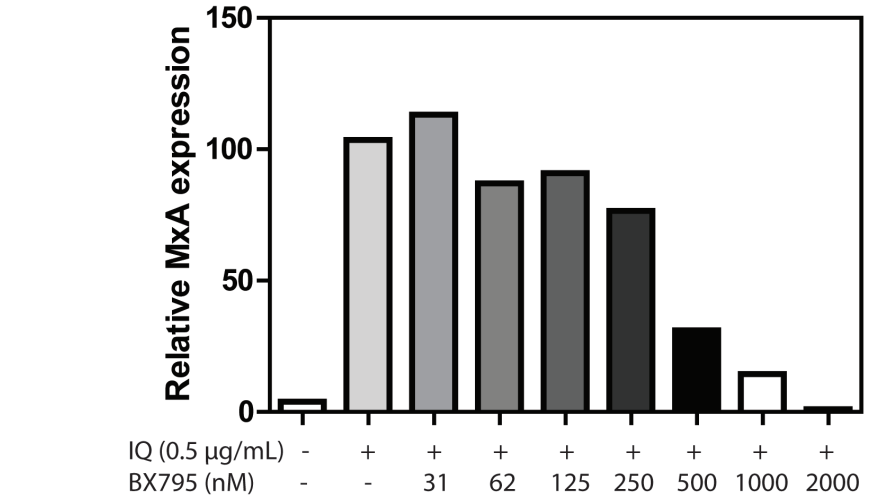
Supplementary table S1. Demographics, characteristics and medication use by participants

	HC (n= 22)	pSS (n=22)	SLE (n=21)	SSc (n=10)
Demographics				
Female (%)[c]	21/22 (96)	20/22 (91)	16/21 (76)	8/10 (80)
Mean age (years)a	55.0 ± 5.9	56.1 ± 12.5	41.4 ± 16.0	53.3 ± 8.8
Patient characteristics				
Disease duration (years)[a]	-	12.7 ± 8.0	18.6 ± 14.6	9.6 ± 7.5
Disease activity[a,b]	-	9.0 (12)	4.0 (6)	-
Medication status (%)				
Corticosteroids (%)[c]	-	3/22 (14)	8/21 (38)	3/10 (30)

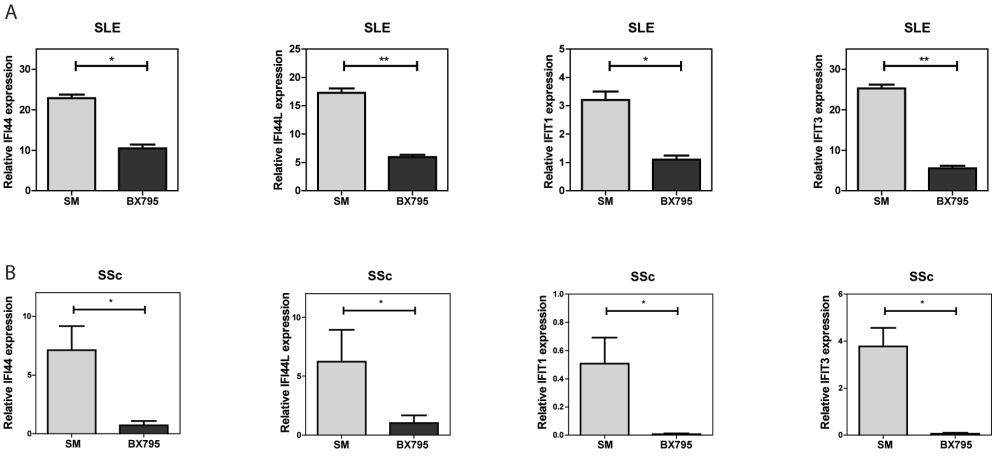
Data are presented as mean ± SD[a], median (IQR)[b] or as number (%)[c] of patients according to data distribution. [c]Disease activity determined by ESSDAI for pSS and SLEDAI for SLE. pSS, primary Sjögren's Syndrome; SLE, Systemic Lupus Erythematosus; SSc, Systemic Sclerosis.



Supplementary figure S1.
A) Gene expression of STAT1 was determined in type I IFN signature positive pSS (n=20), SLE (n=20), SSc (n=8) patients and healthy controls (n=20) **B)** Gating strategy for pDCs. PBMCs were gated after removal of doublets and debris. Within the PBMC fraction CD123/BDCA4+ pDCs were gated. TBK1 **(C)** and MxA **(D)** protein expression in blood-derived plasmacytoid dendritic cells of SLE patients (n=5) and healthy controls (n=6). Protein expression was calculated as 'specific staining (MFI)'-'isotype control (MFI)'. For two group comparisons the Mann-Whitney U test was used and for three or more group comparisons Kruskal-Wallis. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.



Supplementary figure S2.
Titration of BX795 on HC-PBMCs. Relative MxA gene expression after 5 hour incubation of PBMCs of HCs with imiquimod (IQ) (0.5 µg/mL) and increasing concentrations of the TBK1/IKKε inhibitor BX795 (nM).



Supplementary figure S3.
Inhibitory effect of BX795 (1 µM) on the ISGs IFI44, IFI44L, IFIT1 and IFIT3 after 5 hour incubation of unstimulated PBMCs of SLE (n=3) **(A)** and SSc (n=3) patients **(B).**

Chapter 5

Hydroxychloroquine treatment downregulates systemic interferon activation in primary Sjögren's syndrome in the JOQUER randomized clinical trial

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Rheumatology, under review

ABSTRACT

Objective Hydroxychloroquine (HCQ) is frequently used to treat primary Sjögren's syndrome (pSS), however evidence for its efficacy is limited. HCQ blocks interferon (IFN) activation, which is present in half of the pSS patients. The effect of HCQ treatment on the expression of IFN-stimulated genes (ISGs) was studied in pSS. Furthermore, HCQ-treated patients were stratified based on IFN activation and differences in disease activity and clinical parameters were studied.

Methods Expression of ISGs and IFN scores were determined in 77 patients, who were previously enrolled in the placebo-controlled JOQUER trial. Patients were treated for 24 weeks with 400 mg/d HCQ or placebo.

Results HCQ treatment reduced IFN scores and expression of ISGs compared to the placebo-treated group. HCQ reduced ESR, IgG and IgM levels independently of the patients' IFN activation status. No differences in ESSDAI or ESSPRI scores were observed after HCQ treatment, even after IFN stratification.

Conclusion Treatment for 24 weeks with HCQ significantly reduced type I IFN scores and ISG-expression compared to the placebo-treated group. HCQ reduced several laboratory parameters, but failed to improve clinical response. This suggests that in pSS, type I IFN is associated to some laboratory parameters abnormalities, but not related to the clinical response.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease with characteristic local dryness of the eyes and mouth. Additionally, systemic complications are frequently observed and include multi-organ involvement and fatigue. There is no cure for pSS and treatment options focus on symptom relieve, prevention of systemic damage and improvement of quality of life. Hydroxychloroquine (HCQ) is an immunomodulatory drug listed as first line treatment in The Sjogren's Syndrome Foundation Clinical Practice Guidelines, and is usually prescribed for arthralgias, myalgias and sometimes for fatigue [1]. Evidence regarding the efficacy of HCQ however is limited [2]. The JOQUER randomized placebo-controlled trial (Gottenberg et al. JAMA 2014, clinicaltrials.gov identifier NCT00632866) assessed the efficacy of a 24-week HCQ (400 mg/d) treatment in patients with pSS [3]. Disease activity (as assessed by EULAR Sjögren's syndrome disease activity index (ESSDAI)) and patients reported symptoms of dryness, pain or fatigue (assessed by the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI)) did not improve in HCQ-treated pSS patients compared to placebo treatment.

Systemic upregulation of the expression of Toll-like receptor (TLR)7, chronic activation of the type I interferon (IFN-I) pathway and upregulation of IFN-stimulated genes (ISGs) is present in a subgroup of pSS patients [4, 5]. Among the ISGs are several RNA- and DNA-sensing receptors [6]. Triggering of these sensors by their ligands will result in further stimulation of IFN-I production and induction of a pathogenic loop. HCQ blocks among others TLR7 activation, thereby preventing production of IFNs and induction of ISGs via this route [7]. Here we investigated the effect of HCQ treatment on expression levels of ISGs in whole blood RNA of pSS patients enrolled in the JOQUER trial. Patients were stratified in IFN-I positive or negative. Subsequently, the effect of HCQ treatment on disease activity and objective and subjective clinical parameters in IFN-I positive and negative pSS patients was studied.

MATERIALS AND METHODS

Study design

The study design of the JOQUER trial is described in Gottenberg et al. 2014 [3]. The protocol was approved by the review board of Hôpital Bichat (Paris, France) and the study conducted according to the principles of the Declaration of Helsinki. In short, between weeks 0 and 24 patients were randomly assigned to receive oral HCQ (400 mg/d) or an indistinguishable placebo. Between weeks 24 and 48 all patients received HCQ as

this drug might be more efficacious after long term usage and is already commonly prescribed in daily practice.

Blood collection and real-time quantitative PCR

Blood was collected at baseline and week 24 in PAXgene tubes for whole blood RNA analysis. Total RNA was isolated from PAXgene tubes and reverse-transcribed to cDNA. For calculation of relative expression, samples were normalized to expression of the household gene Abl. Relative expression values were determined from normalized CT values using $2^{\hat{}-\Delta\Delta}$CT method (User Bulletin, Applied Biosystems).

Calculation of the IFN score

The IFN score was defined by the relative expression of 5 indicator genes IFI44, IFI44L, IFIT1, IFIT3, MXA as previously described [8]. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. The IFN score was calculated per subject representing the sum of these standardized scores. Patients were divided in groups being positive or negative for systemic IFN-I activation, using a threshold of $mean_{HC} + 2 \times SD_{HC}$.

Questionnaires, laboratory parameters and objective measures of dryness

Acquirement of clinical data and questionnaires is described in Gottenberg et al. 2014 [3]. In short, the collected questionnaires included: ESSPRI and ESSDAI to study disease activity; 36-item Medical Outcomes Study Short-Form Health Survey 36 (SF-36) to study quality of life and Hospital Anxiety and Depression (HAD) scale to study psychological discomforted. Schirmer test score and unstimulated salivary flow were measured as objective measures of dryness and several laboratory parameters were included like serum IgG, IgA, and IgM levels, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) measured in each clinical center.

Statistics

Quantitative variables were described as mean (SD) when normally distributed and as median (25th-75th percentile) when non-normally distributed. Constrained longitudinal analysis was used to compare differences in change in values between the 2 treatment groups for quantitative variables as previously described [3]. Non-parametric analyses of paired data between baseline and the time point of interest was performed using the Wilcoxon signed-rank test.

RESULTS

HCQ reduced the IFN score and gene expression of the IFN-I-inducing pathway

Treatment for 24 weeks with HCQ significantly reduced systemic IFN-I scores in whole blood RNA compared to the placebo group (table 1). In the IFN-I-inducing pathway, systemic levels of TLR9 and MyD88, but not TLR7 were significantly reduced. There was a trend towards lower IRF7 levels, however this did not reach statistical significance. HCQ treatment reduced systemic expression of several ISGs-encoding for RNA- and DNA-sensing receptors (IFIH1, DDX58, EIF2AK2, IFI16 and ZBP1) compared to the placebo group.

HCQ treatment reduced laboratory parameters, but not disease activity

Differences in HCQ response between patients with or without systemic IFN activation were studied. HCQ reduced ESR, IgG and IgM levels similarly in patient with and without systemic IFN activation (table 2). There was no correlation between the change of the IFN score and the change of these secondary outcomes (data not shown). Neutrophil and lymphocyte counts were significantly reduced in the IFN-negative group after HCQ treatment. No difference was observed in the quality of life (assessed by the SF-36) or psychological discomfort (assessed the HAD scale). Additionally, HCQ treatment did not affect ocular or oral dryness measured by Schirmer's test or unstimulated saliva flow analysis neither in IFN-positive nor in IFN-negative pSS patients. Neither in the IFN-positive nor IFN-negative subgroup did HCQ treatment improve ESSDAI or ESSPRI scores (or its subdomains) (figure 1).

DISCUSSION

In this study we showed that HCQ treatment was able to reduce the IFN-I score and ISG-expression in whole blood of patients enrolled in the JOQUER trial. Stratification based upon the presence of absence of IFN activation showed that HCQ reduced ESR, IgG and IgM in patients with and without IFN activation. No effect of HCQ treatment on disease activity was observed, irrespective of the IFN activation status.

HCQ inhibits endosomal TLR signaling and previous studies showed that HCQ treatment resulted in a decreased production of IFNα in SLE [9, 10] and reduced IFN scores in antiphospholipid syndrome (APS) [11]. In this study we show that 24-week HCQ treatment reduced IFN-I scores in pSS and downregulated ISG-expression levels. In a

Table 1. IFN score and molecules in IFN-I pathway, per treated group

Parameter	Placebo		HCQ		Difference in change from baseline score (week 0), adjusted for baseline score, mean (95% CI)	P-value
	No	Mean (SD)	No	Mean (SD)		
Type I IFN score						
0	40	9.23 (8.22)	37	9.44 (9.20)		
24	40	8.87 (9.66)	36	3.69 (8.64)	-5.24 (-7.76 to -2.72)	<0.0001
0, median (25th -75th percentile)[a]	40	11.05 (1.35 to 17.24)	37	12.26 (0.45 to 16.71)		
24, median (25th -75th percentile)[a]	40	12.54 (-0.41 to 16.83)	36	2.63 (-4.75 to 16.71)		
TLR7						
0	40	0.51 (0.21)	37	0.66 (0.34)		
24	40	0.39 (0.20)	36	0.39 (0.16)	-0.05 (-0.13 to 0.03)	0.188
TLR9						
0	40	0.57 (0.21)	37	0.59 (0.37)		
24	40	0.38 (0.18)	36	0.29 (0.21)	-0.09 (-0.16 to -0.03)	0.004
MyD88						
0	40	24.34 (8.27)	37	21.80 (8.90)		
24	40	22.37 (11.48)	36	16.91 (5.43)	-3.99 (-7.64 to -0.35)	0.032
IFIH1						
0	40	1.36 (0.84)	37	1.47 (1.15)		
24	40	1.41 (1.23)	36	0.9 (0.60)	-0.53 (-0.92 to -0.13)	0.010

Table 1. Continued

Parameter	Placebo		HCQ		Difference in change from baseline score (week 0), adjusted for baseline score, mean (95% CI)	P-value
	No	Mean (SD)	No	Mean (SD)		
DDX58						
0	40	20.13 (11.25)	37	18.89 (14.59)		
24	40	22.63 (17.18)	36	14.36 (9.90)	-7.34 (-12.43 to -2.25)	0.005
IFI16						
0	40	12.70 (5.82)	37	12.36 (6.77)		
24	40	13.87 (9.38)	36	8.76 (4.62)	-4.87 (-7.80 to -1.94)	0.001
ZBP1						
0	40	16.33 (9.29)	37	15.95 (11.87)		
24	40	14.95 (10.97)	36	9.07 (6.47)	-5.70 (-8.86 to -2.55)	0.001
EIF2AK2						
0	40	7.27 (5.29)	37	7.61 (6.66)		
24	40	11.55 (10.01)	36	6.12 (4.95)	-5.63 (-8.93 to -2.34)	0.001
IRF7						
0	38	0.40 (0.41)	36	0.50 (0.73)		
24	40	0.50 (0.74)	36	0.28 (0.28)	-0.23 (-0.50 to 0.04)	0.090
0, median (25th-75th percentile)[a]	38	0.29 (0.09 to 0.54)	36	0.26 (0.10 to 0.54)		
24, median (25th -75th percentile)[a]	40	0.28 (0.13 to 0.60)	36	0.18 (0.10 to 0.35)		

a. Where the standard deviations exceeded the mean values, medians (25th – 75th percentile) are reported as well

Table 2. Patient-related outcomes and laboratory parameters of HCQ-treated pSS patients stratified on systemic IFN activation

Parameter, by week	IFN-negative pSS			IFN-positive pSS		
	No	Median (IQR)	P-value	No	Median (IQR)	P-value
SF-36, physical health component						
0	10	46.9 (37.8 - 59.4)		15	71.5 (44.2 - 85.7)	
24	14	42.5 (34.3 - 69.1)	0.919	10	62.3 (40.1 - 84.0)	0.575
SF-36, mental health component						
0	17	56.6 (43.4 - 65.4)		14	71.7 (46.5 - 78.4)	
24	16	64.4 (44.5 - 71.0)	0.289	11	75.8 (54.4 - 89.8)	0.173
HAD-anxiety						
0	16	10.0 (7.0 - 11.0)		15	9.0 (5.0 - 12.5)	
24	17	7.0 (5.0 - 11.0)	0.053	16	9.0 (5.0 - 12.0)	0.219
HAD-depression						
0	17	6.0 (4.0 - 9.5)		15	5.0 (1.5 - 8.0)	
24	17	6.0 (2.5 - 9.8)	0.932	15	6.0 (1.0 - 8.5)	0.368
Schirmer's test, mm						
0	17	9.5 (0.0 - 16.25)		15	8.5 (5.0 - 15.75)	
24	15	6.5 (4.25 - 15.25	0.878	14	10.5 (4.25 - 20.0)	0.932
Unstimulated Salivary flow, mL/min						
0	17	0.7 (0.3 - 2.3)		15	0.5 (0.3 - 0.6)	
24	15	1.3 (0.5 - 1.7)	0.972	14	0.4 (0.3 - 0.7)	0.766
ESR, mm						
0	18	8.5 (4.25 - 16.5)		19	27.0 (11.5 - 99.7)	
24	16	6.0 (4.0 - 12.0)	0.035	16	14.5 (9.25 - 27.0)	0.004
C-reactive protein, mg/L						
0	18	4.0 (1.1 - 6.0)		18	4.0 (0.85 - 10.7)	
24	17	4.0 (1.0 - 5.0)	0.310	17	4.0 (3.0 - 6.0)	0.600

Table 2. Continued

Parameter, by week	IFN-negative pSS			IFN-positive pSS		
	No	Median (IQR)	P-value	No	Median (IQR)	P-value
IgG, g/L						
0	18	11.3 (9.4 - 12.9)		16	16.8 (13.5 - 21.0)	
24	18	9.9 (8.6 - 12.9)	0.035	17	16.0 (12.7 - 19.3)	0.023
IgA, g/L						
0	18	2.2 (1.5 - 3.4)		17	2.9 (2.2 - 3.8)	
24	18	2.1 (1.4 - 3.3)	0.924	18	2.8 (2.1 -3.8)	0.316
IgM, g/L						
0	18	0.74 (0.58 - 1.43)		17	1.42 (1.05 - 1.43)	
24	18	0.74 (0.54 - 1.20)	0.017	18	1.0 (0.87 - 2.0)	0.010
Neutrophils (x10^9/l)						
0	18	3.2 (2.4 - 4.2)		19	2.66 (2.02 - 5.08)	
24	18	2.8 (2.0 - 3.5)	0.028	18	2.3 (1.73 - 3.78)	0.089
Lymphocytes (x10^9/l)						
0	18	1.6 (1.3 - 1.9)		19	1.6 (1.2 - 2.2)	
24	18	1.4 (1.2 - 1.8)	0.021	18	1.5 (1.3 - 1.7)	0.811

Abbreviations: pSS, primary Sjögren's syndrome; SF-36, 36-item Medical Outcomes Study Short-Form Health Survey; HAD, Hospital Anxiety and Depression scale; ESR, erythrocyte sedimentation rate; Ig, immunoglobulin.

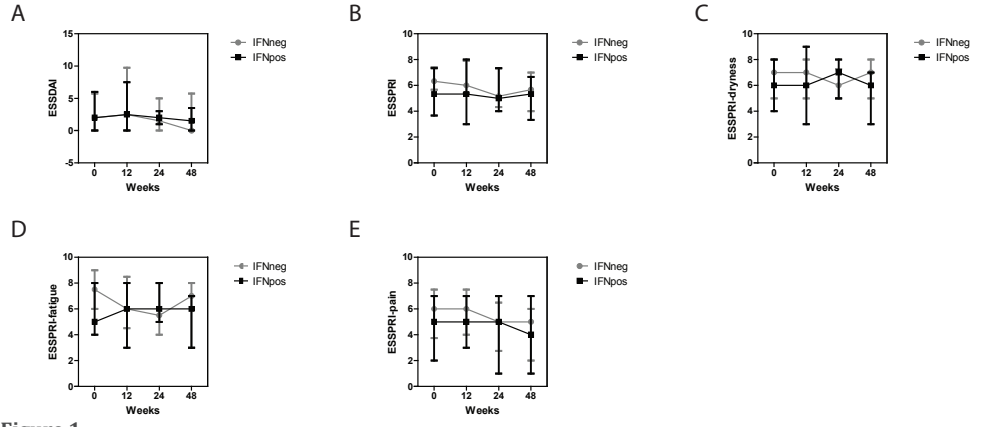

Figure 1.
Disease activity of HCQ-treated pSS patients with (n=19) or without (n=18) IFN activation at baseline in the JOQUER trial. Median values of the EULAR Sjögren's Syndrome EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) (A) Patients Reported Index (ESSPRI) (B), and its subdomains dryness (C), fatigue (D) and pain (E) are reported at week 0, 12, 24 and 48.

cross-sectional study with pSS patients using HCQ for longer periods we previously observed a trend towards reduced IFN-I scores in the HCQ-users group compared to non-HCQ users, but this did not reach statistical significance [8]. So short term HCQ treatment reduces IFN-I scores, but whether this effect remains after long-term treatment is unclear. An explanation for the variable effect of long-term HCQ treatment could be the possible contribution of other pathways than the TLR route that induce IFN expression. Recent data indicate a contribution of RNA- and DNA-sensing pathways to IFN-I production, which are not blocked by HCQ [6]. Therefore, a heterogeneity in induction routes of IFN activation in IFN-positive patients might cause the variable effect of HCQ in this group.

Here the data show that although HCQ decreased the IFN-I signature, there was no effect on disease activity in this pSS cohort. Even in patients with a positive IFN signature the only effect of HCQ treatment was a reduction of several laboratory parameters. This suggests that some laboratory parameters in pSS might be dependent of IFN-I, but are not related to the clinical response. Interestingly, in a recent positive phase 2 study of baricitinib, an inhibitor of JAK1 and JAK2, in SLE a reduction in the IFN-I signature was observed. But this reduction was not related to the clinical response [12]. This suggests that in SLE the clinical response to baricitinib was not linked to the decrease of the IFN-I signature. Likewise, it was recently announced that the first phase 3 study in SLE with the anti-IFNAR1 antibody anifrolumab failed to achieve its primary end-point. Conversely, in the positive phase 2 study of ustekinumab in SLE there was a positive correlation between the decrease of type II IFN and the clinical response [13].

HCQ is prescribed to patients with mild disease, rather than to patients with severe systemic disease manifestations. This study includes mainly patients with low systemic involvement, reflected in low ESSDAI scores. This is representative for the patient group HCQ is prescribed to in clinical practice. A downside of inclusion of patients with low systemic disease is that it is hard to see improvement of clinical parameters such as measures for disease activity, because they are not highly elevated at baseline. Additionally, it can take up to six months before patients notice the effect of HCQ, hence the treatment time of 24 weeks might be too short.

HCQ is sometimes prescribed to treat fatigue in pSS patients. Pro-inflammatory mechanisms and IFN-I may be linked to fatigue as seen in chronic infections or cancer [14, 15]. However, it is much less clear in autoimmune diseases, thus in pSS, IFN-positive patients are not more fatigued than IFN-negative patients [8]. In the present study, the decrease of the IFN-I signature after HCQ treatment was not linked to a decrease of fatigue assessed by the ESSPRI. Evidence for the use of HCQ to treat fatigue is weak and mainly based on uncontrolled studies. A recent systematic review on the use of HCQ in pSS reported no effect of HCQ on fatigue [2].

CONCLUSION

HCQ treatment reduced the IFN-I score and lowered expression levels of several ISGs in whole blood RNA of patients enrolled in the JOQUER trial. Although HCQ treatment reduced several laboratory parameters, there were no effects on disease activity irrespective of IFN activation status of the patient. This suggests that in pSS IFN-I is associated with some laboratory parameters abnormalities, but not to the clinical response.

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Chapter 6

Interferon activation in primary Sjögren's syndrome: recent insights and future perspective as novel treatment target

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ABSTRACT

Introduction: Primary Sjögren's syndrome (pSS) is one of the most common systemic autoimmune diseases. At the moment, there is no cure for this disease and its etiopathology is complex. Interferons (IFNs) play an important role in the pathogenesis of this disease and are a potential treatment target.

Areas covered: Here we discuss the role of IFNs in pSS pathogenesis, complications encountered upon studying IFN-induced gene expression, and comment on the current knowledge on easy clinical applicable 'IFN signatures'. The current treatment options targeting IFNs in pSS are summarized and the perspective of potential new strategies discussed.

Expert commentary: The authors provide their perspective on the role of IFNs in pSS and how this knowledge could be used to improve pSS diagnosis, provide new treatment targets, to monitor clinical trials and to stratify pSS patients in order to move towards precision medicine.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is after rheumatoid arthritis (RA) the most common systemic autoimmune disease and affecting primarily post-menopausal women. pSS is characterized by focal lymphocytic infiltrations in salivary and lachrymal glands. Decreased secretory function of the glands leads to symptoms of dry eyes (keratoconjunctivitis sicca/xerophthalmia) and mouth (xerostomia). In approximately one-third of the patients the symptoms of dryness are accompanied by systemic complications including multi-organ involvement and severe fatigue [1-3]. The etiopathology of the disease is still largely unknown and current treatment options are mainly targeting the symptomatic dryness. Interferon (IFN) activation is found in a large subset of pSS patients. These patients show upregulation of a set of IFN-stimulated genes (ISGs) also called the 'IFN signature'. These upregulated genes influence multiple biological processes likely to play a role in the pathogenesis pSS. In this article, we summarize recent data on the role of IFNs in pSS pathogenesis and review the latest treatment strategies targeting IFNs.

ROLE OF IFN IN THE PATHOGENESES OF PSS

Interferons

IFN was first described in 1957 as a factor capable to interfere with the proliferation of viruses [4]. IFNs are produced upon stimulation of the pattern-recognition receptors (PRRs) expressed by multiple immune and non-immune cells followed by the activation of innate and adaptive immunity. Three different types of IFNs have been described: type I, type II and type III. The type I IFNs are the largest group and can be subdivided in 5 classes, including 12 IFNα proteins, as well as IFNβ, IFNε, IFNκ, and IFNω [5]. All these proteins are encoded by genes on chromosome 9. The plasmacytoid dendritic cell (pDC), can synthesize up to 109 IFN molecules within 24 h after stimulation and thereby is considered the most potent producer of type I IFN [6]. Type I IFNs bind to the receptor for type I IFN (IFNAR) leading to upregulation of up to two thousand ISGs. Type I IFN activity is commonly measured in patients with systemic autoimmune diseases using these ISGs to determine if patients have an 'IFN signature' or to calculate an IFN score. Type II IFN (IFNγ), and the more recently described type III IFN (IFNλ1, IFNλ2 and IFNλ3; also known as IL-29, IL-28A and IL-28B), signal via different receptors and have a low degree of homology with type I IFN. However, all these IFN types signal downstream of the different IFN binding receptors via the JAK-STAT pathway and induce largely overlapping genes [7-9]. IFNγ is located on chromosome 12 and type III IFN on chromosome 19.

Type I IFN is that all there is?

Several observations support a role for activation of the IFN system in the pathogenesis of pSS. Treatment with IFNs, for example to treat viral hepatitis, induced pSS like symptoms in some cases [10, 11]. Furthermore, mouse models for pSS demonstrated that activation of the IFN system leads to accelerated salivary gland hypofunction, which could be improved by blocking the IFNAR [12, 13]. In human pSS patients, transcriptional analysis showed upregulation of type I IFN-induced gene expression locally in the salivary glands as well as systemically in peripheral blood mononuclear cells (PBMCs), isolated monocytes and B cells [14-18]. Furthermore, the 'IFN type I signature' was associated with higher disease activity and an increased presence of autoantibodies [19].

It is difficult to distinguish type I from type II IFN-induced gene expression due to largely overlapping expression patterns. Multiple methods to distinguish the two have been described in literature. Hall et al. stimulated a human submandibular gland (HSG) epithelial cell line with IFNα and IFNγ [20]. Using microarray, genes exclusively upregulated by IFNα and IFNγ at specific time points were selected. GBP1 and GBP2 were identified as specific probes for IFN type II expression by salivary gland epithelial cells. It was more difficult to identify probes specific for type I IFN stimulation as most transcripts were induced by both IFNα and IFNγ. IFIT3 and MDA5 were selected as probes for type I IFN activity as these transcripts were induced more strongly by IFNα then IFNγ at early time points. Subsequently, Hall et al. demonstrated that 58% of the pSS patients showed IFN activation in the salivary gland using these probes. Three distinct IFN patterns were distinguished: type I-predominant (29%), type II-predominant (35.5%), and mixed type I and II IFN (35.5%). Clinically there were no differences between these groups, except for higher focus scores in the type II-predominant group [21].

Also systemically efforts have been made to distinguish type I IFN from type II-induced gene expression. Chiche et al. observed three strongly upregulated IFN-annotated modules (M1.2, M3.4 and M5.12) in peripheral blood transcriptomic data from systemic lupus erythematosus (SLE). Each of these modules has a distinct activation threshold [22]. The M1.2 transcriptional module was more induced by type I IFN than type II IFN, while both M1.2 and M3.4 transcripts were upregulated by IFNβ. M3.4 and M5.12 were similarly induced by type I and type II IFNs. Additionally, they showed that M1.2 was always induced first, next M3.4 appeared and finally M5.12. M5.12 was never induced without M1.2 or M3.4 expression. Using this approach in 2 large pSS cohorts, a similar pattern of expression was observed [23]. Over 50% of the patients were positive for M1.2 indicated systemic upregulation of type I IFNs. Less than 33% showed M5.12 induced expression, a module induced by both type I and type II. As this module was only induced in the presence of M1.2, no patients exclusively expressed type II IFN systemically. This is consistent with literature showing that systemically type

I IFN is predominantly upregulated over type II IFN [24]. These studies indicate that the type I and II IFN activation pattern in blood is different compared to the salivary glands. These studies also demonstrated that the induced ISGs are dependent on the type of IFN, concentration and duration of the stimulation. An interesting question which remains unanswered is how the local salivary gland and systemic blood IFN activation patterns correlate.

Recently, another IFN scoring system was developed for SLE. Genes were selected from M1.2, M3.4 and M5.12, with addition of IFI27 and IFI6 and subjected to factor analysis [25]. In total 7 factors were present, however a simpler solution with 2 factors still explained 84% of the variance. These factors were called IFN score A and B. B cells were stimulated with IFNα and IFNγ for six hours and ISG expression induced by these two stimuli was analyzed. Although many ISGs showed to be responsive to both IFNα and IFNγ, most genes in IFN score A and B were mainly upregulated in response to IFNα. Interestingly, most IFNγ responsive genes were also responsive to IFNα, albeit this at a lower level and dependent on the time of stimulation. Most IFNα induced ISGs were not induced by IFNγ or only a minimal induction was observed upon IFNγ stimulation. This again indicates the difficulty to find genes specifically induced by IFNγ. Additionally, it also shows that ISG expression is dependent on the cell type as observed by the differences between HSG and B cells after IFN stimulation. As the genes in both scores are particularly IFNα responsive, the clustering of ISGs between the factors was possibly due to non-IFN pathways. Furthermore, they showed differences in upregulation of IFN score A and B comparing SLE, RA and healthy controls indicating upregulation of different ISGs depending on the disease possibly reflecting differences in pathogenic mechanisms. This observation underlines that to assess an IFN signature in a particular disease multiple ISGs should be measured. In a follow-up paper this group describes that this IFN scoring method can be used to predict progress toward autoimmune-related connective tissue disease in an at-risk population [26].

Data on type III IFNs in pSS are very limited. Recently, expression of type III IFN was described in salivary gland tissue of pSS patients [27]. Interestingly, the receptor IFNλR1 was particularly strongly expressed by pDCs. In the sera of pSS patients only IFNλ1 was detected, however this could also be due to low sensitivity of available detection methods. Type III IFNs have similar anti-viral and immunoregulatory properties as type I IFNs, although its effects might be more localized. The role of type III IFNs in pSS pathogenesis remains to be further elucidated.

Triggers for type I IFN production

pDCs have an important role in antiviral immunity, however they are also frequently indicated as culprits in IFN positive autoimmune diseases like pSS. This rare blood

cell population (0.2-0.8% of the peripheral blood cells) expresses endosomal Toll-like receptor 7 (sensing single stranded RNA), TLR9 (sensing dsDNA) and multiple cytosolic receptors to sense cytosolic RNA (RIG-like receptors (RLRs)) or DNA (DNA sensing receptors (DSRs)). Upon triggering, these three different receptor pathways induce type I IFN expression. Evidence in several autoimmune diseases including pSS supports a role for the TLR route in the production of type I IFNs [28-34]. Necrotic or apoptotic cells release nucleic acids and due to increased damage and/or reduction in removal of these nucleic acids, self-RNA and -DNA accumulates in the tissues. In pSS, autoantibodies targeting these nucleic acids are present. Båve et al. showed that serum from pSS patients combined with apoptotic cells was able to induce IFNα production by pDC [28]. Lövgren et al. more specifically demonstrated that immune complexes (ICs) containing SLE and pSS autoantigen-associated U1 small nuclear RNA (U1 snRNA) and hY1RNA can be taken up via the FCγ receptor IIa and induce type I IFN expression. Mavragani et al. described a different source of endogenous nucleic acids [33]. Long interspersed nuclear element-1 (LINE-1; L1), is a family of retroelements coming from a viral origin that long ago integrated into our mammalian genome. Increased L1 mRNA expression levels were detected in salivary gland tissue from pSS patients compared to tissue from healthy or sicca controls and this correlated with upregulation of type I IFNs. Transfection of pDCs and monocytes with a plasmid carrying L1 induced IFNα production and ISG expression. Subsequent addition of a blocker for TLR7/8 or cytosolic nucleic acid sensors prevented IFN production and ISG upregulation indicating that L1-elements can induce IFN production via TLR-dependent and -independent mechanisms. Recently, it was shown more specifically that RLRs, RIG-I and MDA5, are involved in L1 sensing [35].

A different source of nucleic acids are neutrophil extracellular traps (NETs). Extracellular structures composed of decondensed chromatin and antimicrobial molecules, are released in a process called NETosis. Aberrancies in NETosis are described in multiple autoimmune diseases, although they are not described in detail yet in pSS [36-40]. But their frequent description in SLE pathogenesis, which partly resembles pSS, suggests a potential role for NETosis in pSS. A different source of TLR ligands could be the content of exosomes. Exosomes are small membrane-bound vesicles (30–100 nm) that are secreted by different types of cells [41]. They are formed after inwards budding of endosomal membranes. Thereby they produce multivesicular bodies, which can be released after fusion with the plasma membrane. These exosomes can contain proteins, mRNAs and miRNAs. Exosomes isolated from SLE patients were able to induce production of type I IFN and proinflammatory cytokines in a TLR-dependent pathway [42]. More recently, Salvi et al. showed IFNα production after stimulation of cells with exosomes isolated from SLE plasma in a TLR7-dependent way [43]. However, as the content of these exosomes is unclear, it is difficult to compare studies. Salvi et al. showed

more specifically that microRNAs isolated from exosomes were able to induce IFNα production in a TLR7-dependent way, but a contribution of the RLR and DSR pathway cannot be ruled out.

Cytosolic DSR are not well studied in pSS. However, as DNA sensing is an important pathway leading to production of IFNs and this pathway is already implicated in SLE pathogenesis, these molecules are of great interest. There are many different DSR, which signal via a variety of downstream molecules [44]. In particular signaling via de STING-IRF3 pathway is important to induce type I IFN production. Mouse studies have demonstrated that treatment with a STING agonist, resulted in elevated levels of type I IFNs and proinflammatory cytokines systemically and locally in the salivary glands. Furthermore, these mice showed signs of salivary gland inflammation and hypofunction [45]. This indicates that activation of STING can initiate pSS-like disease in mice. Furthermore, IFI16, a DNA sensor that is known to bind STING, is upregulated in pSS [46]. Antibodies that target IFI16 can be found in pSS patients and are associated with markers for severe disease [47]. We found elevated mRNA expression of several DSR in monocytes of pSS and childhood-onset SLE patients with a positive type I IFN score (IFNpos) [48]. Therefore the role of DSRs as contributors to IFN activation in pSS should be considered.

IFNs shape the immune response in pSS

As most cell types express the IFNAR, these cytokines can have a large array of different effects. An important role for type I IFN is the induction of a priming state, which affects the production and regulation of proinflammatory cytokines and other mediators [49]. Monocytes differentiate into dendritic cells (DCs) upon triggering by IFN type I. Furthermore, IFNs stimulate immature DCs to express chemokines and costimulatory molecules, facilitating homing to secondary lymphoid organs where these cells can activate adaptive immunity. Macrophages increase phagocytic properties upon exposure to IFNs stimulating adaptive immunity [50].

B-cell activating factor (BAFF) (also known as BLYS) has been linked to pSS pathogenesis. BAFF is upregulated in response to type I and II IFNs in monocytes and promotes the survival of B cells. In addition to monocytes, also macrophages, dendritic cells and salivary gland epithelial cells (SGECs) can express BAFF in response to IFNs [51-53]. Transgenic mice overproducing BAFF (BAFF-Tg) show enhanced B-cell proliferation, increased spontaneous germinal center (GC) reactions, enhanced autoantibody production and an increased number of ICs [54]. Furthermore, salivary gland destruction and a decreased saliva production, mimicking pSS pathophysiology, is seen in this mouse model [55]. In pSS, besides innate cells also B, T and epithelial cells were shown to be able to produce BAFF [56, 57] and BAFF levels were increased

in salivary gland tissue, saliva and serum [58, 59]. IFNs can affect B cells indirectly via BAFF, or directly by promoting differentiation into plasmablasts and stimulation of class switching [49, 60]. Activation of autoreactive B cells in pSS patients leads to the production of multiple autoantibodies like antinuclear antibodies (ANA), rheumatoid factor (RF), anti-SSA and anti-SSB [61, 62]. Increased B cell numbers are also observed in salivary glands of pSS patients. There these B cells sometimes form organized structures resembling GCs, so-called GC-like structures. BAFF sustains these GC-like structures [63]. The presence of these GC-like structures is associated with lymphoma, which develops in 5-10% of the patients and is one of the most severe complications in pSS [64-68]. As there is a link between IFNs and B cells, Nezos et al. studied if IFNs could predict lymphoma development. They showed that a high IFNγ, low IFNα mRNA ratio in salivary gland tissue in pSS patients was associated with lymphomagenesis [24], but overall IFN type I or type II scores could not discriminate between non-lymphoma and lymphoma patients.

IFNγ is found in increased levels in pSS patients implicating T cells, NK cells and NKT cells in the pathogenesis of pSS. T cells present in the salivary gland infiltrates in pSS are likely to be a source of IFNγ. These T cells can be activated by pDC directly or indirectly after IFN-priming of DCs. Additionally, T cells can be activated by SGECs. These cells express class I and II MHC molecules, costimulatory molecules, TLRs and adhesion molecules and can act as non-professional antigen presenting cells (APCs) [53]. In the salivary glands, CD4+ T cells predominate over CD8+ T cells in pSS. Of the CD4+ T cells both Th1 and Th17 cells are implicated in pSS pathogeneses and can produce IFNγ. IFNγ was shown to upregulate CD80 (B7.1) and CD86 (B7.2) molecules on local APC and SGECs [69] thereby possibly contributing to increased local T-cell activation. In addition, IFNγ induces the production of IP10 and Mig, which are T-cell-attracting cytokines, inducing a positive feedback loop that sustains inflammation [70]. IFNγ additionally contributes to SGEC cell death by inducing upregulation of several pro-apoptotic molecules [71, 72]. In addition to IFNγ, Th17 cells produce their signature cytokine IL-17, which is found upregulated in salivary glands of pSS patients and was shown to induce salivary gland dysfunction in mice [73-75]. Both Th17 and Th1 cells support further differentiation of B cells in to autoreactive plasma and memory cells [76]. Follicular helper T cells (Tfh), which can also produce IFNγ and IL-17, have recently shown to be increased in pSS [77]. Furthermore, Tfh cells produce several B-cell survival signals including BAFF and IL-21. IL-21 is also a potent activator for Th17 cells, again stimulating a feed-forward loop. SGECs of pSS patients were shown to be able to differentiate naïve T cell to a Tfh phenotype and are thereby likely additional contributors to pSS pathogenesis [78].

The observed IFNγ activity might also indicate the presence of NK cells, however the literature on NK cells in pSS is limited. Systemically, Izumi et al. indicated an overall

reduction in NK cell number and function [79]. A different study by Rusakiewicz et al. found systemically no difference in NK numbers, but did find an increase in CD56+ NK cells [80]. In the salivary glands only low numbers of infiltrating CD56+ NK were demonstrated [81]. On NK cells several activating receptors are present. Among these receptors are NKp30 and NKp46. Polymorphisms are described in NCR3/NKp30 which regulate cross talk between NK cells, DCs and IFNγ production. Rusakiewicz et al. describe increased circulating levels of NCR3/NKp30. Additionally, they describe an increase number NKp46+ cells in the minor salivary glands, while NKp30 expression was low in NK cells residing in the salivary gland. Izumi et al. found systemically increased levels of NKp46+ and not in NKp30+ cells. The low number of studies, different markers for NK cells and locations (blood or salivary gland) make it difficult make conclusions about the role of NK cells in pSS. Further studies are needed to elucidate the exact role of NK cells in pSS.

Lastly, IFNγ producing NKT cells were found systemically to be decreased in pSS [82]. They are thought to play a role in regulating entry of autoreactive B cells into GCs, where a decreased number of NKT cells is associated with expansion of autoreactive B cells. In SLE, NKT cells are additionally thought to play a role in regulating Th1/Th2 balance and were shown to affect IL-17 production and DC maturation [83]. However not much is known about these cells in pSS.

Regulatory mechanisms affected by IFNs

Chronic activation of the immune system, response to self-peptides and failure of feedback mechanisms are implicated in pSS. Regulatory T cells (Tregs) play an important role in maintaining homeostasis by regulating proliferation and controlling effector functions of T lymphocytes and other immune cells. Literature on the numbers of Tregs in pSS is showing conflicting results. However, the function of Tregs appears to be normal [84-88]. In the salivary glands the number of Tregs has been found to correlate with the focus score [84, 87]. IFNs can affect Tregs via the induction of indoleamine 2,3-dioxygenase (IDO). IDO is an enzyme which catabolizes the important metabolic conversion of tryptophan (Trp) to kynurenines. IDO is encoded by two homologous genes IDO1 and IDO2. IDO1 is upregulated by IFNs and catalyzes the conversion of Trp into kynurenine (Kyn), which is the first and rate-limiting step. Subsequently, Kyn is further degraded into kynurenic acid (KA), 3-hydroxy-anthranilic acid (HAA), quinolinic acid (QA), niacin, and other catabolites. Trp depletion is a signal to suppress immunogenic functions and induce tolerance by shifting towards Treg activation. Additionally, several catabolites of the Kyn pathway are suppressing T-cell activation and activate Tregs. IDO1 gene and protein levels have been shown to be elevated in serum, pDCs, monocytes and T cells of pSS patients [89-92]. We showed in particular upregulation of IDO1 mRNA

levels in CD14+ monocytes and elevated Kyn/Trp ratios in serum of IFNpos pSS patients compared to patients without an IFN signature (IFNneg) [93]. Catabolites in the Kyn/Trp pathway are elevated in the blood of pSS patients [94]. Literature on whether this also correlates with increased numbers of Tregs is conflicting [90, 93]. Possibly the upregulation of IDO and activation of the Kyn/Trp pathway is an attempt of the immune system to restore the balance and induce tolerance in pSS. Future studies aiming to proof this should be initiated as the IDO system provides an opportunity to interfere in aberrancies in the immune system.

A different regulatory pathway to prevent overactivation of the immune system is the regulation by costimulatory molecules. Costimulatory molecules are required to provide the 'second signal' to activate T cells. These costimulatory molecules are important regulators as they can have stimulatory and inhibitory effects on T cells. IFNs can affect these costimulatory molecules. Engagement of the T-cell receptor (TCR) induces CD80 and CD86 expression on APCs via CD28 on T cells and protects T cells from apoptosis and anergy. TCR engagement additionally induces the phosphorylation of cytotoxic T lymphocyte antigen 4 (CTLA-4), which will compete with CD28 for binding to CD80 or CD86. As CTLA-4 has a higher affinity than CD28 a block of this costimulatory signal will prevent further stimulation. In addition, CTLA-4 mediated mechanisms induce IDO and upregulation of the Kyn/Trp pathway in costimulatory molecule-expressing APCs [95]. Several haplotypes in CTLA-4 are described and associated with susceptibility to pSS and other autoimmune diseases [89, 96-98], although this was questioned by others [99].

PD-1 is another costimulatory receptor, which inhibits T-cell receptor signaling. PD-1 on T cells interacts with its ligands PD-L1 and PD-L2 [100, 101]. Both type I and II IFN have been shown to affect the expression of PD-1 and its two ligands. In mouse models for pSS blockade of PD-L1 was shown to accelerate pSS development [102, 103]. This suggests that stimulation of this pathway might also be an interesting target for the treatment of pSS. Furthermore, in SLE anti-PD1 antibodies have been observed in association with disease activity [104]. This is of particular importance as antibodies blocking CTLA-4 and PD-1 are used to promote T-cell activation as a treatment for various cancers. Interestingly, in these trials among the immune-related adverse event (irAE) reported are the development of autoimmunity and sicca symptoms [105, 106]. This suggests that inhibition of costimulatory molecules might drive a Sjögren syndrome-like autoimmune response towards the salivary glands. Therefore, attention should be paid for using checkpoint inhibitors in patients with pSS or other autoimmune diseases.

IFNS AND THE IFN PATHWAY AS TREATMENT TARGET AND THE IFN SIGNATURE FOR STRATIFICATION

Biologics in pSS and the IFN signature as biomarker

Systemic treatment for pSS is prescribed for high disease activity or involvement of specific organ systems. Table 1 provides an overview of therapies with treatment potential in pSS and indicates the use of activation of the IFN pathway for stratification. As IFNs play an important role in pSS pathogenesis, targeting IFNs using monoclonal antibodies (mAb) could be a potential strategy to treat pSS. IFN targeting mAb have been developed but are not tested yet in pSS patients. Data however is available from trials in SLE patients. Sifalimumab and rontalizumab are mAb and IFNα-kinoid is a therapeutic vaccine, all targeting IFNα. Rontalizumab improved disease activity and reduced flares in SLE patients with low type I IFN signatures, but not in patients with high IFN signatures [107, 108]. Sifalimumab showed some improvement of disease activity in SLE, however, treatment did not completely normalize the type I IFN signature [109, 110]. IFNα-kinoid induced polyclonal anti-IFNα activity and led to a decrease of IFN scores in SLE patients [111]. These therapies do not block other type I IFNs, which could explain the lack of response in some patients. Anifrolumab is a mAb targeting the IFNAR and thus blocks the activity of all type I IFN subtypes and showed in SLE encouraging results. Interestingly, it was particularly effective in patients with high IFN signature scores [112]. Recently, a mechanistic study showed that anifrolumab reduced surface expression of the IFNAR and was able to block upregulation of ISGs [113]. Furthermore, this drug inhibited proinflammatory cytokine induction and reduced upregulation of costimulatory molecules on pDCs. Lastly, in a co-culture system of pDCs and B cells, blockade of the IFNAR suppressed plasma cell differentiation. These promising results indicate that anifrolumab could be beneficial also in pSS. IFNγ targeting Ab also have not been studied in pSS. Although showing positive effects in RA, in SLE they were not able to reduce disease activity [114, 115].

Therapies like rituximab that target CD20 on B cells have been tested in pSS. Although earlier studies showed effective B cell depletion and improvement of symptoms of dryness and autoantibody levels [116-120], two large recently performed randomized controlled trials failed to confirm these findings [121, 122]. Perhaps stratification of pSS patients on IFN activation could help to identify subgroups who could benefit from treatment with rituximab, like in RA where IFN scores have shown to be predictive for rituximab responders [123-125]. Epratuzumab, which targets CD22 on B cells was found to enhance IL-10 production and blocks Blimp1-dependent B-cell differentiation after TLR7 stimulation in SLE [126]. In pSS, this treatment showed limited improvement

Table 1. Overview of biologics and small molecule inhibitors with potential for pSS treatment and use of IFN pathway for stratification

Name	Type	Target	Clinical trial in pSS*	Clinical trial in SLE/RA*	Genes measured	Effect upon stratification on IFN pathway activation	Reference
Sifalimumab/ MEDI-545	mAb	IFNα	no	SLE, phase 2b	*IFI27*, *IFI44*, *IFI44L*, and *RSAD2*	Particularly beneficial in IFNhigh group, no additional effect of drug in IFNlow group in compared to standard therapy in SLE	
Rontalizumab/ rhuMAb interferon-α	mAb	IFNα	no	SLE, phase 2	*HERC5*, *EPSTI* and *CMPK2*	Improved disease activity and reduced flares only in IFNlow group in SLE	[107]
IFNα-kinoid/ IFN-K	therapeutic vaccine	IFNα	no	SLE, phase 2b	*MX1*, *LY6E*, *IFI27*, *OAS1*, *IFIT1*, *IFI6*, *IFI44L*, *IFIT3*, *OAS2*, *ISG15*, *LAMP3*, *RSAD2*, *IFI44*, *OAS3*, *USP18*, *RTP4*, *HERC5*, *SIGLEC1*, *EPSTI1* and *SPATS2L*	Treatment was effective in decreasing the expression of transcripts associated with B cell activation in peripheral blood in SLE patients with an IFN signature, not in patients without a signature	[111]
Anifrolumab	mAb	IFNAR	no	SLE, phase 2b; RA phase 2	*IFI27*, *IFI44*, *IFI44L*, and *RSAD2*	Particularly beneficial in IFNhigh group, no additional effect of drug in IFNlow group in compared to standard therapy in SLE	[112]
Rituximab	mAb	CD20	Yes, phase 2	SLE, phase 3; RA, phase 3	L*Y6E*, *HERC5*, *IFI44L*, *ISG15*, *MxA*, *MxB*, *EPSTI1* and *RSAD2*	Low expression of ISG predicted better response in RA	[123-125]
Epratuzumab	mAb	CD22	no	SLE, phase 2	-	-	
Belimumab/ Benlysta	mAb	BAFF	Yes, phase 2	SLE, approved; RA phase 2	-	High type I IFN scores at baseline were associated with better response in pSS	[131, 132]
VAY736	mAb	BAFF receptor	Yes, phase 2	RA, phase 2	-	-	
Abatacept/ Orencia	fusion molecule with Ig	CTLA-4	Yes, phase 3	SLE, phase 3; RA, phase 3	-	-	

Table 1. Continued

Name	Type	Target	Clinical trial in pSS*	Clinical trial in SLE/RA*	Genes measured	Effect upon stratification on IFN pathway activation	Reference
Hydroxychloroquine/ Plaquenil	Blocking SMI	TLR7/9	Approved	SLE, approved; RA, approved	-	-	
Tofacitinib	SMI	JAK1/3	Yes, phase 2 (local application (eye))	SLE, phase 1b/2; RA, approved	-	-	
Filgotinib	SMI	JAK1	Yes, phase 2	SLE, phase 2; RA, phase 3	-	-	

*www.clinicaltrials.gov
Abbreviations: pSS, Primary Sjögren's syndrome; SLE, Systemic Lupus Erythematosus; RA, Rheumatoid arthritis; mAb, monoclonal antibody; SMI, small molecule inhibitor; IFN, interferon; IFNAR, type I interferon receptor; BAFF, B-cell activating factor; TLR, Toll-like receptor; JAK, Janus kinases

[127]. Recently, epratuzumab showed improvement on clinical outcomes only in patients with SLE and an associated diagnosis of Sjögren's syndrome (secondary SS) and not in patients with SLE without additional SS diagnosis [128]. Randomized placebo-controlled studies in primary and secondary SS are needed to confirm these observations.

Activated innate cells are crucial to initiate adaptive immunity. As previously mentioned, important factors contributing to activation of adaptive immunity are BAFF and costimulatory molecules. Belimumab is a mAb targeting BAFF. Several studies using this drug have been performed in pSS and show some positive results [129-131]. Particularly patients with upregulation of type I IFN and subsequently higher BAFF levels were good responders to belimumab [131, 132]. Additionally, silencing of BAFF was shown to suppress generation of Th17 cells [133]. Therefore BAFF targeting therapies could be interesting to block pathogenic Th17 generation. In addition to targeting BAFF itself, mAb are available targeting the BAFF receptor (BAFF-R). In an early phase trial targeting BAFF-R, a trend towards improvement of pSS disease activity was observed although this was not significant [134]. At the moment data on the effectivity of BAFF-R mAb is limited. Abatacept is a fusion molecule of IgG-Fc and CTLA-4, blocking CD80 and CD86 ligands thereby interfering with T cell activation. Abatacept has been tested in pSS and was shown to reduce Tfh cells, GC formation and improve secretory function [135-137]. These results show that BAFF or costimulatory molecules are potentially promising to treat pSS.

Conventional drugs targeting the IFN pathway

A lot of effort has been put into the development and testing of biological treatments for pSS, however these types of drugs have several downsides. A major downside are the high costs, the effects of long-term usage is unknown and often frequent injections for longer periods of time are required. To treat rheumatic diseases there are already multiple conventional disease-modifying antirheumatic drugs (DMARDs) available of which the safety is well-studied and that are less expensive than biologics. Recently the use of DMARDs in pSS was reviewed and the conclusion was that DMARDs in pSS are poorly studied in controlled trials, but do shown potential [138]. One of the DMARDs that is frequently prescribed in pSS is hydroxychloroquine (HCQ). HCQ was shown to bind nucleic acids, thereby blocking TLR7/9-binding epitopes and preventing production of type I IFNs and proinflammatory cytokines [139, 140]. As HCQ mainly targets APC, this is an interesting drug because these cells are important drivers of sustained T- and B-cell activation and therefore HCQ potentially blocks the chronic immune activation. Although HCQ is successfully used in other systemic autoimmune diseases [141, 142], its efficacy in pSS remains questionable [143-150]. Differences in treatment duration, dosage, inclusion criteria and outcome measures make it difficult to study the effect of

HCQ in pSS. As HCQ affects TLR7 activation, we studied the effect of HCQ on TLR7 and type I IFN signaling pathways in samples from the JOQUER clinical trial and observed a reduction of many molecules involved in this pathway, while there was no clinical improvement [148].

Type I, II and III IFN signal via JAK/STAT molecules and could therefore also be a treatment target in pSS [151]. Interestingly, tofacitinib, a JAK1/3 inhibitor, is already approved to treat RA, psoriatic arthritis and ulcerative colitis (www.drugs.com). Tofacitinib was tested in a phase 2 trial in the form of eye drops in pSS and was safe and improved dryness in the eyes and reduced expression levels of proinflammatory cytokines in the patients' tears [152]. Filgotinib, a selective JAK1 inhibitor, reduced STAT1 and STAT3 phosphorylation, BAFF levels and B cells in the salivary glands in a mouse model for pSS [153]. Furthermore a positive effect on salivary flow was observed. At the moment oral application of filgotinib, is being tested in a phase 2 clinical trial according to www.clinicaltrials.gov in pSS, but the study has not been completed yet.

Identified novel treatment targets

The complex pathogenesis of pSS consists of an interplay between innate and adaptive immunity, which is bridged by IFNs. Biologics targeting the IFNAR, thereby blocking type I IFN signaling show positive results, however they are very expensive and not tested in pSS so far. As there are three pathways: TLR, RLR and DSR leading to the production of type I IFNs, blocking these pathways would also be an interesting strategy. Blocking of the TLR pathway by HCQ is already described here but showed no clear clinical effect yet, possibly due to the contribution of the RLR and DSR pathway. Both of these pathways signal via TANK-binding kinase 1 (TBK1). TBK1 is an ubiquitously expressed protein belonging to the IκB kinase (IKK) family. TBK1 and its closely related homologue IKKε have the ability to phosphorylate IFN regulator factor (IRF) 3 and 7, leading to their translocation into the nucleus and production of type I IFNs. Increased mRNA levels of TBK1 were found in several immune populations isolated from PBMCs of SLE patients and in particular in myeloid cells [154]. Additionally, elevated phosphorylation of TBK1 was described in pDC isolated from IFNpos pSS patients and patients suffering from SLE and systemic sclerosis [155]. This indicates TBK1 as a potential treatment target. An advantage of targeting TBK1 is that there are small molecule inhibitors available. Small molecule inhibitors are less expensive than biologics, can be taken orally, are easy to deliver and pharmacologically stable [156]. Furthermore, TBK1 inhibitors are already used to treat cancer and several inflammatory conditions where their safety has been proven. Recently, it has been suggested that also in metabolic disorders TBK1 inhibitors might be of use [157]. Because of its potential in several conditions, the industry has taken a great interest in this molecule and there are many patented inhibitors of TBK1

available [156, 158]. In rare monogenetic disorders where TBK1-dependent signaling is chronically upregulated, inhibitors of TBK1 have shown favorable effects [154, 159]. We have recently shown in pSS and several other systemic autoimmune diseases that treatment with TBK1 blockers was able to reduce the spontaneous type I IFN signature by PBMCs of these patients *in vitro* [48, 155]. Further research is needed to test the full potential of TBK1 inhibitors in pSS.

Another interesting treatment target is STING. This molecule signals upstream of TBK1 and is activated in the presence of cytosolic DNA by cyclic GMP-AMP synthase (cGAS) and IFI16. As earlier described, studies have demonstrated a role for STING in initiating pSS-like disease in mice. In mouse models for SLE, STING inhibition shows potential to suppress inflammation. However, STING knock out mice are autoimmune-prone and develop signs of increased immune activation [160, 161]. Therefore, STING might also have regulatory roles besides its function in DNA sensing, which should be taken into account while developing STING inhibitors.

Lastly, there are several other potential treatments for pSS all in various stages of clinical trials. Like molecules intervening in the TLR pathway (IRAK4 inhibitor, PF-06650833 and PI3Kσ inhibitors, CDZ173 and Seletalisib (UCB5857)) [162, 163] and strategies targeting pDCs directly as well as studies using therapeutic RNases (RSLV-132) to prevent pDC activation (www.clinicaltrials.org). Finally, blockers for CD40 co-stimulation (CFZ533) to prevent T-cell activation, low dose of IL2 to induce Tregs and BTK inhibitors (Tirabrutinib) preventing B-cell signaling are under investigation as treatments for pSS. Although the results most of these trials are not available yet, it does indicate the interest of the industry in IFN targeting strategies and the need for understanding of disease pathogenesis of pSS to develop treatments for pSS.

CONCLUSION

pSS is a systemic autoimmunity disease with a high prevalence primarily affecting woman. Treatment is still mainly symptomatic but specific treatments based upon studies of the pathogenicity of pSS are on the horizon. The role of type I IFNs in pSS has been acknowledged for over a decade, however recently type II and III IFNs appear to play a role in the pathogenesis of this disease as well. As IFNs are elevated in a subgroup pSS patients and can affect both immune and non-immune cells, they are an interesting treatment target. Several novel biological and molecular strategies to target IFNs itself or molecules in their signaling pathways are in various stages of clinical trials. The efforts to improve the understanding of the disease mechanisms underlying pSS will hopefully in the near future provide effective treatment strategies.

EXPERT COMMENTARY

pSS is a complex and heterogeneous disease, hence the need for stratification to identify groups who could benefit from specific treatment. IFNs offer an interesting possibility for stratification as they play an important role in the pathogenesis of this disease and there are large subgroups with chronic upregulation of type I, type II IFNs and potentially also type III IFNs. A question that still remains is whether there is a relation between systemic IFN activation in the blood and local IFN activation in the salivary glands. In order to answer this question blood and salivary gland tissue must be collected simultaneously from the same patient. If there is a good correlation between local and systemic IFN activation then the lip biopsy, which can be painful for the patient, might not be necessary anymore for those patients that have systemically a positive IFN signature. We described easy assessable signatures for IFN activation in peripheral blood, which could be introduced into clinical practice to provide more information [164]. Recently, standardized consensus guidance for the labial salivary gland histopathology has been described thanks to the efforts of an European workgroup [165]. These guidelines will help to correlate gland and systemic pathology. When local and systemic IFN correlate, a peripheral blood IFN signature test can serve as extra confirmation for the diagnosis of pSS. In addition, the IFN signature is an interesting biomarker which should be taken along in trials targeting molecules which affect IFN signaling. In several trials described above the IFN signature has already proven to identify specific subgroups which are responding to a certain therapeutic. Until now it is unclear if IFNneg or IFNlow pSS patients have a totally different pathogenic mechanism or alternatively have the same mechanism but did not reach the high IFN activation levels of the IFNpos patients. Additionally, considering the heterogeneity in pSS potential other signatures could also be present. In order to effectively study this, collaborations are important to include large numbers of patients as well as standardization of the methods used. This will lead to stratification and ultimately precision medicine. We would also like to highlight the potential use of small molecule inhibitors. There are many biologics being tested for the treatment of pSS at the moment; however, only about one third of the pSS patients have systemic complications. As these treatment options are very expensive it might be worthwhile to look at the effect of small molecule inhibitors which are already developed for other diseases and might be additionally successful to treat pSS. As these molecules are a lot more affordable and already proven to be safe they have great potential.

FIVE-YEAR VIEW

Understanding of pSS disease pathogenesis is rapidly expanding and the role of IFNs and use of the IFN signature is more commonly acknowledged. However, therapy at the moment is still mainly symptomatic. Fortunately many different treatments are under investigation for the use in pSS and an extensive set of drugs has proven to be safe. Increased knowledge of underlying mechanisms as the basis for stratification will help us to move towards personalized medicine. This will not only improve the quality of life of the patients but might also reduce healthcare costs. Because of the interplay between many immune cells in pSS pathogenesis combination therapy might be beneficial. The combination of belimumab and rituximab has been studied in pSS and currently another trial is being performed using these two mAb together [166] (www.clinicaltrials.gov). In SLE combination of these drugs already showed beneficial effects over the use of these drugs separately [167, 168]. Another interesting combination would be to block IFN signaling in combination with T- or B-cell inhibiting therapies. We would like to highlight the importance of the IFN signature for monitoring of treatments. Often ESSDAI and ESSPRI are used to monitor improvement of disease features, however these measurements seem not very sensitive to change. Therefore, treatment might have a beneficial effect but remains undetected. Another point is that only 1/3 of the pSS patients have systemic manifestations and for the other patients the ESSDAI cannot be used to monitor disease activity. Stratification based upon underlying pathophysiological mechanisms and selection of relevant biomarkers to monitor treatment will be key in the future for clinical trials.

KEY ISSUES

pSS is a systemic autoimmune disease characterized by dryness of the eyes and mouth. This is accompanied by systemic complications including vasculitis, arthritis, multi-organ involvement (often affecting the lung or kidney) and fatigue.

Interferons (IFNs) play an important role in pSS pathogenesis. Systemically type I IFNs are upregulated in 57% of the pSS patients. A subset of these patients additionally has type II IFN activation. There are indications that type III IFNs might also be upregulated systemically.

Large overlap in type I and II IFN-induced gene expression patterns makes it difficult to distinguish between these two IFNs.

Plasmacytoid dendritic cells are the main producers of type I IFN. There are three pathways leading to induction of IFN type I: toll-like receptor pathway, RIG-I- receptor pathway and the DNA sensing pathway.

Both innate and adaptive immunity are involved in the pathogenesis of pSS and IFNs play an important role connecting these two arms of the immune system.

IFNs also induce regulatory factors like IDO and coinhibitory molecules. In some patients these inhibitory signals might be less strong than the stimulatory signals. Activation of these inhibitory pathways provides potential for pSS treatment.

Systemic therapy with biologics shows potential as future treatment, but the costs are a drawback. Small molecule inhibitors targeting molecules in the IFN pathway have shown great promise.

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Chapter 7

Fatigue in Sjögren's syndrome: a search for biomarkers and treatment targets

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ABSTRACT

Background Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, where patients often suffer from fatigue. Biological pathways underlying fatigue are unknown. In this study aptamer-based SOMAscan technology is used to identify potential biomarkers and treatment targets for fatigue in pSS.

Methods SOMAscan® Assay 1.3k was performed on serum samples of healthy controls (HCs) and pSS patients characterized for interferon upregulation and fatigue. Differentially expressed proteins (DEPs) between pSS patients and HC or fatigued and non-fatigued pSS patients were validated and discriminatory capacity of markers was tested using independent technology.

Results Serum concentrations of over 1300 proteins were compared between 63 pSS patients and 20 HCs resulting in 58 upregulated and 46 downregulated proteins. Additionally, serum concentrations of 30 interferon positive (IFNpos) and 30 interferon negative (IFNneg) pSS patients were compared resulting in 25 upregulated and 13 downregulated proteins. ELISAs were performed for several DEPs between pSS patients and HCs or IFNpos and IFNneg all showing a good correlation between protein levels measured by ELISA and relative fluorescence units (RFU) measured by the SOMAscan. Comparing 22 fatigued and 23 non-fatigued pSS patients, 16 serum proteins were differentially expressed, of which 14 were upregulated and 2 were downregulated. Top upregulated DEPs included neuroactive synaptosomal-associated protein 25 (SNAP-25), alpha-enolase (ENO1) and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1). Furthermore, the proinflammatory mediator IL36a and several complement factors were upregulated in fatigued compared to non-fatigued pSS patients. ROC analysis indicated that DEPs showed good capacity to discriminate fatigued and non-fatigued pSS patients.

Conclusion In this study we validated the use of aptamer-based proteomics and identified a novel set of proteins which were able to distinguish fatigued from non-fatigued pSS patients and identified a so-called 'fatigue signature'.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a common systemic autoimmune disease, characterized by lymphocytic infiltrations in salivary and lachrymal glands. This is accompanied by sicca symptoms of the eyes and mouth and frequently also extraglandular manifestations [1-3]. Fatigue is one of the most common extraglandular manifestation in pSS and is associated with a poor quality of life [4-9]. Fatigue affects up to 70% of pSS patients, while approximately 20% of healthy adults are affected [10-13].

The biological basis of fatigue is largely unknown, however proinflammatory mechanisms are thought to play a role. Interferons (IFNs) are proinflammatory cytokines, which play a pivotal role in the pathogenesis of pSS and are systemically upregulated in 57% of the pSS patients [14]. Elevated levels of IFNs induce increased expression of IFN-stimulated genes in the salivary glands, peripheral blood mononuclear cells (PBMCs), isolated monocytes and B cells of pSS patients [15-19]. This so-called 'IFN type I signature' is associated with higher disease activity and higher levels of autoantibodies. In addition, mutations affecting IFN signaling are observed in TREX, IRF5, STAT4 and PTPN22W and are associated with pSS [20-26]. There is evidence for a link between IFNs and fatigue. Patients receiving IFNα treatment for viral hepatitis or melanoma can develop severe fatigue [27, 28]. However, we and others have previously shown that there was no correlation between systemic upregulation of IFNs and fatigue in pSS patients [13, 29].

Because fatigue is a common problem in pSS, it is important to identify pathways underlying this fatigue. Here we use a proteomics approach to identify pathways related to fatigue. We used the aptamer-based SOMAscan technology, a highly multiplexed proteomic assay that queries 1300 proteins in serum for protein biomarker discovery and identification of serum proteomic signatures and possible treatment targets for fatigue in pSS.

METHODS

Patients and methods

PSS patients and healthy controls (HC) were recruited at the Erasmus Medical Centre, Rotterdam, the Netherlands. All pSS patients fulfilled the 2002 American-European Consensus Group classification criteria [30] and were free of symptoms of viral infection at inclusion. HC did not suffer from autoimmune disease and did not use corticosteroids. Written informed consents were obtained from all participants in the

study, in compliance with the Helsinki Declaration. Medical Ethical Review Committee of the Erasmus MC approved this study.

Blood collection

Blood was collected in clotting tubes for serum preparation and in PAXgene RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) for whole blood RNA analysis.

Real-time quantitative PCR and calculation of IFN score

Total RNA was isolated from PAXgene tubes and reverse-transcribed to cDNA. For calculation of relative expression, samples were normalized to expression of the household gene Abl [31]. Relative expression values were determined from normalized CT values using 2^-ΔΔCT method (User Bulletin, Applied Biosystems). The IFN score was defined by the relative expression of 5 genes, IFI44, IFI44L, IFIT1, IFIT3, MXA. $Mean_{HC}$ and SD_{HC} of each gene in the HC-group were used to standardize expression levels. IFN scores per subject represent the sum of these standardized scores, calculated as previously described [31, 32]. Patients were divided in groups being positive or negative for the IFN score using a threshold of $mean_{HC} + 2 \times SD_{HC}$.

Proteomic analysis of serum protein concentrations

Serum protein concentrations were measured using the SOMAscan platform. SOMAscan utilizes single stranded DNA-based protein affinity reagents called SOMAmers (Slow Off-rate Modified Aptamers) [33, 34]. The SOMAscan 1.3k kit was used following manufacturer's protocol, measuring over 1300 proteins in 65 µl of serum. Intra-run normalization and inter-run calibration were performed according to SOMAscan assay data quality-control procedures as defined in the SomaLogic good laboratory practice quality system. Data from all samples passed quality-control criteria.

Measurement of complement, immunoglobulin levels and auto-antibodies

C3, C4 and IgG were measured as described previously [35]. Anti-SSA and anti-SSB were determined by EliA (Thermo Fisher Scientific), confirmed with ANA profile immunoblot (EuroImmun) and re-confirmed where needed by QUANTA Lite ELISA-kit (INOVA).

Assessment of Fatigue and Depressive symptoms

Fatigue was assessed using the Dutch version of the multidimensional fatigue inventory (MFI) [36]. As a cut-off the 25 percentile highest (fatigued group) and lowest scores (non-fatigued group) were used resulting in the inclusion of 45 patients. The

Dutch-validated Center for Epidemiologic Studies Depression (CES-D) was used to study depression and anxiety [37, 38].

Statistics

SOMAscan was performed to identify differences in quantitative binding of proteins to aptamers. Data were analyzed using empirical Bayes moderate t-test by the limma Bioconductor package in the R environment [39-41]. P-values were corrected for multiple hypothesis testing using Benjamini-Hochberg method (FDR< 0.05). Differential expression was calculated on normalized $\log_{10}$ intensities. Visualization of differentially expressed proteins (DEPs) between pSS and HC and fatigued and non-fatigued pSS patients was based on ^{2}Log transformed binding intensities and geometric means were calculated for HC and pSS patients.

Independent T-test was used to compare means and the Mann-Whitney U test was used to compare medians. Categorical data were compared using Fisher's exact test and correlations were assessed using Spearman's rho (r_s). In order to determine the discriminatory capacity of markers receiver operating characteristics (ROC) curves and areas under the curves (AUCs) were calculated. Statistical analysis and visualization of DEP was performed using Instem/Omniviz, R version 3.4.3 bioconductor package limma version 3.34., IBM SPSS 24.0 (SPSS, Chicago, IL, USA) and Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA), 9.

RESULTS

Differential protein expression in serum of pSS and interferon positive patients

Characteristics of pSS patients and HC are summarized in supplemental table S1. Using the SOMAscan multiplex proteomic assay, in total 104 serum proteins were differentially expressed between pSS patients and healthy controls after correction for multiple testing. Of these proteins 58 were upregulated and 46 were downregulated. A heatmap representing the most significant DEPs (2LogFC>0.5) is shown in figure 1A and indicates a clear distinction between pSS and healthy controls (figure 1A). Figure 1B shows in a volcano plot for the same DEPs. Top upregulated DEPs include Fcγ receptor 3B, a receptor binding immune complexes which are often observed in pSS, the interferon-inducible protein ISG15 and several female hormones including follicle-stimulating hormone (FSH) and human chorionic gonadotropin (HCG). A list of significantly up- and downregulated proteins is shown in supplementary table S2 (in this thesis the top 25 up- and downregulated proteins are shown).

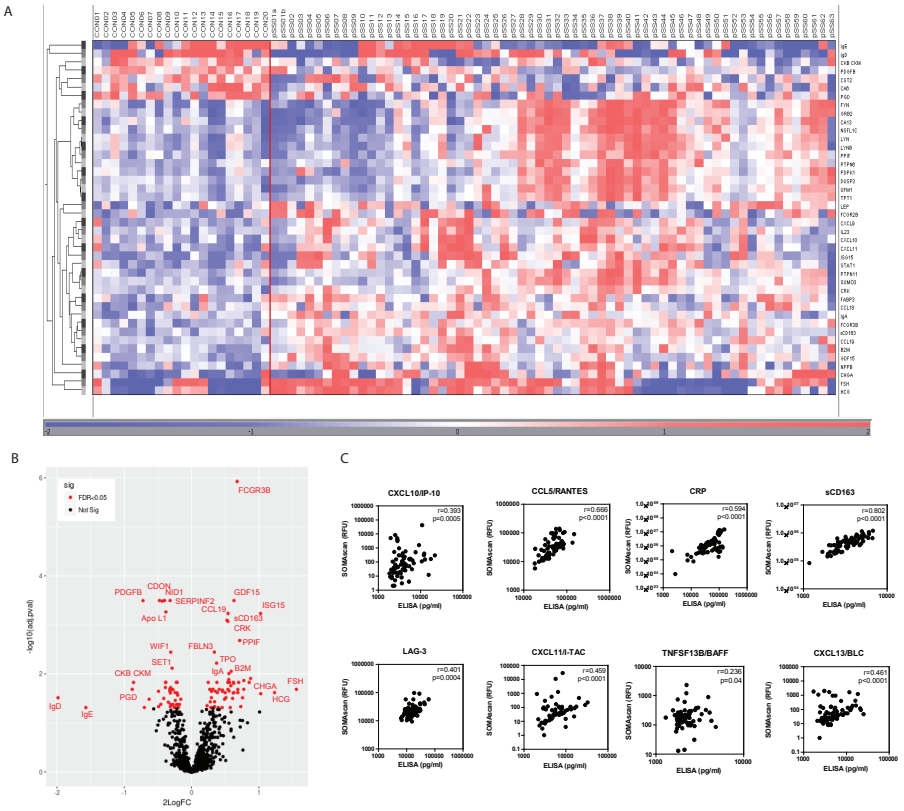

Figure 1. Differential protein expression in serum of pSS and HCs
Heatmap of differentially expressed proteins measured by SOMAscan technology in serum samples of pSS patients (pSS) (n=63) and healthy controls (CON) (n=20) clustered unsupervised within the groups **(A)** and volcano plot **(B)** visualizing the same DEPs. The correlation between RFU determined by SOMAscan and protein levels determined by ELISA is shown in **(C)**.

Additionally, IFN positive (IFNpos) and IFN negative (IFNneg) pSS patients were compared. Characteristics of IFNpos and IFNneg pSS are summarized in supplemental table S3. IFNpos patients showed significantly higher IgG levels, higher frequency of anti-SSA and anti-SSB autoantibodies and lower C3 complement levels compared to IFNneg patients. Comparing protein expression between IFNpos and IFNneg patients 38 proteins were DEPs of which 25 were upregulated and 13 were downregulated. As expected many interferon-inducible proteins were found upregulated including ISG15, CXCL11/I-TAC, CXCL10/IP-10, OAS1 and TNFSF13B/BAFF. Volcano plots and a full list of all significantly up- and downregulated proteins between IFNpos and IFNneg pSS patients is shown in supplementary figure 1 and supplementary table S4.

To validate the SOMAscan data, ELISAs were performed for several DEPs between pSS patients and HCs or IFNpos and IFNneg pSS patients. Proteins for validation were

selected based upon availability of reliable ELISAs. The selected proteins included CXCL10/IP-10, CCL5/RANTES, CRP, sCD163, LAG-3, CXCL11/I-TAC, TNFSF13B/BAFF and CXCL13/BLC. All protein levels measured by ELISA significantly correlated with relative fluorescence units (RFUs) determined by the SOMAscan (Figure 1C).

Fatigued pSS patients are characterized by a differential serum protein expression pattern

Characteristics of fatigued and non-fatigued pSS patients are summarized in table 1. In total 16 serum proteins were differentially expressed between fatigued and non-fatigued pSS patients, of which 14 were upregulated and 2 were downregulated in fatigued patients. Top upregulated DEPs included neuroactive synaptosomal-associated protein 25 (SNAP-25), alpha-enolase (ENO1) and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1). Furthermore, the proinflammatory mediator IL36a and several complement factors were upregulated in fatigued compared to non-fatigued pSS patients. A heatmap representing the DEPs is shown in figure 2A. When unsupervised clustering of patients was performed 15 of the 22 fatigued patients (~68%) clustered together and only one non-fatigued patients clustered with this group. This grouping of fatigued pSS patients indicated a signature for fatigue in pSS. A volcano plot of the DEPs is shown in figure 2B and a full list of all DEPs, Log Fold changes and (adjusted) p-values is depicted in table 2 and supplementary table 5.

As hydroxychloroquine (HCQ) is sometimes used to treat fatigue, differential protein expression was additionally determined after exclusion of patients who used HCQ. No differences were found compared to the analyses including HCQ users (data not shown). Additionally, dimensions of fatigue were compared between HCQ users and non-users and no differences were observed (supplementary figure 2).

The SOMAscan data of DEPs were validated by ELISAs when these were available. ELISAs for ENO1 and epidermal growth factor (EGF) showed significant correlations with RFUs determined by SOMAscan (figure 2C). In addition, C3 and C4 RFUs were compared to C3 and C4 complement levels determined through routine diagnostics at the Erasmus MC, clinical chemistry lab by Immage nephelometer. Proteins selected for validation showed good correlation with protein levels determined by ELISA and Immage nephelometer. Fatigued pSS patients showed higher complement levels and lower IFN scores than non-fatigued patients (figure 2D). There was no difference in European League Against Rheumatism Sjögren's Syndrome Disease Activity Index (ESSDAI) score, although there was a trend towards higher ESSDAI scores in the fatigued patients, which had higher scores in the articular and pulmonary domain (data not shown).

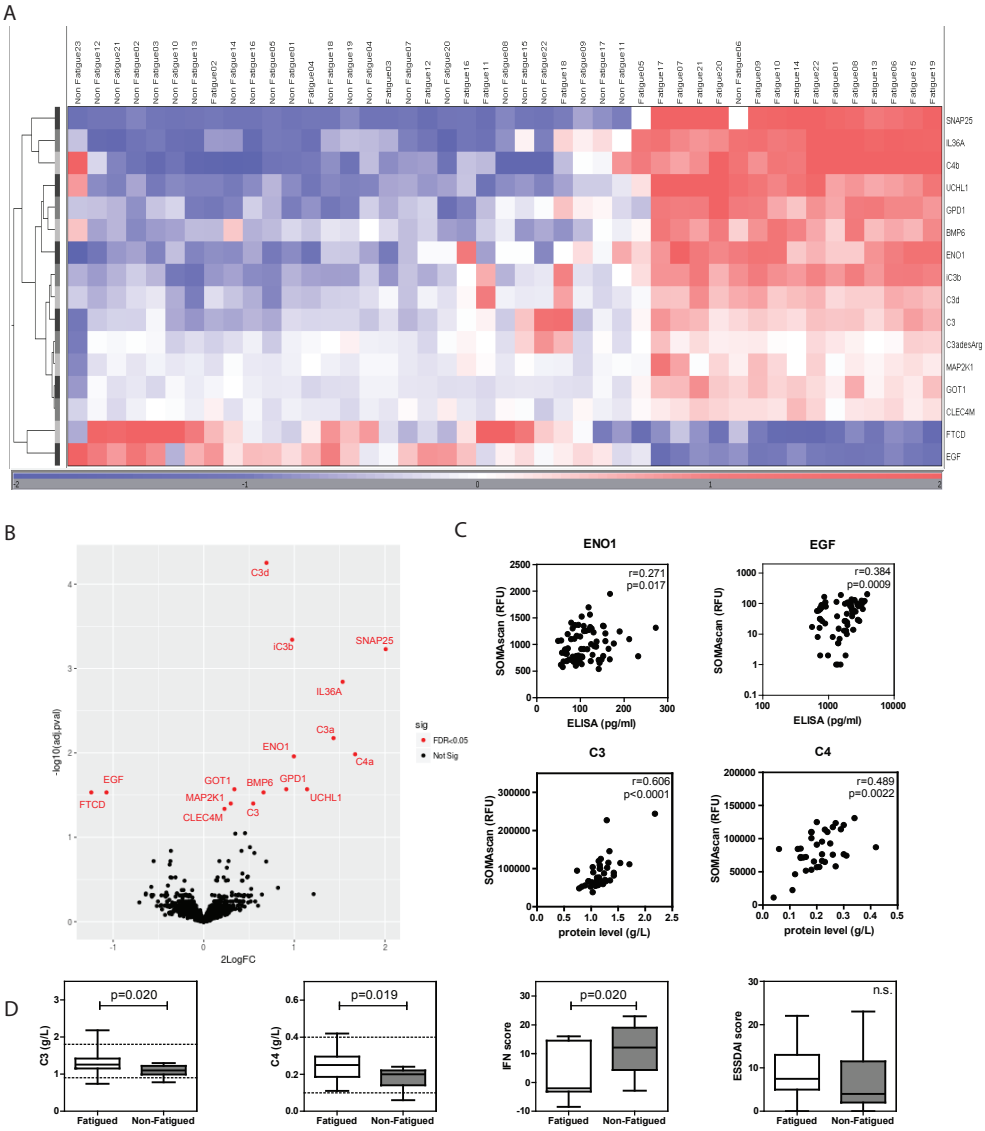

Figure 2. Differential protein expression in serum of fatigued pSS patients
Heatmap showing the unsupervised clustering of differentially expressed proteins between fatigued (n=22) and non-fatigued (n=23) pSS patients **(A)** and volcano plot **(B)** visualizing the same DEPs. **(C)** Correlation between RFU determined by SOMAscan and protein levels determined by ELISA (for ENO1 and EGF) and Immage nephelometer (for C3 and C4). **(D)** Comparison of complement levels, IFN and ESSDAI score between fatigued and non-fatigued pSS patients.

Table 1. Characteristics fatigued and non-fatigued pSS patients*

	Fatigued (n=22)	Non-Fatigued (n=23)	Significance
Demographics			
Female (%)	22/22 (100)	21/23 (91)	n.s.
Mean age (years)	58.7 ± 11.3	57.3 ± 12.4	n.s.
Disease duration (years)	11.0 (15.5)	11.5 (16.8)	n.s.
ESSDAI	7.5 (8.0)	4.0 (9.5)	n.s.
IFN score	3.6 (9.4)	11.2 (8.7)	p=0.020
CES-D	27.0 (18.0)	6.0 (4.0)	p<0.0001
MFI			
General fatigue	20.0 (1.5)	10.0 (6.0)	p<0.0001
Physical fatigue	19.0 (3.0)	8.0 (5.5)	p<0.0001
Mental fatigue	17.0 (5.5)	7.0 (5.0)	p<0.0001
Reduced motivation	17.0 (5.0)	6.0 (4.0)	p<0.0001
Reduced activity	18.0 (4.0)	7.0 (5.0)	p<0.0001
Medication status (%)			
Pilocarpine	11/22 (50)	8/23 (35)	n.s.
Hydroxychloroquine	14/22 (64)	15/23 (65)	n.s.
Corticosteroids	1/22 (5)	1/23 (4)	n.s.

Data are presented as mean ± SD, median (IQR) or as number (%) of patients according to data distribution.
* patients are selected from the cohort by a cut-off the 25 percentile highest (fatigued group) and lowest (non-fatigued group) MFI scores.

ESSDAI, the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index; IFN, interferon; MFI, multiple fatigue inventory

Predictive value of markers for fatigue in pSS

The predictive potential of the proteins found to be differentially expressed between fatigued and non-fatigued pSS patients was studied. In order to do this ROC curves were calculated for the most DEPs (2LogFC>1) including SNAP25, complement factors C4a/C4b and C3a, IL36a, UCHL1, ENO1, EGF and formimidoyltransferase-cyclodeaminase (FTCD) (figure 3A). Additionally, the corresponding boxplots are shown (figure 3B). ROC analysis yielded AUC values between 0.752 and 0.845 confirming a robust discriminatory capacity between fatigued from non-fatigued patients pSS patients using these proteins (table 3).

Table 2. Differentially expressed proteins between fatigued and non-fatigued pSS patients

	2LogFC	FDR	Function
Upregulated protein			
SNAP25	2.01	5.90E-04	Presynaptic plasma membrane protein involved in the regulation of neurotransmitter release. Restricted expression in brain.
C4b	1.67	1.04E-02	Basic form of complement factor 4, part of the classical activation pathway.
IL36A	1.53	1.44E-03	Cytokine that can activate NF-kappa-B and MAPK signaling pathways to generate an inflammatory response.
C3a	1.43	6.68E-03	C3a is an anaphylatoxin released during activation of the complement system.
UCHL1	1.14	2.69E-02	Belongs to the peptidase C12 family. This enzyme is a thiol protease that hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin. This gene is specifically expressed in the neurons and in cells of the diffuse neuroendocrine system.
ENO1	1.00	1.10E-02	Alpha-enolase, glycolytic enzyme. Alpha-enolase has also been identified as an autoantigen in Hashimoto encephalopathy.
iC3b	0.98	4.56E-04	Proteolytically inactive product of the complement cleavage fragment C3b that still opsonizes microbes, but cannot associate with factor B.
GPD1	0.91	2.69E-02	Member of the NAD-dependent glycerol-3-phosphate dehydrogenase family. The encoded protein plays a critical role in carbohydrate and lipid metabolism.
C3d	0.69	5.60E-02	302-amino-acid fragment in the alpha chain of C3b.
BMP6	0.66	2.94E-02	Secreted ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. Ligands of this family bind various TGF-beta receptors leading to recruitment and activation of SMAD family transcription factors that regulate gene expression.
C3	0.55	3.98E-02	Complement component C3 plays a central role in the activation of the complement system.
GOT1	0.34	2.69E-02	Glutamic-oxaloacetic transaminase is a pyridoxal phosphate-dependent enzyme which exists in cytoplasmic and mitochondrial forms, GOT1 and GOT2, respectively. GOT plays a role in amino acid metabolism and the urea and tricarboxylic acid cycles.
MAP2K1	0.30	3.98E-02	The protein encoded by this gene is a member of the dual specificity protein kinase family, which acts as a mitogen-activated protein (MAP) kinase kinase. MAP kinases, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals.
CLEC4M	0.23	4.60E-02	Involved in the innate immune system and recognizes numerous evolutionarily divergent pathogens ranging from parasites to viruses

Table 2. Continued

	2LogFC	FDR	Function
Downregulated protein			
FTCD	-1.24	2.94E-02	The protein encoded by this gene is a bifunctional enzyme that channels 1-carbon units from formiminoglutamate, a metabolite of the histidine degradation pathway, to the folate pool.
EGF	-1.07	2.94E-02	Member of the epidermal growth factor superfamily.

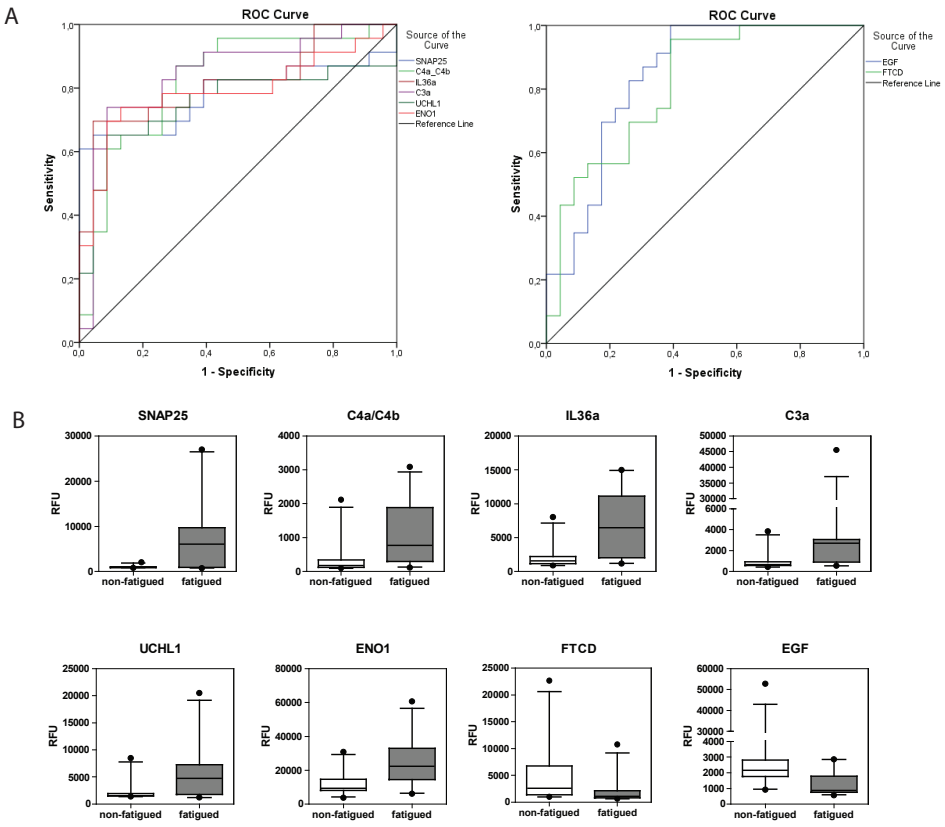

Figure 3. Discriminatory capacity of markers for fatigue in pSS
(A) ROC curves of positively and negatively predictive proteins (2LogFC>1) for fatigue in pSS. **(B)** Boxplots of differentially expressed proteins between fatigued (n=22) and non-fatigued (n=23) pSS patients.

Table 3. Area Under the ROC Curve for markers for fatigue in pSS

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
SNAP25	.781	.075	.001	.634	.927
C4a_C4b	.824	.064	.000	.699	.949
IL36a	.819	.065	.000	.692	.945
C3a	.845	.061	.000	.724	.965
UCHL1	.752	.078	.003	.599	.906
ENO1	.790	.071	.001	.650	.930
EGF	.837	.061	.000	.718	.957
FTCD	.811	.064	.000	.686	.936

[a]Under the nonparametric assumption
[b]Null hypothesis: true area = 0.5

DISCUSSION

PSS is a heterogeneous disease with complex pathogenesis. Traditional proteomic approaches of lachrymal or salivary fluids have shown increased expression of inflammatory and immune response-related proteins [42]. Furthermore, gene expression profiling of pSS blood also revealed systemic upregulation of immune related pathways, like the IFN pathway and B cell receptor signaling pathway [18, 43, 44]. Using SOMAscan technology we were able to identify upregulation of similar pathways as described using other proteomic techniques. To our knowledge one other study used SOMAscan technology to study pSS [45], although a more limited number of proteins were measured. Nishikawa et al. identified several DEPs in serum of pSS patients compared to serum of HCs and DEPs were linked to disease activity measured by ESSDAI score. When we compared pSS patients with HC we identified sets of upregulated proteins such as CD163, CXCL10, TNFSF15, FSH, CXCL11 and β2-microglobulin, that were in agreement with previously published data [45]. In summary, we identified similar upregulated pathways as identified with other microarray platforms [15-19] and found similar upregulated proteins with the same technique in a different cohort of pSS patients [45] indicating the SOMAscan technology as a reliable method for the discovery of biomarkers for fatigue in pSS.

Fatigue is the most prevalent extraglandular symptom in pSS of which we do not know the biological basis. Since fatigue is often seen in conditions where the immune system is dysregulated, proinflammatory mechanisms have been thought to play a role. Previous attempts, however, to find a link between proinflammatory signatures in serum

or tissue and fatigue have failed [46-48]. Previous studies even showed decreasing levels of several proinflammatory cytokines like IP-10/CXCL10, TNFα, LTα and IFNγ in fatigued pSS patients [48]. Furthermore, we previously described a negative trend between IFNs and fatigue [29]. In our current multiplexed proteomic analysis we show the coordinated upregulation of a set of proteins of which some are involved in inflammation including IL36a and complement factors. IL36a is a pro-inflammatory cytokine belonging to the IL-1 family and induces maturation of dendritic cells and drives Th1 and Th17 responses in CD4+ T cells [49]. This cytokine was previously shown to be overexpressed in the salivary glands and serum of pSS patients [50]. Upregulation of this cytokine is also seen in other diseases like psoriasis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and fibromyalgia [49, 51, 52]. In addition to IL36a, several complement factors were upregulated in fatigued pSS patients compared to non-fatigued patients. Quantification of the complement levels, however, showed that all values were in the normal range, but the non-fatigued patients lean towards reduced complement levels. Reduced complement levels are often associated with more severe disease manifestations, vasculitis and lymphoma in pSS [53].

Interestingly, among the "fatigue-signature" proteins were several proteins which have functions in the brain like SNAP-25, UCHL1 and ENO1. SNAP-25 protein is a SNARE protein, critical in neurotransmitter release [54]. Aberrancies in this protein are described in several neurological, cognitive and psychological disorders like Alzheimer's disease and fibromyalgia [55-58]. Also UCHL1 is particularly abundant in the brain, where it is critical for proper function of the ubiquitin-proteasome system in neurons [59]. Reduced levels of this gene have also been linked to among others Parkinson and Alzheimer's disease [59-62]. ENO1 is a glycolytic enzyme which can be expressed in the brain, but other tissues can also express this protein and it has a wide variety of functions (reviewed in [63]). This protein has also been implicated in Alzheimer's disease. Interestingly, data indicate that ENO1 acts as an autoantigen in several autoimmune diseases. Antibodies against ENO1 have been described in Hashimoto's encephalopathy, Behçet's disease, Crohn's disease, rheumatoid arthritis [64-68] Recently, antibodies against citrullinated ENO1 (Anti-CEP-1) peptides have also been observed in pSS [69] and this raises the question if such autoantibodies associate with fatigue. Although aberrancies in all these proteins have been linked to a variety of conditions they have never been described in the context of fatigue.

EGF and FTCD were both significantly reduced in fatigued patients compared to non-fatigued patients. EGF is found in many secretions including saliva. After binding to the EGF receptor it regulates epithelial cell proliferation and survival and therefore is thought to have protective effects. EGF has previously been shown to be reduced in tears [70], salivary glands [71, 72] and saliva of pSS patients and correlates with progression

of intraoral manifestations [73, 74]. FTCD is a metabolic enzyme, which is primary active in the liver and kidneys. However, recently a study described additional neurological effects [75]. So far none of these proteins have been linked to fatigue.

Glycerol-3-phosphate dehydrogenase [NAD(+)] (GPD1), bone morphogenetic protein 6 (BMP6), aspartate aminotransferase (GOT1), dual specificity mitogen-activated protein kinase kinase 1 (MAP2K1) and C-type lectin domain family 4 member M (CLEC4M) were additionally found slightly elevated in fatigued pSS patients compared to non-fatigued patients. These proteins have a variety of metabolic and immunological functions and GPD1, BMP6 and GOT1 also have functions in the brain. However, it is unclear how these proteins could contribute to fatigue.

Recently, proteomics performed on CSF revealed a signature for fatigue in pSS patients [76]. In this abstract they describe similar as in our study upregulation of molecules in the complement system. Overall most discriminatory proteins between fatigued and non-fatigued pSS patients were involved in innate immunity, cellular stress defense and/or function in the central nervous system. It would be interesting to compare the proteins found differentially expressed in the CSF of fatigued pSS patients with the proteins we found in the serum.

A limitation of this study is that we were not able to validate all DEPs between fatigued and non-fatigued patients because there were no sensitive ELISAs available for these proteins. However in this study we showed that when ELISAs were available, DEPs identified by SOMAscan showed good correlation with protein levels measured using different techniques indicating the reliability of the technology. Another limitation of this study is the cross-sectional design and limited number of patients in the fatigue vs. non-fatigue comparison. Furthermore, there could be underlying confounding comorbidities leading to fatigue in some patients.

CONCLUSION

In this study we validated the use of aptamer-based multiplex proteomics and identified a novel set of proteins which were able to distinguish fatigued from non-fatigued pSS patients and identified a so-called “fatigue-signature”. Overall these proteins were involved in inflammatory mechanisms and have neurological and metabolic functions. More studies are necessary to validate these proteins as markers for fatigue in pSS.

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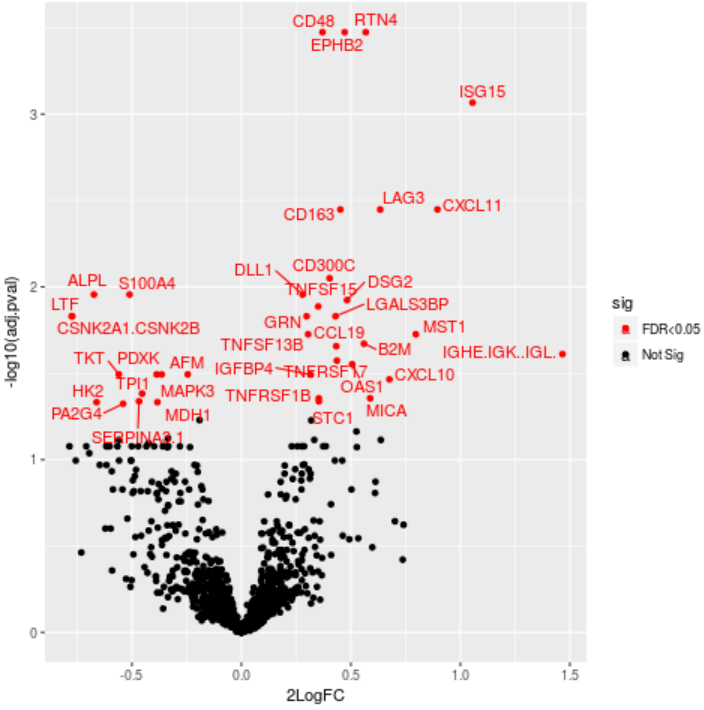
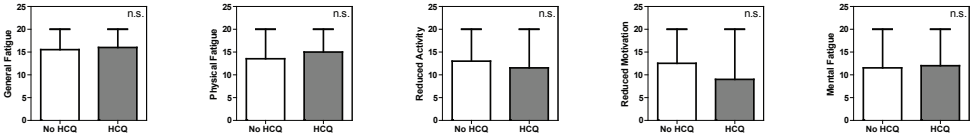
SUPPLEMENTARY FILES



Supplementary figure S1.
Volcano plot of differentially expressed proteins between interferon positive (n=30) and interferon negative (n=30) pSS patients.



Supplementary figure S2.
Comparison of dimensions of fatigue measured by the multiple fatigue inventory questionnaire between HCQ (n=40) and non-HCQ (n=22) treated pSS patients.

Supplementary table S1. Characteristics of patients and healthy controls

	HC (n=20)	pSS (n=63)
Demographics		
Female (%)	17/20 (85%)	59/63 (94%)
Mean age (years)	35.6 ± 16.2	60.1 ± 12.4
Disease duration (years)	-	12.1 ± 8.4
Clinical manifestations		
Ocular symptoms	-	63/63 (100%)
Oral symptoms		63/63 (100%)
Anti-SSA positivity	-	46/63 (73%)
Anti-SSB positivity	-	31/63 (49%)
ESSDAI	-	8.4 ± 7.0
Medication status (%)		
Pilocarpine	-	26/63 (41%)
Hydroxychloroquine	-	41/63 (65%)
Corticosteroids	-	4/63 (6%)

Data are presented as mean ± SD, median (IQR) or as number (%) of patients according to data distribution. HC, Healthy controls; pSS, primary Sjögren's Syndrome; ESSDAI, the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index

Supplementary table S2. Differentially expressed serum proteins between pSS patients and HC

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGeneID	Entrez Gene Symbol	2LogFC	FDR
Upregulated proteins								
3032-11_2	SL000428	Follicle stimulating hormone	FSH	P01215. P01225	1081 2488	CGA FSHB	1.56	0.0206
4914-10_1	SL001766	Human Chorionic Gonadotropin	HCG	P01215. P01233	1081 1082	CGA CGB	1.23	0.0241
8476-11_3	SL002762	Chromogranin-A	CgA	P10645	1113	CHGA	1.03	0.0254
14151-4_3	SL015510	Ubiquitin-like protein ISG15	UCRP	P05161	9636	ISG15	1.02	0.0006
3813-3_2	SL006913	Tyrosine-protein kinase Fyn	FYN	P06241	2534	FYN	0.88	0.0124
5464-52_3	SL003792	Growth factor receptor-bound protein 2	GRB2 adapter protein	P62993	2885	GRB2	0.86	0.0149
4250-23_3	SL006268	NSFL1 cofactor p47	NSF1C	Q9UNZ2	55968	NSFL1C	0.78	0.0136
3310-62_1	SL010460	Low affinity immunoglobulin gamma Fc region receptor II-b	FCG2B	P31994	2213	FCGR2B	0.78	0.0206
7655-11_3	SL002785	N-terminal pro-BNP	N-terminal pro-BNP	P16860	4879	NPPB	0.76	0.0306
3420-21_2	SL004869	Carbonic anhydrase 13	Carbonic anhydrase XIII	Q8N1Q1	377677	CA13	0.73	0.0465
5248-68_2	SL005793	Peptidyl-prolyl cis-trans isomerase F. mitochondrial	Cyclophilin F	P30405	10105	PPIF	0.71	0.0021
3381-24_2	SL010500	Tyrosine-protein kinase Lyn. isoform B	LYNB	P07948	4067	LYN	0.7	0.0149
3038-9_2	SL003326	C-X-C motif chemokine 11	I-TAC	O14625	6373	CXCL11	0.69	0.0241

Supplementary table S2. Continued

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGeneID	Entrez Gene Symbol	2LogFC	FDR
8484-24_3	SL000498	Leptin	Leptin	P41159	3952	LEP	0.69	0.0208
3311-27_1	SL008609	Low affinity immunoglobulin gamma Fc region receptor III-B	FCG3B	O75015	2215	FCGR3B	0.68	1.18E-06
9188-119_3	SL003188	C-X-C motif chemokine 9	MIG	Q07325	4283	CXCL9	0.63	0.0241
4374-45_2	SL003869	Growth/differentiation factor 15	MIC-1	Q99988	9518	GDF15	0.63	0.0003
10370-21_3	SL004396	Signal transducer and activator of transcription 1-alpha/beta	STAT1	P42224	6772	STAT1	0.63	0.0155
3836-51_2	SL010528	Ubiquitin-fold modifier 1	UFM1	P61960	51569	UFM1	0.62	0.0149
4141-79_1	SL003183	C-X-C motif chemokine 10	IP-10	P02778	3627	CXCL10	0.62	0.0245
3453-87_2	SL006917	Tyrosine-protein kinase Lyn	LYN	P07948	4067	LYN	0.61	0.0316
3485-28_2	SL000283	Beta-2-microglobulin	b2-Microglobulin	P61769	567	B2M	0.59	0.0088
3397-7_4	SL010616	Tyrosine-protein phosphatase non-receptor type 11	SHP-2	Q06124	5781	PTPN11	0.58	0.0175
4460-8_2	SL006998	3-phosphoinositide-dependent protein kinase 1	PDPK1	O15530	5170	PDPK1	0.57	0.0487
10365-132_3	SL005184	Interleukin-23	IL-23	P29460. Q9NPF7	3593 51561	IL12B IL23A	0.57	0.0149

Supplementary table S2. Continued

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGeneID	Entrez Gene Symbol	2LogFC	FDR
Downregulated proteins								
4916-2_1	SL000460	Immunoglobulin D	IgD	P01880	3495 50802 3535	IGHD IGK IGL	-1.98	0.0306
4135-84_2	SL000461	Immunoglobulin E	IgE	P01854	3497 50802 3535	IGHE IGK IGL	-1.57	0.0487
4187-49_2	SL000247	6-phosphogluconate dehydrogenase. decarboxylating	6-Phosphogluconate dehydrogenase	P52209	5226	PGD	-0.88	0.0206
3714-49_2	SL000382	Creatine kinase M-type:Creatine kinase B-type heterodimer	CK-MB	P12277 P06732	1152 1158	CKB CKM	-0.86	0.0149
4149-8_2	SL000537	Platelet-derived growth factor subunit B	PDGF-BB	P01127	5155	PDGFB	-0.72	0.0003
3352-80_3	SL010288	Carbonic anhydrase 6	Carbonic anhydrase 6	P23280	765	CA6	-0.7	0.0485
4324-33_2	SL008516	Cystatin-SA	CYTT	P09228	1470	CST2	-0.63	0.0329
3364-76_2	SL006910	Cathepsin L2	Cathepsin V	O60911	1515	CTSV	-0.49	0.0226
4125-52_2	SL003680	Advanced glycosylation end product-specific receptor. soluble	sRAGE	Q15109	177	AGER	-0.49	0.0465
4499-21_1	SL000535	Platelet-derived growth factor subunit A	PDGF-AA	P04085	5154	PDGFA	-0.48	0.0003
5358-3_3	SL008574	Osteomodulin	OMD	Q99983	4958	OMD	-0.46	0.0417

Supplementary table S2. Continued

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGeneID	Entrez Gene Symbol	2LogFC	FDR
4541-49_2	SL014092	Cell adhesion molecule-related/ down-regulated by oncogenes	CDON	Q4KMG0	50937	CDON	-0.44	0.0003
3303-23_2	SL004438	Cystatin-M	Cystatin M	Q15828	1474	CST6	-0.41	0.0316
3213-65_2	SL000640	Nidogen-1	Nidogen	P14543	4811	NID1	-0.4	0.0003
5316-54_3	SL000558	Prothrombin	Prothrombin	P00734	2147	F2	-0.39	0.0189
3175-51_5	SL006610	A disintegrin and metalloproteinase with thrombospondin motifs 13	ATS13	Q76LX8	11093	ADAMTS13	-0.39	0.0149
2475-1_3	SL004010	Mast/stem cell growth factor receptor Kit	SCF sR	P10721	3815	KIT	-0.38	0.0149
11510-31_3	SL005699	Apolipoprotein L1	Apo L1	O14791	8542	APOL1	-0.38	0.0005
3179-51_2	SL009213	Lysosomal protective protein	Cathepsin A	P10619	5476	CTSA	-0.35	0.0378
3535-84_1	SL004367	Dickkopf-related protein 1	DKK1	O94907	22943	DKK1	-0.32	0.0206
3344-60_4	SL000272	Antithrombin-III	Antithrombin III	P01008	462	SERPINC1	-0.32	0.0227
3024-18_2	SL000250	Alpha-2-antiplasmin	a2-Antiplasmin	P08697	5345	SERPINF2	-0.32	0.0003
3035-80_2	SL004354	Interleukin-19	IL-19	Q9UHD0	29949	IL19	-0.32	0.0213
4328-2_2	SL013490	Brother of CDO	BOC	Q9BWV1	91653	BOC	-0.32	0.0465
3633-70_5	SL008193	Nidogen-2	NID2	Q14112	22795	NID2	-0.31	0.0245

Supplementary table S3. Characteristics of IFNpos and IFNneg pSS patients

	IFNpos (n=30)	IFNneg (n=30)	P-value
Demographics			
Female (%)	28/30 (93%)	28/30 (93%)	n.s.
Mean age (years)	58.0 ± 13.7	61.4 ± 10.3	n.s.
Disease duration (years)	12.6 ± 9.4	11.9 ± 7.4	n.s.
Clinical manifestations			
Anti-SSA positivity	30/30 (100%)	14/30 (47%)	<0.0001
Anti-SSB positivity	23/30 (77%)	7/30 (23%)	<0.0001
C3	1.25 (0.18)	1.12 (0.32)	p=0.033
C4	0.23 ± 0.08	0.19 ± 0.07	n.s.
IgG	16.6 (6.3)	10.4 (3.4)	<0.0001
ESSDAI	9.0 (8.1)	8.2 (6.2)	n.s.
Medication status (%)			
Pilocarpine	15/30 (50%)	10/30 (33%)	n.s.
Hydroxychloroquine	18/30 (60%)	21/30 (70%)	n.s.
Corticosteroids	1/30 (3%)	3/30 (10%)	n.s.

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

HC, Healthy controls; pSS, primary Sjögren's Syndrome; ESSDAI, the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index

Supplementary table S4: Differentially expressed serum proteins between IFNpos and IFNneg pSS patients

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGene ID	Entrez Gene Symbol	2LogFC	FDR
Upregulated proteins								
4135-84_2	SL000461	Immunoglobulin E	IgE	P01854	3497. 50802. 3535	IGHE IGK IGL	1.47	0.0245
14151-4_3	SL015510	Ubiquitin-like protein ISG15	UCRP	P05161	9636	ISG15	1.06	0.0009
3038-9_2	SL003326	C-X-C motif chemokine 11	I-TAC	O14625	6373	CXCL11	0.9	0.0036
4407-10_1	SL005202	Hepatocyte growth factor-like protein	MSP	P26927	4485	MST1	0.8	0.0188
4141-79_1	SL003183	C-X-C motif chemokine 10	IP-10	P02778	3627	CXCL10	0.68	0.0343
5099-14_3	SL005195	Lymphocyte activation gene 3 protein	LAG-3	P18627	3902	LAG3	0.63	0.0036
2730-58_2	SL005199	MHC class I polypeptide-related sequence A	MICA	Q29983	4276	MICA	0.59	0.0441
5105-2_3	SL005208	Reticulon-4 receptor	Nogo Receptor	Q9BZR6	65078	RTN4R	0.57	0.0003
3485-28_2	SL000283	Beta-2-microglobulin	b2-Microglobulin	P61769	567	B2M	0.56	0.0213
10361-25_3	SL014684	2’-5’-oligoadenylate synthase 1	OAS1	P00973	4938	OAS1	0.51	0.028
5071-3_3	SL004857	Desmoglein-2	Desmoglein-2	Q14126	1829	DSG2	0.48	0.0119
5077-28_3	SL007179	Ephrin type-B receptor 2	EPHB2	P29323	2048	EPHB2	0.47	0.0003
5028-59_1	SL005764	Scavenger receptor cysteine-rich type 1 protein M130	sCD163	Q86VB7	9332	CD163	0.45	0.0036
2665-26_2	SL004672	Tumor necrosis factor receptor superfamily member 17	BCMA	Q02223	608	TNFRSF17	0.44	0.0267

Supplementary table S4. Continued

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGene ID	Entrez Gene Symbol	2LogFC	FDR
4922-13_1	SL003189	C-C motif chemokine 19	MIP-3b	Q99731	6363	CCL19	0.43	0.022
5000-52_1	SL006522	Galectin-3-binding protein	LG3BP	Q08380	3959	LGALS3BP	0.43	0.0148
5066-134_3	SL014270	CMRF35-like molecule 6	CLM6	Q08708	10871	CD300C	0.4	0.0089
3292-75_1	SL010450	CD48 antigen	CD48	P09326	962	CD48	0.37	0.0003
4930-21_1	SL005789	Stanniocalcin-1	Stanniocalcin-1	P52823	6781	STC1	0.35	0.0459
3152-57_1	SL001800	Tumor necrosis factor receptor superfamily member 1B	TNF sR-II	P20333	7133	TNFRSF1B	0.35	0.0441
2968-61_1	SL004686	Tumor necrosis factor ligand superfamily member 15	TNFSF15	O95150	9966	TNFSF15	0.35	0.013
2950-57_2	SL005171	Insulin-like growth factor-binding protein 4	IGFBP-4	P22692	3487	IGFBP4	0.32	0.0322
3059-50_2	SL004327	Tumor necrosis factor ligand superfamily member 13B	BAFF	Q9Y275	10673	TNFSF13B	0.31	0.0188
4992-49_1	SL007173	Granulins	GRN	P28799	2896	GRN	0.3	0.0148
5349-69_3	SL006970	Delta-like protein 1	DLL1	O00548	28514	DLL1	0.28	0.0111

Supplementary table S4. Continued

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGene ID	Entrez Gene Symbol	2LogFC	FDR
Downregulated proteins								
3427-63_2	SL007324	Casein kinase II subunit alpha	CSK21	P68400	1457	CSNK2A1	-0.77	0.0148
2780-35_2	SL000496	Lactotransferrin	Lactoferrin	P02788	4057	LTF	-0.77	0.0148
2795-23_3	SL002077	Alkaline phosphatase. tissue-nonspecific isozyme	Alkaline phosphatase. bone	P05186	249	ALPL	-0.67	0.0111
13130-150_3	SL007272	Hexokinase-2	HXK2	P52789	3099	HK2	-0.66	0.0465
4306-4_2	SL003655	Transketolase	Transketolase	P29401	7086	TKT	-0.56	0.0321
4258-15_2	SL008331	Proliferation-associated protein 2G4	PA2G4	Q9UQ80	5036	PA2G4	-0.54	0.0475
14116-129_3	SL004821	Protein S100-A4	S100A4	P26447	6275	S100A4	-0.51	0.0111
2879-9_2	SL000248	Alpha-1-antichymotrypsin	a1-Antichymotrypsin	P01011	12	SERPINA3	-0.47	0.0459
4309-59_3	SL004812	Triosephosphate isomerase	Triosephosphate isomerase	P60174	7167	TPI1	-0.45	0.0414
11098-1_3	SL007953	Pyridoxal kinase	PDXK	O00764	8566	PDXK	-0.39	0.0321
3853-56_1	SL008102	Malate dehydrogenase. cytoplasmic	MDHC	P40925	4190	MDH1	-0.38	0.0465
2855-49_2	SL000409	Mitogen-activated protein kinase 3	ERK-1	P27361	5595	MAPK3	-0.36	0.0321
4763-31_3	SL004742	Afamin	Afamin	P43652	173	AFM	-0.24	0.0321

Supplementary table S5. Differentially expressed serum proteins between fatigued and non-fatigued pSS patients

SeqId	SomaId	TargetFullName	Target	UniProt	EntrezGeneID	EntrezGeneSymbol
Upregulated proteins						
13105-7_3	SL007311	Synaptosomal-associated protein 25	SNP25	P60880	6616	SNAP25
2182-54_1	SL000318	Complement C4b	C4b	P0C0L4 P0C0L5	720 721	C4A C4B
14150-7_3	SL005177	Interleukin-36 alpha	IL-1F6	Q9UHA7	27179	IL36A
2755-8_2	SL003220	C3a anaphylatoxin des Arginine	C3adesArg	P01024	718	C3
5019-16_2	SL002803	Ubiquitin carboxyl-terminal hydrolase isozyme L1	PGP9.5	P09936	7345	UCHL1
11105-171_3	SL003650	Alpha-enolase	Alpha enolase	P06733	2023	ENO1
2683-1_2	SL000456	Complement C3b, inactivated	iC3b	P01024	718	C3
11081-1_3	SL007151	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	GPDA	P21695	2819	GPD1
5803-24_3	SL003362	Complement C3d fragment	C3d	P01024	718	C3
8459-10_3	SL003993	Bone morphogenetic protein 6	BMP-6	P22004	654	BMP6
2754-50_2	SL000312	Complement C3	C3	P01024	718	C3
4912-17_1	SL000280	Aspartate aminotransferase, cytoplasmic	GOT1	P17174	2805	GOT1
2864-2_3	SL003793	Dual specificity mitogen-activated protein kinase kinase 1	MEK1	Q02750	5604	MAP2K1
3030-3_2	SL005158	C-type lectin domain family 4 member M	DC-SIGNR	Q9H2X3	10332	CLEC4M
Downregulated proteins						
9213-24_3	SL018900	Formimidoyltransferase-cyclodeaminase	FTCD	O95954	10841	FTCD
5509-7_3	SL000084	Epidermal growth factor	EGF	P01133	1950	EGF

Chapter 8

General Discussion

SUMMARY AND GENERAL CONCLUSIONS

A subgroup of pSS patients has in addition to systemic upregulation of type I IFNs also type II IFN activation. Patients with additional type II IFN activation have higher levels of IgG, ESR and lower levels of lymphocytes compared to patients with only type I or no IFN activation. Furthermore, these patients also have lower Schirmer's test scores compared to healthy controls. Systemic IFN activation correlates with the biological domain of the ESSDAI, but not to any of the other domains or total ESSDAI score.

In cSLE an IFN signature is present in 57% of the patients. This IFN signature is associated with increased expression of TLR7 and cytosolic nucleic acid binding receptors. Blocking of TBK1 signaling reduced type I IFN induced gene expression indicating that these cytosolic nucleic acid binding receptors possibly contribute to the type I IFN production. TBK1 inhibitors may therefore be a promising treatment target for SLE.

Elevated levels of phosphorylated TBK1 are observed in IFN positive pSS, SLE and SSc patients. The TBK1 inhibitor, BX795, significantly downregulates type I IFN-induced gene expression in PBMCs of patients with IFN positive systemic autoimmunity.

In a placebo-controlled trial treatment for 24 weeks with hydroxychloroquine (HCQ) downregulates IFN scores and ISG expression in pSS patients. Treatment with HCQ decreases ESR, IgG and IgM levels similarly in pSS patients with and without systemic IFN activation. Overall, HCQ treatment does not affect disease activity scores (ESSDAI and ESSPRI) and this effect is similar in pSS patients with and without systemic IFN activation.

Using proteomic 'SOMAscan' technology we identify 14 proteins which distinguish fatigued from non-fatigued pSS patients. These proteins are involved in inflammatory mechanisms and have neurological and metabolic functions.

In this chapter we will discuss the recent developments and implications of the findings described in this thesis. Additionally, the limitations and future perspectives are discussed.

WHAT CAN WE LEARN FROM MODULAR TRANSCRIPTOMICS?

Nowadays a wide range of assays for molecular and cellular profiling are available often resulting in large amounts of data [1]. This could help us identify new relevant biological pathways in an unbiased way. However, because of the large number of comparisons the results are susceptible to noise, which can affect data interpretation.

In order to circumvent this problem and structure the data, coordinately expressing transcripts are described [2]. These transcripts are clustered based on their similarity in disease processes and called 'modules'. The modules are labeled based on their primary function using automated literature cohorts. Using this method several research groups described upregulation of 3 IFN annotated modules (M1.2, M3.4 and M5.12) in SLE [3, 4]. As this modular analysis results in complex datasets, we translated these IFN annotated modules in easily applicable 5 gene signatures in **chapter 2**. We assessed these signatures in 2 pSS cohorts.

Due to the complexity of IFN signaling in different cell types and diseases several other groups have tried to capture this complexity in more easily to determine signatures. De Jong et al. identified an IFNα signature (or CG-A cluster) in SLE patients and IFNβ signature (GC-B cluster) in multiple sclerosis patients based on corresponding clusters of IFN regulated genes [5]. Using a ratio between the clusters the IFN expression was analyzed in several other diseases. This could help to identify underlying mechanisms in several diseases, which are either more IFNα or more IFNβ related. Several other studies described co-clustering sets of type I IFN-associated genes based on different leukocyte populations of SLE patients and HCs [6, 7]. In these studies, the authors describe the genes that differentially regulate type I IFN signaling in a cell-specific manner, indicating an extra level of complexity which cannot be captured in paxgene samples of whole blood. Recently, the group of Vital et al. described another IFN scoring system [8]. Genes were selected from the IFN annotated modules described by Chaussabel et al. (M1.2, M3.4 and M5.12) [1, 4], with addition of the genes IFI27 and IFI6 and subjected to factor analysis. A two-factor module explained 84% of the total variance and the 2 factors were called IFN score A and B. In a follow-up paper this two-factor IFN score was able to predict progression from ANA positivity to the development of SLE or pSS in an at risk population [9].

Grouping patients based on gene expression could help to stratify patients, thereby aiding toward personalization of treatment. This is necessary in heterogeneous diseases like pSS and SLE. However, at the moment the different IFN scoring systems are causing confusion. Standardized assessment of type I IFN activation will make it easier to compare different studies.

Interestingly, the transcriptomic studies identified besides upregulation of IFN annotated modules also upregulation of other modules like a plasmablast and neutrophil module in SLE patients [10]. These signatures were present in respectively 21.2% and 48.8% of the patients, while the IFN modules were present in 84.8% of the study cohort. An increased expression of the plasmablast signature in SLE patients was associated with higher disease activity, particularly in patients with African-American background, and showed to be the most robust marker for disease activity in that cohort. Interestingly,

the neutrophil signature was enriched in patients with renal involvement showing a link between neutrophils and active nephritis. Both the plasmablast and neutrophil signature were additionally found in other transcriptomic studies in SLE [6, 11-14]. These observations show that modular signatures can be used for patient stratification and identify underlying immunopathology [12, 15, 16]. Most of the above described studies have been performed in SLE and it should be realized that the heterogeneity between various SLE cohorts and the differences in medication used by the patients require further confirmation in other cohorts. In pSS, the first multi-omics studies identifying new groups of correlating genes in pSS are starting to be performed, more studies in large cohorts with longitudinal data are necessary to confirm these new gene signatures and study their relation with disease activity.

Clinical applicable assays for interferon activity

Transcriptomic data have helped to identify important pathways underlying disease pathology. However, this technique results in large amounts of data and advanced analyses by bioinformaticians to translate the data is required. The upregulated type I IFN expression observed in many systemic autoimmune diseases has already been translated in a simplified IFN score by measuring several IFN inducible genes and using the standardized expression levels [17-19]. Although IFN activity was observed to be linked to disease activity, the systemic type I IFN score in pSS does not always support this, possibly due to the indirect measurement of IFN activity. Furthermore, lack of an uniform set of ISGs to determine the type I IFN score hampers its introduction in clinical practice. Direct quantification of type I IFNs using common ELISA technology is not possible because of the many different subtypes of type I IFNs. Furthermore, the levels of the individual type I IFN subtypes are very low and cannot be detected using common ELISA technology. Therefore, other protein assays that reflect systemic type I IFN activity and can be easily performed in a diagnostic laboratory will be helpful to introduce the IFN signature in clinical practice.

MxA (Myxovirus-resistance protein 1) is a key mediator of the IFN-induced antiviral response and its gene expression is part of the type I IFN gene signature [17, 18]. In pSS, SLE and SSc the MxA enzyme immunoassay (MxA-EIA), measuring MxA in lysed whole blood, shows a good correlation with the systemic type I IFN score ($r_s>0.75$, $p<0.001$) [20](Huijser et al, submitted for publication). Furthermore, MxA-EIA has a good discriminatory capacity between high and low type I IFN activity with high specificity and sensitivity (AUC=0.938 to AUC=0.991, $p\leq0.007$) (table 1). Therefore, the MxA-EIA could be an affordable and easy tool to access systemic type I IFN activity in a routine diagnostic setting and it might be helpful in clinical decision making in the future.

In SLE several other biomarkers for type I IFN activity like galactin-9 (gal-9), CXCL10 and Siglec-1 have been studied. Gal-9 is a protein that elevates HLA-DR and costimulatory molecule expression during the maturation of DCs. Gal-9 protein levels in the serum correlated with SLE disease activity and type I IFN score (rs=0.70, p<0.001) [21]. However, the sensitivity and specificity were lower than for the MxA-EIA (respectively 84% and 72%; table 1). No literature on the correlation of gal-9 protein levels and type I IFN score in pSS are available yet. In pSS, elevated levels of CXCL10 (or IP10; interferon-γ-inducible protein 10) are described, particularly in the salivary glands. CXCL10 is induced by IFNγ, however it is also inducible by type I IFNs [19, 22]. In 2 SLE cohorts, serum levels of CXCL10 were found to correlate with type I IFN scores, although less strong than MxA or gal-9 (respectively rs=0.52, p<0.001 and rs=0.54, p<0.001) [21, 23]. CXCL10 did not correlate with disease activity in SLE and pSS [21, 24]. Lastly, Siglec-1 (CD169, sialoadhesin) is particularly upregulated by type I IFNs and could therefore be an interesting biomarker for type I IFN activity. It is a surface protein with restricted expression on monocytes and macrophages and is involved in cell-cell interactions and antigen presentation. In SLE and pSS Siglec-1, analyzed by flow cytometry, was shown to correlate with the type I IFN signature and disease activity [20, 25].

Recently, a digital ELISA method that detects IFNα levels at attomolar concentrations (femtograms per milliliter) and has a 5000-fold increase in sensitivity over commercial ELISAs has been described [26]. IFNα protein levels measured in the serum and CSF by this new digital ELISA technology strongly correlated to ISG expression measured [26]. Furthermore, elevated levels of IFNα were observed in SLE patients with higher disease activity and a higher presence of autoantibodies. This technique quantifying IFNα protein levels directly is promising for the future, however whether this technique is suitable in clinical practice and the monitoring of therapies remains questionable as it is still highly laborious and expensive.

Table 1. ROC analyses of easy applicable assays for systemic IFN activation in SLE

	AUC	Sensitivity	Specificity	PPV	NPV	Reference
MxA	0.97	91.5%	100%	100%	83.8%	Huijser et al, submitted for publication
Gal-9	0.84	84%	72%	91%	59%	[21]
CXCL10	0.75	57%	78%	89%	36%	[21]
Siglec-1	n.p.	n.p.	n.p.	n.p.	n.p.	

PPV, positive predictive value; NPV, negative predictive value; n.p., not performed

IMMUNOPATHOGENESIS OF INTERFERON POSITIVE SYSTEMIC AUTOIMMUNITY

Chronic upregulation of type I IFNs is described in several systemic autoimmune diseases among which is pSS. In this thesis we study the immunopathogenesis of type I IFNs in pSS, SLE and SSc. Here we describe three important pathways which can result in chronic type I IFN activation: the toll-like receptors (TLR), RNA sensing Rig-like receptors (RLRs) and DNA sensing receptors (DSR). A schematic representation of the contribution of these three pathways to the induction of type I IFNs is shown in **chapter 3** in figure 1. Chronic activation of type I IFNs, via these three pathways, results in damage to cells and the release of their content. Inadequate clearance of the released cell content, as has been demonstrated in several systemic autoimmune diseases, results in an abundant presence of self-RNA and DNA molecules. Subsequently, these self-nucleic acids can trigger as ligands TLR, RNA and DNA sensing receptors. Additionally, these self-nucleic acids are triggering autoreactive B cells to produce autoantibodies against these self-nucleic acids, leading to the formation of immune complexes (ICs). These ICs subsequently activate pDC via the TLRs followed by induction of type I IFNs. These type I IFNs induce upregulation of costimulatory molecules and production of B-cell activating factors by APCs activating adaptive immunity. This will ultimately result in more damage to the cells and increased self-ligands inducing a self-amplifying loop.

TBK1: role in systemic autoimmunity and beyond

The role of TLRs in systemic autoimmunity is supported by animal models and extensively described in the literature [27-33]. We will focus in this thesis on the more recently described possible contribution of RNA and DNA sensing receptors to systemic autoimmunity and discuss how inhibition of these receptors possibly could interfere in the self-amplifying loop of IFN activation in systemic autoimmunity. RLR and DSR induce type I IFNs via TBK1. TBK1 can also be induced via TLR3 and 4, but in pDCs, considered the culprits of type I IFN production in systemic autoimmune diseases, these TLRs are not present. In **chapter 3** and **4** we describe how blocking of TBK1 function, and its related structural homologue IKKε, was able to reduce expression of type I IFN induced genes [34, 35]. These data indicate a role for RNA and DNA sensing receptors in chronic IFN upregulation in IFNpos systemic autoimmunity and additionally provide a possibility to block this pathogenic signaling.

In addition to its role in type I IFN signaling TBK1 has other functions. TBK1 is widely expressed in both hematopoietic and non-hematopoietic compartments. Germline deletion of TBK1 is embryonically lethal in mice demonstrating an important function

during development [36]. Recently, a role for TBK1 in germinal center (GC) responses and humoral immunity was described. In the GC reaction B cells undergo somatic hypermutation, class switching and affinity maturation in order to form high-affinity antibodies. Only after completion of this process B cells can exit the GCs and function as long-lived B cells or plasma cells [37]. Defects in the GC reaction can lead to a breakdown of B cell tolerance and induction of (auto)immune response. T follicular (T_{FH}) cells play an important role in GC reactions. Loss of these cells has been linked to the development of humeral autoimmunity. Interestingly, TBK1 was recently shown to be important for T_{FH} development [38]. In this study the authors describe how TBK1 associates with the inducible T cell co-stimulator (ICOS) via a conserved motif, IProx. ICOS-ICOSL pairing is important for good T_{FH} development and knock out of ICOS in mice results in deformed GCs, impaired humoral responses and lack of immunological memory [39-42]. TBK1 depletion or alteration of IProx in T cells impaired the differentiation of T_{FH} cells and the development of GCs. This resulted in impairment of B cell differentiation and disruption of the development of antibody responses. In SLE several defects in selection of B cells in GC as well as an increase in T_{FH} cells has been observed [43]. These data indicate that besides the beneficial effects of TBK1 inhibitors on the blocking of type I IFN production in systemic autoimmunity, there might be additional beneficial effects of these inhibitors in the impairment of the production of autoantibodies by autoreactive B cells.

Besides the role of TBK1 in type I IFN signaling and GC formation it also functions in autophagy and cell metabolism. Autophagy is a mechanism to disassemble dysfunctional or unnecessary proteins in cells. TBK1 appears to be involved in autophagy-mediated killing of several bacteria [44-46]. Furthermore, TBK1 has a role in cell metabolism as ligation of TLRs increases glycolysis in DCs via TBK1. Chronic upregulation of TBK1 was shown to suppress mTORC1 activity, leading to dysregulated glycolysis [47, 48]. Recently, TBK1 was identified as an important molecule at the crossroad between energy metabolism and inflammation in adipose tissue [49] and several mice studies indicate a beneficial role for TBK1 inhibitors in obesity-related metabolic dysfunction [50, 51]. Interestingly, TBK1 also appears to be involved in activation of microglia and correlates with the release of proinflammatory cytokines in metabolic brain regions which were linked to insulin resistance [52]. Although the exact mechanisms of TBK1 in glycolysis and neuroinflammation are beyond the scope of this thesis, we need to keep in mind that inhibition of TBK1 can have widespread effects. These data support further studies into the relation between obesity and autoimmunity and the possible role for TBK1 in this relation.

STING: pro- or anti-inflammatory

Several of the members of the large family of cytosolic DNA sensors signal via STING and induce type I IFNs via TBK1 and IRF3 signaling (figure 1A). The important role of STING in the defense against DNA viruses and bacteria has extensively been described [53, 54]. In patients with a gain-of-function mutation in STING (SAVI patients) a chronic upregulation of type I IFNs has been observed [55]. Interestingly, evidence is accumulating for a role of these DNA sensors and the STING pathway in SLE [56]. For pSS this was also confirmed in a mouse model [57]. In this study C57BL/6 mice were injected with a STING agonist, DMXAA (dimethylxanthenone-4-acetic acid), which induced systemic proinflammatory responses. DMXAA treatment resulted in upregulation of proinflammatory cytokines and type I IFN activation in the salivary glands of the mice. Furthermore, the mice developed pSS-like symptoms like salivary gland hypofunction. In this thesis we provide the evidence for a role of this pathway in the pathogenesis of pSS [34]. Defects in DNA degradation and accumulation of nucleic acids can trigger type I IFN production [58]. Elevated levels of dsDNA were found in SLE serum and were shown to induce IFN production via the STING pathway [56, 59]. Interestingly, a recent study indicated a role for IFNα in impairment of degradation of mitochondrial DNA (mtDNA). This can subsequently lead to cytosolic accumulation of mtDNA and production of proinflammatory mediators via STING [60]. These data indicated that accumulation of self-DNA might lead to type I IFN activation via STING, thereby contributing to the pathogenic chronic IFN activation in IFNpos systemic autoimmunity.

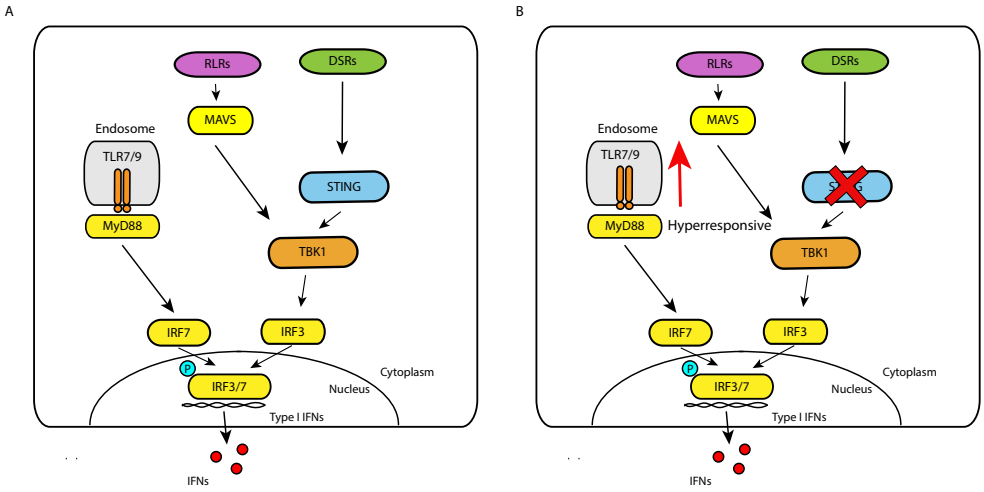


Figure 1. Schematic overview of the TLR, RLR and DSR signaling pathway
(A). Knocking out STING resulted in TLR7 and 9 hyperresponsiveness via unknown pathways **(B)** [61]

Although STING activation can lead to type I IFN signaling, in autoimmune prone MRL-lpr mice knock out of STING resulted to a more severe autoimmune phenotype than in STING sufficient animals [61]. Furthermore, IRF3 (an important transcription factor downstream of STING inducing type I IFN) was not required for this STING-mediated immune suppression, as both the sufficient and KO mice developed similar disease levels. Additionally, intraperitoneal injection of pristane resulted in a more severe TLR-dependent inflammatory response in STING−/− mice than in STING sufficient mice (figure 1B). These data indicate that STING also has immunosuppressive properties. There are a number of potential anti-inflammatory mechanisms described. One might be the induction of M2 (anti-inflammatory and wound healing) macrophages, which STING was able to induce via STAT6 signaling [62, 63]. Furthermore, STING was also shown to inhibit dsDNA-triggered signaling of JAK1-STAT1 via SHP-1 and SHP-2 phosphorylation [64]. Also STING deficiency is associated with a reduction in Treg numbers in the secondary lymphoid organs. This effects is possibly mediated via IDO (this molecule will be discussed later) [61]. There are also other potential suppressors of type I IFNs described downstream of STING, like NIK and p52 [65]. More research is necessary to better understand the delicate balance between pro- and anti-inflammatory functions of STING and how they relate to the pathogeneses of systemic autoimmunity.

Inflammasome activation in systemic autoimmunity

Activation of DSR might contribute via a different pathway to systemic autoimmunity. Two of these DSRs, IFI16 and AIM2, have been shown to trigger activation of the inflammasome. This is a multimodular complex, which after oligomerization stimulates caspase-1 activation and leads to the production of the proinflammatory cytokines IL-1β and IL-18. Both in pSS and SLE activation of the inflammasome is thought to be involved in the pathogeneses [66-68]. IFI16 is a unique DNA sensor as it can shuttle between the nucleus and cytoplasm and is able both to stimulate type I IFN production and inflammasome activation. Elevated levels of IFI16 and anti-IFI16 antibodies are described in several systemic autoimmune diseases, including pSS [69-73]. Triggering via AIM2 also results in inflammasome activation, but does not lead to type I IFN production. Whether AIM2 is involved in the pathogeneses of systemic autoimmunity is unknown. In lupus-prone mice, AIM2 knock down is described to both activate and inhibit SLE disease progression [74, 75]. A recent study looking at inflammasome activation in PBMCs of pSS patients did not reveal elevated levels of AIM2 transcripts compared to HCs [66]. Interestingly, they do find upregulation of inflammasome-related genes ASC/PYCARD, the NLRP3 (a different type of inflammasome backbone), pro-caspase1, pro-IL-1β and pro-IL-18 in pSS patients compared to HCs. This NLRP3 inflammasome activation was particularly observed in pSS patients with a high disease

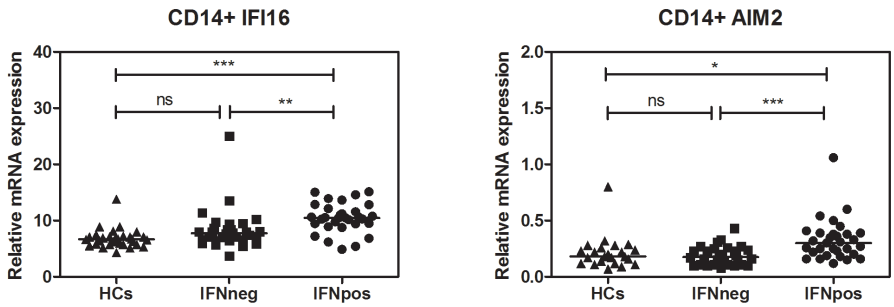


Figure 2. Significant upregulation of IFI16 and AIM2 mRNA levels in monocytes of IFN positive pSS patients (unpublished results)

activity. In our cohort we unfortunately did not study NLRP3, however when looking at IFI16 and AIM2 mRNA levels in monocytes of IFN positive pSS patients we find a small but significant upregulation of both (figure 2). This suggests the presence of intracellular and extracellular deposits of proinflammatory DNA in pSS, which triggers the activation of these inflammasomes. There are also indications that the type I IFN pathway and AIM2 inflammasome pathway inhibit each other [76, 77]. These results suggest that activation of DNA sensors can besides pathogenic triggering of type I IFNs also result in inflammasome activation, although it is still unclear how these pathways are exactly regulated in systemic autoimmune diseases.

Relation between systemic and local IFN score?

In this thesis we focus on systemic upregulation of IFNs, however also locally in the salivary glands of pSS patients upregulation of both type I and type II IFN has been observed [78, 79]. Data on a possible correlation between local versus systemic IFN activation are limited. Salivary gland analysis of pSS patients revealed a predominant type II activation pattern, while systemically activation of type I IFNs is more prevalent [17, 19, 78]. This indicates that local and systemic IFN activation patterns within the same patient may differ. To study this correlation peripheral blood and minor salivary gland tissue must be obtained simultaneously from the same patients. We performed a small pilot study on peripheral blood samples (obtained from the SICCA Registry) from pSS patients of which the salivary glands were previously analyzed for IFN activation [78, 79]. In this labial salivary gland (LSG) tissue local upregulation of IFN type I (α) and II (γ) was determined by IFIT3 and GBP1 protein expression. We analyzed the monocytes of these patients, collected at the same time as the biopsies, and determined the IFN type I and IFN type I+II signatures that are described in **Chapter 2** (figure 3A).

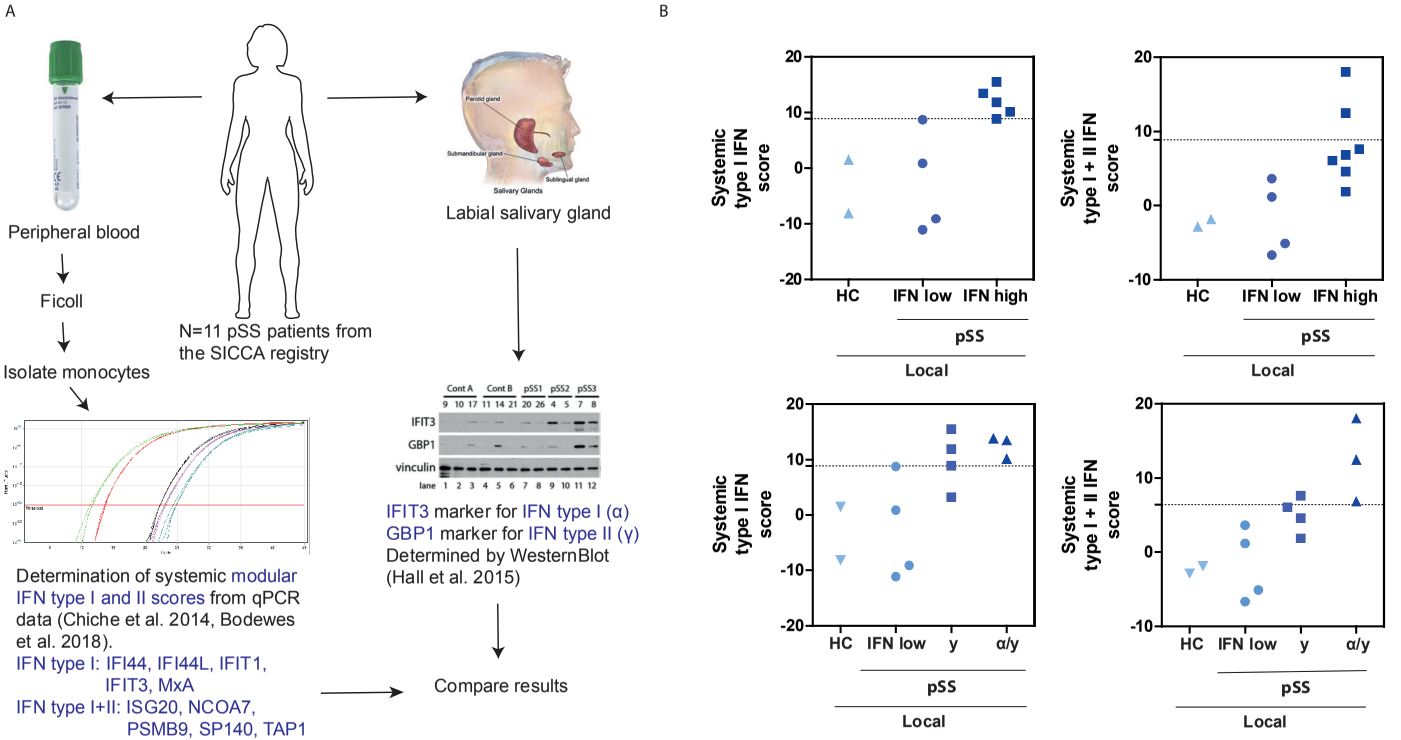


Figure 3. Relation local and systemic IFN activation
Schematic overview of the set-up of this small pilot study **(A)**. Systemic type I IFN and I+II IFN activation (monocytes in peripheral blood) and local upregulation of IFN type I (α) and II (γ) (salivary gland) of pSS patients (n=11) **(B)**. Dotted lines indicate the cut-off for systemic positivity of IFN score.

In salivary gland tissue IFN levels were classified as IFN high for the presence of IFN type I or II and IFN low indicated for a low expression of IFN type I or II. Interestingly, pSS patients with local glandular IFN activation also had systemically higher IFN scores (figure 3B). This small study indicates that systemic IFN activation might therefore be a useful biomarker to monitor local inflammation. Future studies are required to unravel the relationship between local and systemic upregulation. If the systemic IFN activation is a good reflection of the local IFN activation assessment of the systemic IFN signature could prevent (repeated) salivary gland biopsies for instance in clinical trials.

BIOLOGICAL PATHWAYS UNDERLYING FATIGUE IN PSS

Fatigue is one of the most common extraglandular manifestation in pSS and is associated with a poor quality of life [80-85]. It affects up to 70% of pSS patients, while approximately 20% of healthy population is affected [86-89]. Fatigue is a complex, multifactorial phenomenon with a variable severity defined as "an overwhelming sense of tiredness, lack of energy and feeling of exhaustion" [90]p@. It is a common compliant in the general population [91], but also a prominent symptom of many diseases, such as pSS [92]. In the recently published clinical practice guidelines for pSS, the recognition of fatigue, pain and cognitive dysfunction in pSS are the unmet needs according to patients [93]. Despite the increasing interest in fatigue research, the underlying biological basis of fatigue remains poorly defined [92]. There are several important components like cytokines thought to contribute to fatigue in pSS patients.

A role for cytokines in fatigue?

Pro-inflammatory mechanisms have been suggested to play a central role in the development of fatigue. This suggestion is supported by the observation of fatigue in conditions with immune dysregulation, such as the post-infective syndrome [94]. During inflammatory states or infection, proinflammatory cytokines act on the brain and induce a behavioral response called 'sickness behavior' [95]. This behavioral response is characterized by drowsiness, loss of appetite, decrease in activity and withdrawal from social interaction [96]. In humans fatigue could be considered part of this response. In pSS several proinflammatory cytokines like IL-6, IL-1β, TNF-α, IL-10 and type I IFNs, which are in the literature linked to fatigue, are upregulated. However, in pSS serum concentrations of these cytokines did not correlate with fatigue levels (assessed using the MFI questionnaire) [97]. A more recent study described an inverse relationship between fatigue scores and four pro-inflammatory cytokines (IP-10, TNFα, LTα and IFNγ) [88]. Additionally, we and others have shown that pSS patients with systemic upregulation

of IFNs are not more fatigued compared to patients without IFN type I upregulation [98, 99]. Although there might still be a role for cytokines in fatigue induction, there is no correlation between direct measures of cytokines in the serum or their induced gene expression and fatigue as measured by questionnaires indicating towards other biological pathways.

Kynurenine pathway: linking peripheral immune activation and neuroinflammation

It is well-established that there is a relationship between immune activation, inflammation of the brain and fatigue and depression [100, 101]. Activation of the peripheral immune system by for instance LPS can induce sickness behavior and activate microglial cells, which are innate immune cells of the brain [102]. An interesting pathway which is thought to be involved in linking peripheral activation of the immune system and neuroinflammation is the kynurenine (KYN) pathway. This pathway is upregulated during an immune activation and catabolites of this pathway can cross the blood-brain-barrier. Interestingly, proteins of the kynurenine pathway have been found elevated in pSS serum [103]. This metabolic pathway represents a major route for the metabolism of tryptophan (Trp) and was recently highlighted as a mechanism of central fatigue [104]. Trp can be converted into KYN by indoleamine 2,3-dioxygenase 1 (IDO1), the related enzyme IDO-2 or tryptophan 2,3-dioxygenase (TDO) (figure 4). TDO is primarily found in the liver, while IDO1 and IDO2 can be produced by many cell types both in the periphery and in the brain. KYN can further be converted into kynurenic acid (KYNA) or via 3-hydroxyanthranilic acid (3-OH-AA) converted quinolinic acid (QA) or picolinic acid. These kynurenines have been shown to affect neuronal function via among others N-methyl-D-aspartate (NMDA) receptors and can induce local inflammation in the brain [105]. Furthermore, Trp also acts as a precursor for the synthesis of serotonin, which affects mood and cognitive functioning. Therefore, depletion of Trp can lead to deletion of serotonin. Although it should be realized that the synthesis of serotonin only accounts for a small part of Trp metabolism, while over 90% is metabolized in the kynurenine pathway. Many central nervous system disorders like neurogenerative disorders (Parkinson's and Alzheimer's disease), psychiatric disorders (major depression, schizophrenia, ADHD), infectious diseases (HIV-associated cognitive disorder) and autoimmune disorders (multiple sclerosis) are associated with imbalances of the KYN pathway. Aberrances in the kynurenine pathway are also observed in systemic autoimmune diseases [103, 104, 106, 107].

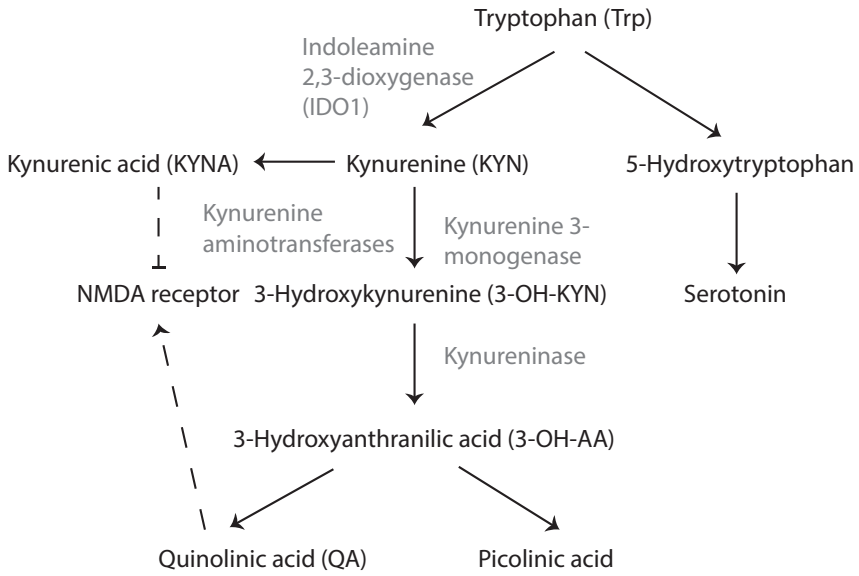


Figure 4. Schematic overview of tryptophan catabolic pathway

Dysregulated IDO activity in pSS

The first and rate limiting step in the kynurenine pathway is catabolized by IDO. IDO can be affected by IFNs, LPS and several proinflammatory cytokines like TNFα, IL-1, 2 and 6 [108, 109]. In pSS patients with systemic IFN activation increased IDO1 mRNA levels were observed in CD14+ monocytes. Furthermore, in the serum of IFNpos pSS patients higher KYN/Trp ratios, as a measure for IDO activity, were found compared to IFN negative pSS patients or HCs [103]. In addition to IDO1, also other enzymes in this pathway were dysregulated and IDO activity has been linked to pSS disease activity [107, 110]. In SLE KYN/Trp ratios were elevated in patients with a high disease activity, while there were no differences between overall SLE patients and HCs [104]. The KYN/Trp ratio only weakly correlated with severe fatigue.

Kynurenine pathway and fatigue

Activation of the immune system and production of proinflammatory cytokines lead to depletion of Trp and production of KYN. KYN is readily transported over the blood-brain barrier where microglia, astrocytes and perivascular macrophages generate neuroactive kynurenines [100]. KYNA is an antagonist and QA an agonist of the NMDA receptor. KYNA can furthermore inhibit α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and α7 nicotinic acetylcholine receptors (α7nAChRs) receptors and overall is thought to have neuroprotective effects [111]. QA has mainly

neurotoxic effects and is a proinflammatory mediator. In pSS kynureninase, the enzyme which converts 3-hydroxykynurenine (3-OH-KYN) into 3-OH-AA leading to an increase in QA, is upregulated while kynurenine aminotransferases leading to the production of KYNA are downregulated [112]. These data indicate a shift toward more neurotoxic catabolites in pSS.

Imbalance of NMDA and AMPA receptors is thought to affect cognitive function and has been suggested to be involved in development of fatigue [113-115]. Upon induction of central fatigue in a rat model, Trp and KYN were shown to be taken up into the brain and metabolized into KYNA in presynaptic neurons of the hypothalamus, hippocampus, and cerebral cortex [116]. In humans increased levels of plasma KYN were associated with exhaustion of athletes and in hemodialysis patients plasma KYN levels correlated with worse fatigue and depression scores [106, 117]. These data suggest a role for the KYN pathway in fatigue. Interestingly leflunomide, an anti-inflammatory drug recently tested as a treatment for pSS, blocks the conversion of KYN to 3-OH-KYN and shifts the balance to the production of neuroprotective KYNA [118]. Preliminary data indicate that leflunomide treatment was found to reduce general fatigue in pSS patients [119]. Overall these studies indicate that the kynurenine pathway might be involved in fatigue but future studies are needed to confirm this.

Hypothalamus-pituitary-adrenal axis

In addition to the kynurenine pathway a dysregulated hypothalamus-pituitary-adrenal (HPA) axis is proposed to be linked to fatigue [92, 96]. Many symptoms often reported in pSS resemble that of hypoadrenalism such as fatigue, depression, arthralgia and myalgia indicating that an altered HPA axis could be involved in pSS pathogenesis. The HPA axis is regulated by cortical, autonomic and sensory input. Proinflammatory cytokines are described to affect this axis both directly and indirectly [120-122]. Interestingly, in pSS a hypofunctional HPA axis is described [122]. In other diseases such a dysregulation is linked to fatigue and depression [123, 124]. However, in pSS so far no causative role for dysregulation of this pathway in fatigue has been described indicating the need for more in depth studies.

Proteomics to identify pathways underlying fatigue

Proteomics is the large-scale study of proteins in biological samples and recently new proteomic techniques are becoming available. The lack of knowledge on the biological basis of fatigue indicates the need for novel technologies. So far a single abstract using label-free shotgun mass spectrometry to investigate cerebrospinal fluid (CSF) of patients with pSS described the differential expression of 15 proteins when comparing. Most proteins detected in this study have a function in the regulation of innate immunity,

cellular stress defense or the central nervous system [125]. In **chapter 7** we used a novel proteomics technology to study fatigue in pSS using the SOMAscan platform. Here we determined over 1300 proteins in serum samples of fatigued and non-fatigued pSS patients and identified the differential expression of 14 proteins. These proteins are involved in inflammatory mechanisms and had neurological and metabolic functions. Interestingly, comparing our list of proteins with those detected in CSF, complement factors were found in both studies. Although in our study it is likely that complement levels are reduced in non-fatigued patients as opposed to elevated levels in fatigued patients. These new proteomic technologies will hopefully give more insight in the biological mechanisms underlying fatigue.

Limitations of measures for disease activity in pSS

An important limitation in studying the pathogenesis of pSS in relation to disease activity is the lack of good measures to detect differences in disease activity. This a problem we encountered when analyzing the data of **chapter 5**. To study systemic disease activity in pSS the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) is used [126]. Although described to be sensitive to change, the use of this tool for longitudinal analysis is controversial [127-129]. The ESSDAI consists of 12 domains, of which 11 are related to organ involvement and has one biological domain reflecting B-cell activity. The score is supposed to reflect disease activity and not damage, therefore only manifestations should be scored that are 'new' or have worsened [130]. However, when a patient manifestation persists until the next evaluation point this manifestation may not be scored as active. So although there is no change in the patient's condition, the disease activity score will reduce. A paper discussing the ESSDAI's sensitivity to change mentions this problem [128]. In this particular study the limitation of the ESSDAI scores was solved by determination of the scores without a reference to a previous assessment. This confusion indicates that an uniformal guideline to score the ESSDAI is necessary and will help to correctly interpret the data.

The ESSDAI is a tool designed to asses systemic disease activity, however only around 30% of the pSS patients have systemic activation of their disease. In trials testing new medication in pSS, primarily patients with high ESSDAI scores (often over 5) are included. If the medication is supposed to interfere specifically with the systemic manifestation of pSS this set up is correct, however only the minority of the pSS patients will benefit. For trials testing medication for the general pSS population (with ESSDAI scores usually below 5) a change in ESSDAI score cannot be used, because the score will be already low at baseline. Therefore, in clinical trials for pSS it is important to keep in mind the intended pSS population and use appropriate outcome measures to determine whether the treatment is effective.

FUTURE PERSPECTIVE

HDACs: a new mechanism to block IFN production

A possible mechanism targeting IFN production by pDCs (not described in **chapter 6**) is the use of histone deacetylase inhibitors. Histone deacetylases (HDAC) are epigenetic regulators of gene transcription. By the removal of acetyl groups from histones and other nuclear proteins they induce chromatin condensation and suppression of transcription [131]. HDACs can be grouped into four: class I (involving HDAC1-3 and 8), class II (involving HDAC4-7, 9 and 10), class III (also known as sirtuins) and class IV (HDAC11) [132]. Trichostatin A (TSA) is an inhibitor of class I and II HDACs and was shown to inhibit the production of type I IFN and other proinflammatory cytokines of activated pDCs [131]. Furthermore, TSA inhibited IFNα production by pDC stimulated with serum from SLE patients. Recently, we observed an upregulation of HDAC 6, 8 and 9 in whole blood of pSS patients (figure 5) and both TSA and two HDAC6 specific inhibitors were able to block the imiquimod induced MxA expression in PBMC cultures (unpublished data). Although these data are still preliminary, they indicate the potential of these drugs, already used in other clinical conditions, as a possible future treatment for pSS and other IFN positive systemic autoimmune diseases.

Is there a role for gut microbiota in development and progression of systemic autoimmunity?

Recent insights show that intestinal microbiota shape the immune response in- as well as outside the gut. Studies in germ-free animals have shown extensive deficits in the gut-associated lymphoid tissues (GALT) and antibody production. This suggests that in the absence of the normally present microbiota, or so-called commensals, normal development and function of the immune system is hampered. This close interplay between the immune system and gut microbiota led to the study of the role

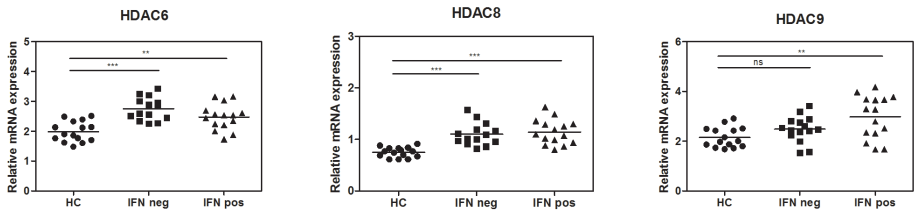


Figure 5. Relative expression of histone deacetylases (HDACs) in PAXgene samples of 15 healthy control (HC), 15 IFN negative (IFN neg) and 15 IFN positive (IFN pos) pSS patients

of commensals in autoimmunity [133]. In mouse models for RA, a significant reduction in disease activity in the absence of commensal bacteria in association with a reduction of Th17 cells was observed. Upon monocolonization of these mice with segmented filamentous bacteria (SFB), levels of Th17 cells increased in the lamina propria and the spleen and the arthritic phenotype was restored [134].

On the other side there are also protective effects of commensals on autoimmunity described. The non-obese diabetic (NOD) mice is a model for type I diabetes, but also develops lymphocytic infiltrations in the salivary and lacrimal glands mimicking pSS pathogenesis [135]. In this model diabetes progression is exacerbated when the animals are housed in germ-free conditions, particularly in females [136]. Interestingly, MyD88$^{-/-}$ NOD were protected from diabetes, but only in the presence of commensals as animals housed under germ-free conditions did develop diabetes. This indicates that exposure to bacterial antigens and infection decreased the risk of NOD mice to develop diabetes. However, how the gut microbiota prevent autoimmunity in distant organs is largely undefined. Systemically, microbial metabolites were shown to stimulate systemic Treg functions in NOD mice [137]. Furthermore, a recent study revealed an important interaction of gut-microbiota-derived metabolites on the control of the local immune response in the pancreas of NOD mice [138]. Whether there was also an effect on the salivary and lacrimal glands was not studied. Collectively, these mouse models show that microbiota and their metabolites can affect immune function, positively and negatively [139]. Human studies show some alterations in gut microbiota in patients with SLE or pSS compared to HCs [140-143]. But whether there is a role for intestinal microbiota in the pathogenesis of systemic autoimmunity in humans is still unknown and more studies are needed to study these complex interactions.

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Addendum

ABBREVIATIONS

ANAs	anti-nuclear antibodies
Anti-SSA/B	anti-Sjögren's syndrome-associated autoantigen A/B
APC	antigen-presenting cell
APRIL	a proliferation-inducing ligand
APS	antiphospholipid syndrome
BAFF	B-cell activating factor
BCR	B-cell receptor
BDCA	blood dendritic cell antigen
C	complement
CD	cluster of differentiation
CES-D	center for epidemiologic studies depression
cGAS	cyclic GMP-AMP synthase
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DEP	differentially expressed proteins
DMARDs	disease-modifying antirheumatic drugs
DNA	deoxyribonucleic acid
DSR	DNA-sensing receptors
EIA	enzyme immune assay
ELISA	Enzyme-Linked ImmunoSorbent Assay
ESR	erythrocyte sedimentation rate
ESSDAI	EULAR Sjögren's syndrome disease activity index
ESSPRI	EULAR Sjögren's syndrome patient-reported index
EULAR	European League Against Rheumatism
FACS	fluorescence activated cell sorting
FcR	Fc receptor
FOXP3	forkhead box P3
GAS	IFNγ-activated sites
GC	germinal center
HAA	3-hydroxy-anthranilic acid
HADS	hospital anxiety and depression scale
HC	healthy control
HCQ	hydroxychloroquine
HDAC	histone deacetylases
HLA	human leukocyte antigen
ICOS	inducible T cell co-stimulator
ICs	immune complexes
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFNAR	type I IFN receptor
Ig	immunoglobulin
IKKε	inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL	interleukin
IRF	IFN regulatory factor
ISGs	IFN-stimulated genes
ISREs	IFN-stimulated response elements
JAK	janus kinase
KYN	kynurenine
KYNA	kynurenic acid
mAB	monoclonal antibodies

MAVS	mitochondrial antiviral signaling protein
MFI	multiple fatigue inventory
MHC	major histocompatibility complex
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NKT	natural killer T cells
NOD	non-obese diabetic
PAMPs	pattern-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
pDC	plasmacytoid DC
PROFAD-SSI	profile of fatigue and discomfort-sicca symptoms inventory
pSS	primary Sjögren's syndrome
QA	quinolinic acid
RA	rheumatoid arthritis
RF	rheumatoid factor
RFU	relative fluorescence units
RLR	retinoic acid-inducible gene I receptors
RNA	ribonucleic acid
RQ-PCR	real-time quantitative PCR
SD	standard deviation
SGECs	salivary gland epithelial cells
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
SNP	signal nucleotide polymorphism
snRNPs	small nuclear ribonucleoproteins
SOMAmers	slow off-rate modified aptamers
SSc	systemic sclerosis
STAT	signal transducers and activators of transcription
STING	stimulator of interferon genes
TACI	transmembrane activator and CAML interactor
TBK1	TANK-binding kinase 1
Tc	T cytotoxic cell
TCR	T-cell receptor
TDO	tryptophan 2,3-dioxygenase
Tfh	follicular helper T cell
Th	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory Th cell
Trp	tryptophan
VAS	visual analogue scale
WT	wild type

SUMMARY

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by infiltrations of immune cells in the salivary and lachrymal glands. Characteristic symptoms are dryness of the eyes and mouth. Besides these local symptoms, patients can additionally suffer from extraglandular manifestations including fatigue, joint pain, muscle pain and Raynaud's phenomenon. In some patients the disease can also affect internal organs like the lungs or kidneys. Of these extraglandular symptoms, fatigue is the most prevalent affecting around 70% of the pSS patients. Like in most autoimmune diseases, pSS affects mainly females and the average age at diagnosis is between 40 and 60 years. Type I interferon (IFN) upregulation **(chapter 1)** is a hallmark of several systemic autoimmune diseases, and is also observed in systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). In this thesis we aim to unravel the mechanism leading to the increased production of these IFNs and study the efficacy of an inhibitor on the production of IFNs. Furthermore, we study the link between IFNs and fatigue, and explore biological pathways underlying this symptom.

In **chapter 2** we studied in two European pSS cohorts the presence of three IFN annotated modules, previously identified in SLE using modular transcriptional analysis. This analysis revealed that besides the characteristic systemic upregulation of type I IFN, also upregulation of a type II IFN inducible module was present in a subgroup of the pSS patients. This module was never upregulated without the presence of the type I IFN inducible module. Patients with type I plus type II IFN activation showed higher IgG and ESR levels and lower lymphocyte counts compared to only type I IFN positive and IFN negative patients. Furthermore, these patients had lower Schirmer's test scores compared to IFN negative patients. These results indicated that patients with upregulation of multiple IFN inducible modules have a more severe disease activity. These modules also correlated with the biological domain of the ESSDAI (EULAR Sjögren's Syndrome Disease Activity Index), but not the total ESSDAI score. Therefore, the biological domain might be a more sensitive endpoint for IFN targeting therapies.

In **chapter 3** we study the prevalence of type I IFN activation in a cohort of patients with childhood-onset SLE (cSLE). Although the disease pathogenesis is similar to adult-onset SLE, cSLE is often more severe and has a poorer prognosis. A positive IFN score (IFNpos) was detected in 57% of the cSLE patients. Monocytes of these IFNpos cSLE patients showed an upregulated gene expression of Toll-like receptor (TLR)7, RIG-like receptors (RLRs; IFIH1, DDX58, DDX60, DHX58) and DNA-sensing receptors (DSR; ZBP-1, IFI16). Additionally, at the protein level an upregulation of RIG-I and ZBP-1 was observed. *In vitro* addition of a inhibitor for TANK-binding kinase 1 (TBK1) (BX795), which interferes with IFN induction via RNA- and DNA-sensing receptors, was able

to block spontaneous expression of type I IFN stimulated genes (ISGs). These results indicated that IFN positivity in cSLE was associated with an upregulated expression of TLR7, and other RNA- and DNA-sensing receptors. As blocking of TBK1 reduced ISG expression, TBK1 may be a promising treatment target for cSLE.

In **chapter 4** the potential role of TBK1 as a treatment target in IFNpos systemic autoimmunity was studied in more detail. Here we show a significant upregulation of phosphorylated TBK1 (pTBK1) in plasmacytoid dendritic cells (pDC) of pSS, SLE and SSc patients. This indicates activation of this molecule in these diseases. Furthermore, TBK1 inhibitors blocked spontaneous ISG expression in peripheral blood mononuclear cells from pSS, SLE and SSc patients. The data described in **chapter 3** and **4** indicate the potential of TBK1-blockers in IFNpos systemic autoimmunity.

Hydroxychloroquine (HCQ) is the most frequently prescribed drug for pSS, however data regarding the efficacy of HCQ are limited. HCQ is a TLR7/9 inhibitor and in **chapter 5** we describe the *in vivo* effect of HCQ on IFN-related gene expression. For this study, samples of the previously published placebo-controlled JOQUER trial were used. Treatment for 24 weeks with HCQ significantly downregulated type I IFN scores, RLR and DSR expression. Stratification based on IFN activation showed a reduction of HCQ on ESR, IgG and IgM levels both in pSS patients with and without IFN activation at baseline of the study. HCQ treatment showed no effect on disease activity (assessed by the ESSDAI and ESSPRI (EURLAR Sjögren's Syndrome Patient Reported Index)) irrespective of the IFN activation status at baseline.

In **chapter 6** we discuss the latest literature on the role of IFNs in the pathogenesis of pSS and describe the limitations in studying IFN-induced gene expression. Additionally, we summarize the treatment options targeting IFNs and comment on the possible role of clinical applicability of 'IFN signatures'.

Chapter 7 focused on fatigue in pSS, which is one of the most frequently described extraglandular manifestations. Although fatigue appears to be related to inflammation and pro-inflammatory cytokines in various other diseases, there are no indications that IFNpos pSS patients are more fatigued than IFN negative patients (**chapter 2**). To study biological pathways underlying fatigue in pSS we used a novel proteomics technique, SOMAscan. Using this technique, we identified several differentially expressed proteins in fatigued pSS patients compared to non-fatigued patients and describe a proteomic signature for fatigue in pSS patients.

In **chapter 8** the overall findings of this thesis are discussed. In this thesis we show additional evidence for a role of RNA- and DNA-sensing receptors in type I IFN activation in patients with IFNpos systemic autoimmunity. The presence of multiple type I IFN-inducing pathways highlights the need for therapies to target all the different activation pathways. As IFN activation did not identify a subgroup of more fatigued pSS patients,

the identification of a novel set of upregulated proteins is a first step to unravel biological pathways underlying this common extraglandular manifestation.

The additional systemic activation of other types of IFNs, as observed in a subset of the patients with systemic autoimmunity, might underlie the clinical heterogeneity observed in these diseases and indicates a tailored treatment to improve management of these systemic autoimmune diseases.

SAMENVATTING

Het primaire syndroom van Sjögren (pSS) is een systemische auto-immuunziekte, die gekenmerkt wordt door ophoping van immuuncellen in de speeksel en traanklieren. Karakteristieke symptomen zijn droogheid van de ogen en mond. Naast deze lokale symptomen hebben patiënten vaak last van vermoeidheid, pijnlijke spieren en gewrichten en het syndroom van Raynaud. Bij sommige patiënten zijn ook de interne organen, zoals bijvoorbeeld de longen of nieren, aangedaan. Vermoeidheid is bij patiënten met pSS de meest voorkomende klacht en ongeveer 70 procent van de patiënten geeft aan vermoeid te zijn. Net als bij de meeste auto-immuunziekten komt pSS vaker voor bij vrouwen dan bij mannen en de gemiddelde leeftijd waarop de diagnose wordt gesteld is tussen de 40 en 60 jaar. Een belangrijk kenmerk bij systemische auto-immuunziekten is een verhoogde hoeveelheid type I interferon (IFN) (**hoofdstuk 1**). Deze verhoogde type I IFN zien we behalve bij een subgroep van de patiënten met pSS ook bij patiënten met systemische lupus erythematosus (SLE) en systemische sclerosis (SSc). In dit proefschrift bestuderen we het mechanisme dat leidt tot de verhoogde productie van IFN en testen het effect van een blokkerende stof op de productie van IFN. Tevens onderzoeken we het verband tussen IFN en vermoeidheid en bestuderen we de biologische processen die tot vermoeidheid kunnen leiden.

In **hoofdstuk 2** onderzoeken we in twee Europese cohorten van pSS patiënten de aanwezigheid van drie door IFN verhoogde modules met genen, die eerder geïdentificeerd zijn in SLE door middel van een zogenaamde modulaire transcriptie analyse. Deze analyse toonde aan dat er naast de karakteristieke verhoging van type I IFN, ook een type II IFN induceerbare module aanwezig was in een subgroep van de patiënten met pSS. Deze module was alleen verhoogd als ook de type I IFN induceerbare module verhoogd was. Patiënten waarbij de type I en II IFN induceerbare module aanwezig was hadden hogere IgG en bloedbezinkingswaarden en een lagere hoeveelheid lymfocyten in vergelijking met patiënten met alleen type I IFN of zonder IFN verhoging. Verder hadden deze patiënten lagere Schirmer's scores, indicatief voor drogere ogen, in vergelijking met patiënten zonder IFN activatie. Deze resultaten geven aan dat patiënten met verhoogd IFN een hogere ziekteactiviteit hebben. Verhoging van de IFN geïnduceerde modules was gecorreleerd met het biologische domein van de ESSDAI (EULAR Sjögren's Syndrome Disease Activity Index), maar niet met de totale ESSDAI score. Daarom is het biologische domein misschien een geschikter eindpunt voor behandelingen die IFN blokkeren.

In **hoofdstuk 3** bestuderen we hoe vaak de type I IFN handtekening voorkomt bij kinderen met SLE. Ondanks dat de ziektepathogenese vergelijkbaar is met patiënten die SLE ontwikkelen op volwassen leeftijd, is de ziekte wanneer deze ontstaat op de kinderleeftijd vaak heftiger en de prognose slechter. Een positieve IFN (IFNpos) score

werd gezien in 57% van de kinderen met SLE. Monocyten van deze IFNpos kinderen met SLE hadden verhoogde expressie van Toll-like receptor (TLR) 7, RNA sensoren (RSR; IFIH1, DDX58, DDX60, DHX58) en DNA sensoren (DSR; ZBP-1, IFI16). Op eiwit niveau was de expressie van RIG-I en ZBP-1 verhoogd. Het *in vitro* toevoegen van een blokker voor TANK-binding kinase 1 (TBK1) (BX795) remt de IFN productie via de RNA- en DNA-sensoren. Dit resulteerde in een verlaagde spontane expressie van genen, die door type I IFN worden aangezet; de zogenaamde type I IFN gestimuleerde genen. Deze resultaten geven aan dat IFN positiviteit in kinderen met SLE geassocieerd is met een verhoogde expressie van TLR7 en RNA- en DNA-sensoren. Blokkeren van TBK1 leidt tot een verlaagde expressie van type I IFN gestimuleerde genen en zou daarom mogelijk een nieuwe behandelmethode kunnen zijn voor kinderen met SLE.

In **hoofdstuk 4** bestuderen we TBK1 blokkers als mogelijke behandeling voor IFNpos systemische auto-immuunziekten. In dit hoofdstuk laten we een significante verhoging van gefosforyleerd TBK zien in plasmacytoide dendritische cellen (pDCs) van patiënten met pSS, SLE en SSc. Dit geeft aan dat TBK1 geactiveerd is in deze cellen. Verder vonden we dat in perifeer bloed mononucleaire cellen van patiënten met pSS, SLE and SSc het remmen van TBK1 de spontane expressie van type I IFN gestimuleerde genen verlaagd. De resultaten beschreven in hoofdstuk 3 en 4 geven aan dat TBK1 blokkers een mogelijke nieuwe behandelmethode zouden kunnen zijn voor patiënten met IFNpos systemische auto-immuunziekten.

Hydroxychloroquine (HCQ) is het meest voorgeschreven medicijn bij patiënten met pSS. Echter is het bewijs voor de effectiviteit van deze medicatie beperkt. HCQ is een ontstekingsremmer, en remt activatie van TLR7 en 9. In **hoofdstuk 5** beschrijven we het *in vivo* effect van HCQ op IFN gestimuleerde genexpressie. Voor deze studie zijn monsters gebruikt uit het eerder gepubliceerde JOQUER onderzoek, waarbij patiënten 24 weken behandeld werden met HCQ of een placebo. Behandeling van 24 weken met HCQ verlaagde de type I IFN activatie en expressie van RNA- en DNA sensoren. IgG, IgM en de bloedbezinkingswaarden waren verlaagd in zowel patiënten met als zonder IFN activatie. Behandeling met HCQ had geen effect op de ziekte activiteit (onderzocht door middel van de ESSDAI en ESSPRI (EURLAR Sjögren's Syndrome Patient Reported Index) score). Er was geen verschil in het effect van de behandeling tussen patiënten met of zonder IFN activatie.

In **hoofdstuk 6** wordt de nieuwste literatuur over de rol van IFN in de pathogenese van pSS besproken en we beschrijven de beperkingen, die verbonden zijn aan het meten van IFN geïnduceerde genexpressie als maat voor type I IFN activatie. Daarnaast geven we een samenvatting van de behandelingsopties, die IFN blokkeren, en beschrijven de klinische toepassing van de bepaling van de IFN activatie status.

In **hoofdstuk 7** bestuderen we de vermoeidheid bij pSS patiënten. Vermoeidheid is het meest voorkomende symptoom naast het hebben van droge ogen en mond. Hoewel er een relatie tussen vermoeidheid, ontstekingen en ontstekingsmediatoren zoals IFN is gevonden in diverse andere ziektebeelden zijn IFNpos patiënten met pSS niet vermoeider dan patiënten zonder IFN activatie (beschreven in **hoofdstuk 2**). Om de biologische processen die kunnen ten grondslag liggen aan vermoeidheid te onderzoeken hebben we gebruik gemaakt van een nieuwe techniek genaamd de 'SOMAscan'. Met deze nieuwe techniek hebben we een aantal eiwitten gevonden, die met name verhoogd zijn in vermoeide pSS patiënten.

In **hoofdstuk 8** worden de resultaten van dit proefschrift bediscussieerd. Hier laten we zien dat RNA- en DNA-sensoren mogelijk een rol spelen bij de type I IFN activatie die we zien bij patiënten met IFNpos systemisch auto-immuniteit. De aanwezigheid van meerdere routes, die leiden tot de productie van IFN geeft aan dat al deze routes geremd zouden moeten worden om de IFN productie uit te zetten in IFNpos patiënten. Aangezien IFN niet zoals eerder verondersteld verhoogd was bij vermoeide pSS patiënten, is de identificatie van een set verhoogde eiwitten in vermoeide pSS patiënten een eerste stap op weg naar het achterhalen van de biologische processen die de vermoeidheid in deze patiëntengroep veroorzaken.

Omdat er in een subgroep van patiënten met pSS naast type I IFN ook andere IFNs verhoogd zijn, zou dit de oorzaak kunnen zijn van de klinische heterogeniteit, die gezien wordt bij patiënten met systemische auto-immuunziekten. Dit geeft ook aan dat een behandeling op maat voor patiënten met systemische auto-immuunziekten in de toekomst mogelijk nodig is.

SAMENVATTING VOOR EEN BREED PUBLIEK

Tijdens het leven worden we constant blootgesteld aan indringers van buitenaf. Het immuunsysteem is belangrijk om ons hiertegen te beschermen en om ervoor te zorgen dat we niet ziek worden. Daarnaast helpt het immuunsysteem het ontstaan van kanker te voorkomen. Het immuunsysteem bestaat uit een fysieke barrière, algemene afweer en specifieke afweer. Tot de fysieke barrière behoren bijvoorbeeld de huid, de slijmvliezen in de luchtwegen, speeksel en maagzuur. Al deze barrières voorkomen dat pathogenen (ziekteverwekkers) het lichaam binnen kunnen dringen. Mocht het toch lukken om het lichaam binnen te dringen, dan is er een volgende verdedigingsmethode om pathogenen op de sporen en te doden: de algemene afweer. Algemene of aspecifieke afweer bevindt zich door het hele lichaam in het bloed, in weefselvloeistof en in de lymfevaten. Dit type afweer herkent bepaalde structuren, die normaal niet in het menselijk lichaam voorkomen, als pathogenen. Deze afweer is echter niet specifiek voor één pathogeen. De fysieke barrière en de algemene afweer vormen samen het aangeboren immuunsysteem. Als laatste verdedigingslijn hebben we de specifieke afweer. Dit type afweer ontwikkel je tijdens je leven en deze vorm van afweer richt zich op één specifieke ziekteverwekker. In tegenstelling tot de aangeboren afweer ontstaan bij dit type afweer ook geheugencellen, waardoor bij een tweede besmetting je lichaam de infectie sneller kan herkennen en onderdrukken.

Helaas komt het voor dat het immuunsysteem niet goed functioneert en dan kunnen er verschillende ziekten ontstaan. Als het immuunsysteem lichaamseigen cellen of stoffen aanziet voor lichaamsvreemd, kan er een auto-immuunziekte ontstaan. De auto-immuunziekte kan orgaan-specifiek zijn. Dan wordt er een immuunrespons gevormd tegen één bepaald orgaan, zoals bijvoorbeeld het geval is bij diabetes type I. De immuunrespons kan ook gericht zijn tegen lichaamseigen cellen, stoffen of moleculen die door het hele lichaam voorkomen. Vaak zijn er dan meerdere organen of weefsels aangedaan en spreken we van een systemische auto-immuunziekten. In dit proefschrift doen we onderzoek naar de pathogenese van een aantal van deze systemische auto-immuunziekten, waarbij we ons met name richten op het syndroom van Sjögren.

Het syndroom van Sjögren wordt gekenmerkt door ophoping van immuuncellen in de traan- en speekselklieren. Patiënten met het syndroom van Sjögren hebben last van droge ogen en een droge mond. Ook zijn deze mensen vaak vermoeid en hebben zij last van pijnlijke spieren en gewrichten. In sommige gevallen zijn zelfs de vitale organen aangedaan, zoals nieren en longen. In meer dan de helft van het aantal patiënten met het syndroom van Sjögren zien we in het bloed een chronische activatie van het immuunsysteem. Een belangrijke stof waar het in dit proefschrift beschreven onderzoek zich op richt is interferon. Interferon is een signaalmolecuul (cytokine) dat door verschillende cellen wordt geproduceerd bij besmetting met een virus. Deze stof 'interfereert' met de vermeerdering van een virus in een cel. Daarnaast zorgt deze stof ervoor dat het immuunsysteem wordt geactiveerd en de infectie wordt bestreden.

Nadat het virus is uitgeschakeld, wordt interferon normaliter weer afgebroken. Echter, bij meer dan de helft van de patiënten met het syndroom van Sjögren is interferon chronisch aanwezig terwijl er geen sprake is van een virusinfectie. Dit geeft aan dat bij deze patiënten het immuunsysteem constant gestimuleerd wordt.

Er zijn meerdere aanwijzingen dat interferon een rol speelt bij het syndroom van Sjögren. Bij sommige ziekten, zoals bijvoorbeeld bij hepatitis, wordt toediening van interferon als behandeling toegepast. Bij dergelijke patiënten kan de behandeling met interferon typische 'Sjögren'-symptomen opwekken. Daarnaast blijkt uit onderzoek in muizen dat stimulatie van de interferonproductie samengaat met een verminderde functie van de speekselklier. Als vervolgens de receptor voor interferon geblokkeerd wordt, verbetert de speekselklierfunctie weer. Dit geeft aan dat interferon de verminderde speekselklierfunctie veroorzaakt in deze muizen. Aangezien het blokkeren van interferon ook bij patiënten met systemische auto-immuunziekten mogelijk een goede behandeling zou kunnen zijn, is het belangrijk verder onderzoek te doen naar het mechanisme dat leidt tot de productie van interferonen en hoe dit zou kunnen worden geblokkeerd.

Hoewel er meerdere interferontypes zijn, richten wij ons in dit proefschrift met name op interferon type I en II. De type I interferonen bestaan uit een grote groep eiwitten. Al deze eiwitten binden aan één receptor en zetten net als in een ketting reactie een hele serie nieuwe moleculen aan: dit noemen we de zogenaamde 'type I interferon-geïnduceerde genen' aan. De expressie van deze genen kan gemeten worden in het bloed. We vergelijken deze expressie met die van gezonde personen en dan weten we of in de patiënt interferon verhoogd is. Dat bij Sjögren patiënten type I interferon verhoogd kan zijn hadden wij al eerder aangetoond, maar nog onbekend is of type II interferon ook verhoogd is. Type II interferon wordt geproduceerd door andere cellen dan type I interferon en dit type heeft een andere receptor. Helaas overlappen de genexpressies die geïnduceerd worden door type I interferon en door type II interferon elkaar grotendeels. Het is belangrijk dat er beter onderscheid tussen type I en type II gemaakt wordt. Andere onderzoekers hebben eerder met behulp van computermodellen groepen van genen geïdentificeerd die door interferonen aangezet worden. Deze groepen genen worden ook wel modules genoemd. Dit onderzoek is gedaan met bloed van patiënten met systemische lupus erythematosus (of verkort lupus). Dit is een andere systemische auto-immuunziekte, waarbij patiënten ook vaak verhoogde interferonactivatie in hun bloed hebben. In hoofdstuk 2 van dit proefschrift bestuderen we deze interferon-geïnduceerde modules, die beschreven zijn in het lupusonderzoek, in 2 grote Europese groepen met Sjögren patiënten. Hierbij vonden we dat er naast verhoogde type I interferonen, in een subgroep van de patiënten ook een type II interferon-induceerbare module aanwezig was. Dit ging gepaard met een verhoging van bloedwaarden die aangeven dat er meer activatie is van het immuunsysteem. Daarnaast hadden deze patiënten ook meer last van droge ogen dan patiënten zonder interferonactivatie in het bloed. Deze resultaten geven aan dat patiënten met het syndroom van Sjögren met behulp van de interferon I

en II activatie kunnen worden ingedeeld in drie groepen: 1) alleen interferon type I, 2) interferon type I+II en 3) zonder interferonactivatie. Wij denken dat het belangrijk is om dit onderscheid te maken om een geschikte behandeling te vinden voor iedere groep.

In hoofdstuk 3 en 4 onderzoeken we een mogelijke behandelmethode voor het remmen van interferonactivatie bij mensen met systemische auto-immuunziekten Hierbij kijken we niet alleen naar patiënten met het syndroom van Sjögren, maar ook naar patiënten met lupus (hierboven al genoemd) en patiënten met systemische sclerosis. In al deze ziektebeelden zien we vaak type I interferonactivatie in het bloed. Deze patiënten noemen we ook wel interferon positief. Om interferon te remmen moeten we eerst weten hoe het wordt geactiveerd. Hierbij richten we ons op een bepaald type bloedcel, namelijk de plasmacytoïde dendritische cel. Dit celtype maakt na activatie een enorme hoeveelheid interferon en wordt gezien als de boosdoener bij interferonactivatie in systemische auto-immuunziekten. Dit celtype is echter vrij zeldzaam en daarom lastig te bestuderen. Daarom kijken we ook naar de monocyt, een ander type bloedcel die ook interferon kan maken na activatie. In de plasmacytoïde dendritische cel zijn er drie routes die kunnen leiden tot interferonactivatie: 1) de Toll-like receptor (TLR7+9), 2) RNA-sensorroute en 3) DNA-sensorroute. In het bloed van interferon positieve patiënten met Sjögren en lupus hebben wij een verhoging van al deze receptoren gevonden. Een verhoogde expressie van TLR7 is al vaker beschreven bij Sjögren en lupus, maar dat RNA- en DNA-sensoren ook verhoogd kunnen zijn is minder bekend. De RNA- en DNA-sensorroute maken deels gebruik van dezelfde signaaleiwitten na activatie. Een belangrijk molecuul hierin is TBK1. Wij hebben gekeken naar het effect van het blokkeren van dit molecuul op de interferonproductie. Hiervoor hebben we in bloed van interferon positieve patiënten met een systemische auto-immuunziekte in een kweek van bloedcellen TBK1 geblokkeerd. Deze blokkade zorgde voor verlaging van interferon-geïnduceerde genen. Dit geeft aan dat RNA- en DNA-sensoren bij kunnen dragen aan de interferonproductie. Bovendien zou het blokkeren van TBK1 een mogelijke nieuwe behandelmethode kunnen zijn voor interferon positieve patiënten met een systemische auto-immuunziekte.

In hoofdstuk 5 bestuderen we het effect van hydroxychloroquine op de interferon-activatie. Hydroxychloroquine is de meest voorgeschreven medicatie bij patiënten met het syndroom van Sjögren, maar er is weinig wetenschappelijk bewijs dat het ook echt werkt. Hydroxychloroquine is een ontstekingsremmer en remt activatie van TLR7 en 9. Dit is zoals hierboven beschreven een van de routes die leidt tot interferonproductie. In dit hoofdstuk onderzoek we bloed dat verzameld is tijdens een klinische studie die onderzoek deed naar de effectiviteit van hydroxychloroquine. Tijdens dit onderzoek werden Sjögren patiënten voor 24 weken behandeld met hydroxychloroquine of een placebo. Aan het begin en eind van de studie is er bloed verzameld. Wij hebben dit bloed onderzocht en vonden dat behandeling met hydroxychloroquine de interferon-geïnduceerde genexpressie verlaagde. Verder vonden we na behandeling met hydroxychloroquine geen verschil in ziekteactiviteit tussen patiënten met of zonder

interferonactivatie. Dit geeft aan dat ondanks dat hydroxychloroquine interferon-geïnduceerde genexpressie verlaagt, dit geen effect heeft op de ziekteactiviteit.

In hoofdstuk 6 beschrijven we de nieuwste literatuur over de rol van interferonen in het syndroom van Sjögren en de verschillende manieren om interferonactivatie te meten en wat de voor- en nadelen van deze methoden zijn. Daarnaast geven we een samenvatting van de behandelingsopties die er zijn om interferon specifiek te blokkeren.

In hoofdstuk 7 bestuderen een veel voorkomende klacht van patiënten met Sjögren, namelijk vermoeidheid. Er is in andere ziektebeelden een verband tussen vermoeidheid, ontstekingen en stoffen zoals interferonen gevonden. Echter tegen de verwachting in laat onderzoek zien dat patiënten met interferonactivatie niet meer vermoeid zijn dan patiënten zonder interferonactivatie (hoofdstuk 2). Daarom gaan we in dit hoofdstuk op zoek naar biologische processen die ten grondslag zouden kunnen liggen aan vermoeidheid. Hierbij maken we gebruik van een nieuwe techniek genaamd 'SOMAscan'. Met deze methode kunnen we 1300 eiwitten onderzoeken in een kleine hoeveelheid bloed. Vervolgens hebben we gekeken welke eiwitten verschillend waren bij vermoeide en niet vermoeide patiënten met het syndroom van Sjögren. We vonden in totaal 14 eiwitten die verschillend waren. De identificatie van deze set eiwitten in vermoeide Sjögren patiënten is een eerste stap op weg naar het achterhalen van de biologische processen die de vermoeidheid in deze patiëntengroep veroorzaken.

In hoofdstuk 8 bediscussiëren we de gevonden resultaten van dit proefschrift. Hier laten we zien dat RNA- en DNA-sensoren bij kunnen dragen aan de chronische interferon activatie die we zien bij interferon positieve patiënten met systemisch auto-immuniteit. De aanwezigheid van meerdere interferonactivatieroutes geven aan dat al deze routes geremd zouden moeten worden om de interferonproductie te blokkeren. Verder laten we zien dat interferon niet verhoogd is bij vermoeide patiënten, zoals eerder verondersteld. Omdat we naast activatie van type I interferonen ook type II interferonen hebben gevonden in het bloed van patiënten met Sjögren, laat dit zien dat er een grote heterogeniteit is binnen dit ziektebeeld. Dit geeft ook aan dat in de toekomst een behandeling op maat, gebaseerd op testen van bijvoorbeeld de interferon activatie in het laboratorium, voor patiënten met systemische auto-immuunziekten nodig is om elke patiënt goed te behandelen.

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CURRICULUM VITAE

Iris Louise Agaath Bodewes was born in Breda, the Netherlands on the 14th of January 1991. She graduated secondary School 'Mencia de Mendoza Lyceum' in 2009 and moved from Breda to Wageningen to study Nutrition and Health at the Wageningen University and Research Centre.

From the start she was interested in medicine and molecular regulation of the human body. She obtained her bachelor degree in 2012 and continued her master in Nutrition and Health following the molecular track. In her master she focused on immunology with research internships in the field of allergy and vaccine research. She obtained her master degree in December 2014.

In 2015 she started her PhD in the field of autoimmune diseases under the supervision of Dr. M.A. Versnel at the Department of Immunology, Erasmus MC, Rotterdam, the Netherlands. She studied the immunopathology of primary Sjögren's syndrome, focusing on interferon activation and fatigue in patients suffering from this disease and other systemic autoimmune diseases. During her PhD she supervised a number of bachelor and master student and assisted in histology teaching for medical and clinical technology students. She presented her research at multiple national and international conferences and symposia in the field of rheumatology and immunology. She also had the opportunity to go abroad on a collaborative working visit to the lab of Prof. dr. Wan-Fai Ng at the Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, UK. After she finishes her PhD she will receive recognition as a SMBWO immunologist.

PHD PORTFOLIO

Name PhD student	Iris Bodewes
Erasmus MC department	Immunology
Research school	Molecular Medicine (MolMed)
PhD period	May 2015 - May 2019
Promotors	Dr. M.A. Versnel
	Prof. Dr. P.D. Katsikis

PhD training

Courses and workshops

2014	Laboratory Animal Science (Article 9)
2015	Introduction Graphpad
2015	SNP course XII: SNPs and Human Diseases
2016	Flow cytometry training by Becton Dickson (BD)
2016	Ingenuity Pathway Analyses
2016	Annual course on Molecular Medicine
2016	Advanced Immunology
2016	Basic course on R
2016	Gene Expression Data Analysis using R
2016	Research Integrity
2017	Biomedical English Writing Course
2017	Photoshop and Illustrator CS6
2017	Diagnostic Data for Dummies
2017	Monocytes: Origins, Destinations, Functions and Diagnostic Targets
2018	Advanced course on Application in Flow Cytometry
2018	Follow-up workshop on Photoshop and Illustrator CS6
2018	Medical Immunology

(Inter)national Scientific meetings and presentations

2016	NVVI annual meeting, Lunteren, Netherlands
2016	Working visit Newcastle University, Prof.dr. W-F. Ng lab

2016	Biomarkers and Targeted Therapeutics in Sjögren's (BATTS) Conference, Oklahoma city, OKL, USA (Oral and poster)
2017	Annual MolMed meeting (Poster)
2017	NVVI annual meeting, Lunteren, Netherlands
2017	EULAR 2017, Madrid, Spain (Oral)
2017	NVVI annual meeting, Noordwijkerhout, Netherlands (Poster)
2018	Annual MolMed meeting (Poster)
2018	14th International Sjögren's Syndrome symposium, Washington, DC, USA (2 Orals and 2 Posters)

Teaching

2015 - 2018	Supervising students (research internships)
2016 - 2018	Histology practicals: Acute and Chronic inflammation, Cellular interaction immune response, Spleen and MALT(2nd year Medical Students and 1st year Clinical Technology)

Grands and Awards

2016 - 2018	Travel grants from the Dutch National Foundation for Sjögren Patients (NVSP)
2016 - 2018	Travel grants from the Erasmus MC Trustfonds
2017	Travel Award EULAR 2017

Other activities

2015 - 2018	Journal club at the Department of Immunology
2015 - 2019	Seminars and minisymposia dept. Immunology Erasmus MC
2017 - 2018	PhD committee member

PUBLICATIONS

Manuscripts published

Iris L.A. Bodewes[#], Peter J. van der Spek[#], Leticia G. Leon, Annemarie J.M. Wijkhuijs, Cornelia G. van Helden-Meeuwsen, Liselotte Tas, Marco W.J. Schreurs, Paul L.A. van Daele, Peter D. Katsikis, Marjan A. Versnel. 'Fatigue in Sjögren's syndrome: a search for biomarkers and treatment targets.' Frontiers in Immunology - accepted for publication.

Erika Huijser, Cornelia G. van Helden-Meeuwsen, Noortje Groot, **Iris L.A. Bodewes**, M. Javad Wahadat, Marco W.J. Schreurs, Paul L.A. van Daele, Virgil A.S.H. Dalm, Jan A.M. van Laar, P. Martin van Hagen, Matti Waris, Sylvia Kamphuis*, Marjan A. Versnel*. 'MxA is a clinically applicable biomarker for IFN type I activation in systemic lupus erythematosus and systemic sclerosis.' Rheumatology - accepted for publication.

Iris L. A. Bodewes[#], Albin Bjork[#], Marjan A. Versnel*, Marie Wahren-Herlenius*, 'Innate immunity and interferons are important players in the pathogenesis of Sjögren's syndrome. ' Rheumatology - accepted for publication.

Iris L. A. Bodewes, Marjan A. Versnel. 'Interferon activation in primary Sjögren's syndrome: recent insights and future perspective as novel treatment target.' Expert Review of Clinical Immunology, 2018; 14(10), 817-829.

Iris L. A. Bodewes, Erika Huijser, Cornelia G. van Helden-Meeuwsen, Liselotte Tas, Ruth Huizinga, Paul L. A. van Daele, Virgil A.S.H. Dalm, P. Martin van Hagen, Marjan A. Versnel. 'TBK1: a key regulator and potential treatment target for interferon positive Sjögren's syndrome, systemic lupus erythematosus and systemic sclerosis.' Journal of Autoimmunity, 2018; 91: 97-102.

M. Javad Wahadat, **Iris L. A. Bodewes**, Naomi I. Maria, Cornelia G. van Helden-Meeuwsen, Annette van Dijk-Hummelman, Eline C. Steenwijk , Sylvia Kamphuis, Marjan A. Versnel. 'Type I IFN signature in childhood-onset Systemic Lupus Erythematosus: a conspiracy of DNA- and RNA-sensing receptors?'. Arthritis Research & Therapy, 2018; 20(4).

Iris L. A. Bodewes, Shereen Al-Ali, Cornelia G. van Helden-Meeuwsen, Naomi I. Maria, Jessica Tarn, Dennis W. Lendrem, Marco W.J. Schreurs, Eline C. Steenwijk, Paul L. A. van Daele, Tim Both, Simon J. Bowman, Bridget Griffiths, The UK Primary Sjögren's Syndrome registry, Wan-Fai Ng*, Marjan A. Versnel* . 'Systemic interferon type I and type II signatures in primary Sjögren's syndrome reveal differences in biological disease activity.' Rheumatology, 2018; 57(5): 921–930.

Manuscripts submitted

Iris L.A. Bodewes, Jacques-Eric Gottenberg, Corine G. van Helden-Meeuwsen, Xavier Mariette, Marjan A. Versnel. 'Hydroxychloroquine treatment downregulates systemic interferon activation in primary Sjögren's syndrome in the JOQUER randomized clinical trial.' Rheumatology - under review.

#Denotes shared first author

*Denotes shared last author