Type I Interferons in Primary Sjögren's Syndrome:

Assays and pathophysiological mechanisms

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The research for this thesis was performed within the framework of the Erasmus MC Postgraduate School Molecular Medicine.

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

The printing of this thesis was supported by Erasmus MC and Nationale Vereniging Sjögrenpatiënten (NVSP).

ISBN: 978-94-91811-31-9

Cover design: Elisa Calamita
Lay-out: Bibi van Bodegom, Daniëlle Korpershoek, Erna Moerland
Printing: Ridderprint | www.ridderprint.nl

Type I Interferons in Primary Sjögren Syndrome:
Assays and pathophysiological mechanisms

Type I interferonen in het primaire syndroom van Sjögren:
Bepalingen en pathofysiologische mechanismen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

dinsdag 7 juni 2022 om 10:30 uur

door

Erika Huijser

geboren te Rotterdam

Erasmus University Rotterdam

PROMOTIECOMMISSIE

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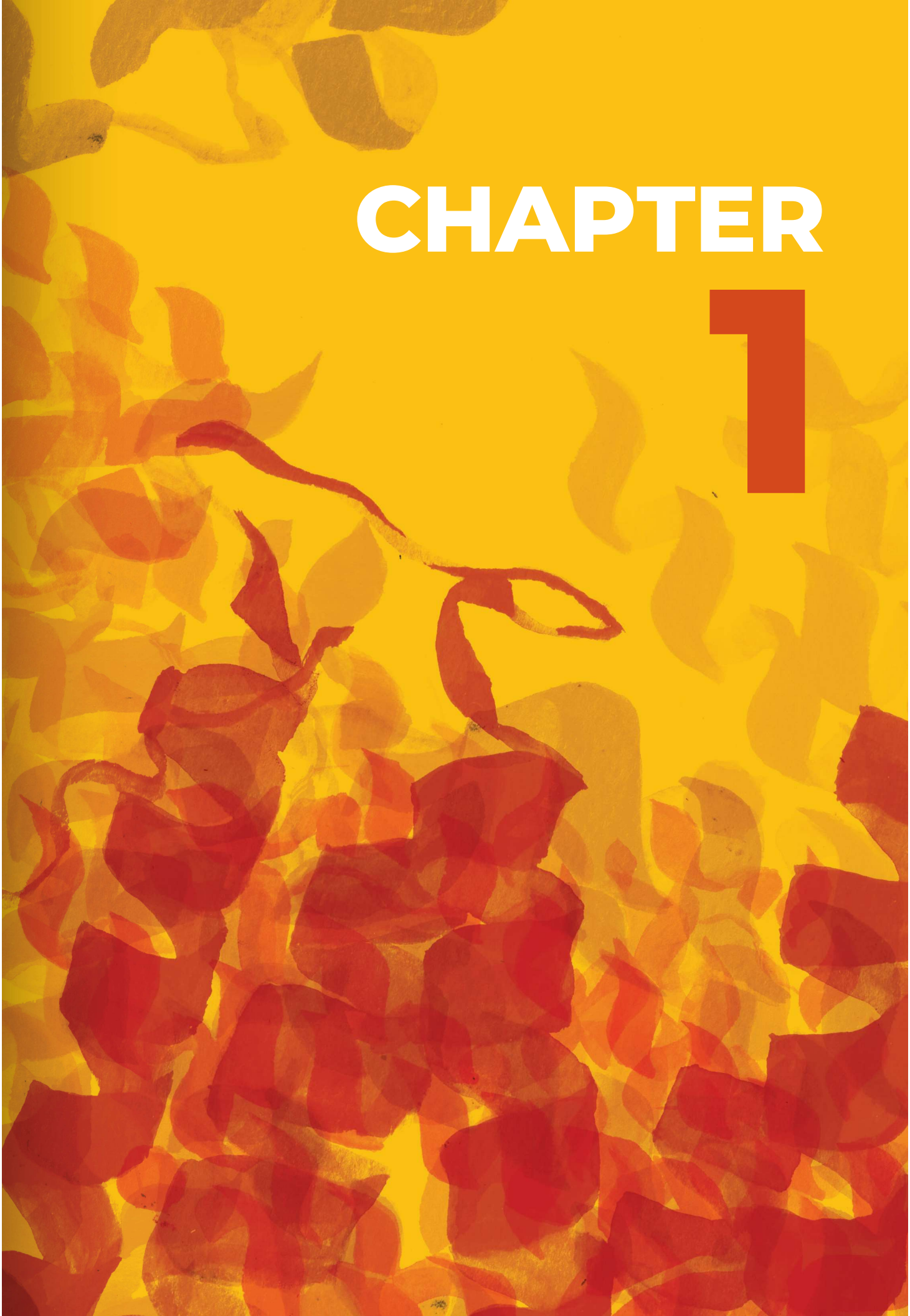
CONTENTS


CHAPTER
1

CHAPTER 1.1

General introduction

SJÖGREN'S SYNDROME

In 1933, the ophthalmologist Henrik Sjögren published his thesis presenting nineteen female patients with combined symptoms of dry eyes, dry mouth, recurrent swelling of the major salivary glands and joint inflammation [1]. Although other physicians already reported similar cases earlier [2-15], Sjögren was the first to detail the clinical and histopathological characteristics of 'keratoconjunctivitis sicca'[1] in a larger group of patients. The term Sjögren's syndrome (SS) was proposed only a few years later and is still used to name this autoimmune exocrinopathy [16].

SS can occur secondary to other autoimmune connective tissue diseases, such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and rheumatoid arthritis [17]. In the absence of these associated rheumatic diseases, SS is classified as primary SS (pSS). The overall prevalence rate of pSS is estimated around 1 in 1700 individuals worldwide [18]. This number may however be underestimated as a recent study suggested substantial underdiagnosis [19]. The disease typically manifests between the ages 40 and 60 and affects approximately ten times more females than males [18].

The clinical presentation of pSS is very heterogeneous. The dryness of eyes, mouth and other mucosal membranes can cause various secondary problems, such as dental decay, corneal damage or infections [20, 21]. Apart from the classical glandular symptoms, a large proportion of patients experience extraglandular disease manifestations. Fatigue, depressive symptoms and chronic pain are prevalent [22-25]. Additionally, different organ systems can be affected including joints, skin, lungs, kidneys and peripheral as well as central nervous system [26]. One of the most serious complications is lymphoma, which occurs in 2-10% of pSS patients [27]. Disease activity in the various organ domains can be monitored using the EULAR Sjögren's syndrome disease activity index (ESSDAI) (Table 1) [28, 29]. The diverse disease features can cause substantial morbidity and mortality and have significant impact on the quality of life [30-37].

AUTOIMMUNE ETIOLOGY

The 'round cell infiltration' that Sjögren observed in the salivary glands of his patients with keratoconjunctivitis sicca is the primary histopathological hallmark of pSS. The presence of this focal mononuclear cell infiltrate surrounding the salivary gland striated ducts represents an important component of the current classification criteria for pSS (Figure 1)

1 *'keratoconjunctivitis' meaning inflammation of the mucous membranes and cornea of the eye and 'sicca' from the Latin word for 'dry'*

Table 1. EULAR Sjögren's syndrome disease activity index (ESSDAI).

Domain	Description	Activity level	Weight
Constitutional	Fever, night sweats or involuntary weight loss	0-2	3
Lymphadenopathy and lymphoma	Lymphadenopathy, splenomegaly or current malignant B cell proliferative disorder	0-3	4
Glandular	Swelling of major salivary glands or lachrymal glands	0-2	2
Articular	Arthralgia with morning stiffness or synovitis	0-3	2
Cutaneous	Erythema multiforma, cutaneous vasculitis, purpura, subacute cutaneous lupus or vasculitis-related ulcers	0-3	3
Pulmonary	Persistent cough due to bronchial involvement or interstitial lung disease	0-3	5
Renal	Tubular acidosis, glomerular involvement with proteinuria / hematuria / renal failure, histological evidence of glomerulonephritis / important interstitial lymphoid infiltrate or cryoglobulinemia-related renal involvement	0-3	5
Muscular	EMG-, MRI- or biopsy-proven myositis	0-3	6
Peripheral nervous system	Active peripheral nervous system involvement shown by nerve conduction study, proven small fiber neuropathy, trigeminal (V) neuralgia or cranial nerve involvement of peripheral origin	0-3	5
Central nervous system	Cranial nerve involvement of central origin, optic neuritis, multiple sclerosis-like syndrome, cerebral vasculitis, seizures, transverse myelitis or lymphocytic meningitis	0-3	5
Hematological	Cytopenia of autoimmune origin with neutropenia, anemia, thrombocytopenia or lymphopenia	0-3	2
Biological	Clonal component, hypocomplementemia, hypergammaglobulinemia, cryoglobulinemia, recent onset hypogammaglobulinemia	0-2	2

[38, 39]. In the early 1960's, dr. Joseph Bunim suggested an autoimmune etiopathology of SS [16]. This hypothesis was substantiated by the identification of rheumatoid factors, 'anti-nuclear factors' (nowadays known as anti-nuclear antibodies; ANA) and increased gamma globulin concentrations in serum from SS patients [2]. Many sera from SS patients also showed autoimmune complement fixation capacity when mixed with human tissue extracts [2], a phenomenon that was also observed in sera from SLE patients. In addition to these autoantibodies directed against generalized antigens, organ-specific autoantibodies against salivary duct epithelium were detected in sera from SS patients, some of which could fix complement [40-42]. In the following decade, independent research groups identified the autoantibodies against the SSA/Ro and SSB/La antigens [43-47]. These autoantibodies are strongly associated with pSS, have important diagnostic value in current clinical practice and are included in the 2016 ACR-EULAR Classification Criteria for primary Sjögren's Syndrome (Table 2) [38]. The presence of autoantibodies in spontaneous murine SS models and the development of salivary and/or lacrimal gland dysfunction in mice immunized with SS-associated autoantigens further support the autoimmune etiopathology of pSS [48-53]. Although the treatment options for pSS are still limited, the knowledge

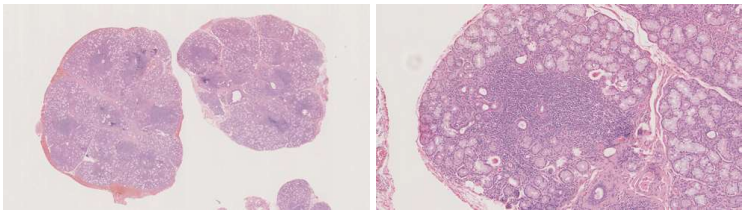


Figure 1. Focal mononuclear cell infiltrates surrounding the ducts in a pSS labial salivary gland.
2x (left) and 10x (right) magnification image of hematoxylin-eosin-stained formalin-fixed paraffin-embedded labial salivary gland biopsy from a pSS patient.

about the pathogenesis of this systemic autoimmune diseases has greatly advanced since 'keratoconjunctivitis sicca' was described almost a century ago.

PATHOGENESIS OF PRIMARY SJÖGREN'S SYNDROME

The current consensus hypothesis on the etiology of pSS is that a combination of environmental and internal factors in a genetically susceptible individual triggers chronic inflammation, both in target organs and systemically. This involves the loss of tolerance to self, the production of autoantibodies, immune infiltration in target organs and dysfunction of exocrine glands.

Etiology of primary Sjögren's syndrome

The exact triggers that cause pSS are unresolved. Most likely these include damage of the salivary gland epithelium that triggers local inflammation, which is exacerbated by hormonal influences and genetic risk factors.

Infections

Infections of the salivary glands, particularly those of viral origin, have been among the leading suspects for decades [54]. Experimental viral infections or stimulation with viral-like components can cause salivary gland inflammation and SS-like symptoms in mouse models [55-60]. Several viruses – such as hepatitis C virus (HCV) and human cytomegalovirus (CMV) – are known to infect human salivary gland epithelial cells [61, 62]. Further supporting a viral etiology, HCV and human immunodeficiency virus (HIV) can cause SS-like symptoms in humans [63, 64]. Importantly, most of these patients do not develop anti-SSA/SSB autoantibodies and the composition of immune cell infiltrates in the salivary glands is usually different from that seen in pSS [65]. Associations between specific viral infections and pSS has been an ongoing debate in literature [54]. Yet, no formal proof exists for a causal role in the initiation of pSS.

Table 2. ACR-EULAR 2016 Classification Criteria for primary Sjögren's syndrome.

Inclusion criteria	
At least 1 symptom of ocular or oral dryness	
Exclusion criteria	
History of head/neck radiation treatment	
Positive PCR for Hepatitis C	
AIDS	
Sarcoidosis	
Amyloidosis	
Graft versus host disease	
IgG4-related disease	
Item	**Score**
Autoantibodies	
Anti-SSA (Ro52/Ro60) seropositive	3
Oral manifestations	
Focal lymphocytic sialadenitis (focus score ≥ 1) in labial salivary gland	3
Unstimulated whole saliva flow rate ≤ 0.1 mL/min	1
Ocular manifestations	
Ocular staining score ≥ 5 (or van Bijsterveld score ≥ 4) on at least 1 eye	1
Schirmer ≤ 5mm/5min on at least 1 eye	1

For classification of pSS, an individual should meet the inclusion criteria, should not have any of the conditions listed as exclusion criteria and should have a total score ≥ 4. Adapted from: Shiboski, *et al.* [38].

Female predominance

Like most autoimmune diseases, pSS affects more females than males. The peak age of pSS onset in women is around the time of menopause [18]. Both observations suggest hormonal influences on pSS development. Indeed, the level of exposure to estrogens negatively associates with pSS and androgen levels are reduced in pSS [66-68]. These steroid hormones are immunomodulatory and affect the function and integrity of salivary gland epithelium [69, 70]. In fact, the enzymatic machinery of salivary gland epithelial cells to metabolize steroid hormone precursors is dysfunctional in pSS reducing the local exposure to steroid hormones [68, 71].

The female predominance of pSS also implies the involvement of the X-chromosome, possibly by a gene dosage effect. Interestingly, males with Klinefelter syndrome, who have an additional copy of the X-chromosome, have a higher risk of developing pSS [72, 73].

Additional genetic risk factors

Genome wide association studies have additionally identified other genetic risk factors of pSS [74-77]. Not surprisingly, the strongest associated variants were found in the major histocompatibility complex (MHC) class II genes encoding the human leukocyte antigens (HLA)-DR and –DQ. Other risk loci encode genes that are important in lymphocyte activation, interaction between B and T cells, and the innate immune type I interferon (IFN) and NFκB signaling pathways. Together, these genetic factors indicate both the innate and adaptive arms of the immune system as key players in the development of pSS.

Adaptive immune system in primary Sjögren's syndrome

The chronic inflammation in pSS that follows the initiation phase involves a complex interaction between cells of the innate and adaptive immune system as well as the cells of the target organs.

B lymphocytes in pSS

Patients with pSS frequently have elevated polyclonal IgG levels, self-reactive antibodies, infiltration of B cells in target organs and an increased risk of B cell lymphoma [78]. These features indicate that (dysregulated) B cell activation is part of pSS pathogenesis. B cells in pSS show signs of general hyperactivity. This has been exemplified by the high expression of BTK in circulating B cells favoring their survival [79]. Additionally, vaccinations induce stronger vaccine-specific antibody responses in pSS patients compared with healthy individuals and in parallel further increase the levels of autoantibodies in pSS [80].

To prevent autoimmune reactions, most autoreactive and polyreactive B cells are removed by self-tolerance checkpoints [81, 82]. Negative selection of self-reactive immature B cells in the bone marrow (central tolerance) and naïve B cells in the periphery (early peripheral tolerance) limits the survival of strongly autoreactive cells in the naïve B cell compartment. The third tolerance checkpoint (germinal center tolerance) deletes self-reactive B cells during memory B cell differentiation and germinal center responses. Accumulation of autoreactive cells has been observed in both naïve and memory B cell compartments in pSS. This indicates impaired regulation of self-tolerance at both the early peripheral and germinal center checkpoints [83]. Whether the accumulation of self-reactive B cells represents a primary cause for pSS development or a result of chronic inflammation remains unsettled.

A significant portion of the focal mononuclear cell infiltrates in pSS salivary glands is constituted by memory B cells and plasma cells [84, 85]. Plasma cells are also present in other affected organs in pSS [86]. Plasma cells in the salivary glands are thought to be the major producers of autoantibodies against SSA/SSB, since the number of infiltrating plasma cells correlates with serum levels of anti-SSA/SSB in pSS [85, 87]. Longitudinal

proteomic analysis of serum anti-SSA antibodies of pSS patients showed a regular replacement of dominant clonotypes[2] over time, suggesting a continuous generation or expansion of short-lived plasma cells [88]. The presence of germinal center-like structures in the salivary glands of 10-30% of pSS patients also support this notion of ongoing local B cell activation and differentiation [89]. The pathogenic effects of the anti-SSA/SSB autoantibodies are unclear [78, 90].

B cells can also be found between the ductal epithelial cells in salivary glands from pSS patients. In contrast to the B cells in the focal infiltrates, B cells in these so-called lymphoepithelial lesions express markers of chronic activation and proliferation but lack the typical memory and plasma cell markers [91, 92]. The non-Hodgkin lymphomas associated with pSS presumably arise from these constantly expanding B cell populations [93, 94]. The importance of B cells in pSS pathogenesis is further highlighted by the inclusion of rituximab (anti-CD20 B cell depleting antibody) in the EULAR recommendations for the management of severe or treatment-refractory pSS [95].

T lymphocytes in pSS

Although the majority of the salivary gland infiltrates in pSS is composed of T cells, the role of these lymphocytes in pSS is far less extensively studied than the involvement of B cells. The strong genetic risk variants in HLA class II genes, which are also associated with anti-SSA/SSB seropositivity [96], implies antigen presentation to CD4+ T cells and T cell help to B cells in pSS. The number of CD4+ T cells in the peripheral blood of pSS patients is reduced, while the remaining circulating CD4+ T cells have an activated phenotype [85, 97], suggesting their migration into tissues. Indeed, CD4+ T cells are abundantly found in salivary gland infiltrates in pSS [85, 98]. CD4+ T cells in glands without germinal center-like structures mostly display T-helper (Th)1- or Th17-phenotypes [99]. Follicular Th cells are found in salivary glands with germinal center responses and their number is also increased in the circulation of pSS patients [100-103]. Analysis of the T cell receptor (TCR) repertoire has found enrichment of specific clones in salivary glands compared with the peripheral blood indicating selective recruitment and/or expansion of T cell clones within the glands [104, 105]. Although this implies local antigen presentation by (restricted) HLA molecules to T cells, it remains elusive which antigens are recognized by these TCRs. Nevertheless, the notion of shared TCR clonotypes between different pSS patients strongly supports involvement of a common antigen. Altogether, in the immunopathogenesis of pSS, CD4+ T cells contribute to inflammation by B cell activation and production of cytokines [106]. Supporting a pathogenic role of T cells and T cell-derived cytokines in pSS, IL-17 deficiency reduced sialadenitis, while adoptive transfer of Th17 cells provoked salivary gland

2 clonotype meaning a unique sequence that arises from gene rearrangement of the B cell receptor

inflammation in SS mouse models [107, 108]. CD8+ cytotoxic T cells are less well studied in pSS, but CD8+ T lymphocytes in infiltrates typically display an activated immunophenotype and may contribute to salivary gland tissue destruction [85].

Innate immune system in primary Sjögren's syndrome

There is ample evidence that activation of innate immune pathways contributes to chronic inflammation in pSS. This section mainly focuses on the cellular components of innate immunity and their contribution to pSS. Cellular innate immunity predominantly functions through pattern recognition receptors (PRRs) that sense the presence of either pathogen-associated molecular patterns or host-derived damage-associated molecular patterns [109]. Although for many years assumed to remain unchanged during lifetime, innate immune cells have the ability to form memory in response to certain inflammatory stimuli, orchestrated by metabolic and epigenetic reprogramming [110, 111]. Unlike adaptive immune memory, innate immune memory – or *trained immunity* – is antigen non-specific, and is characterized by hyperresponsive cytokine production when re-challenged with an (unrelated) inflammatory stimulus.

Dendritic cells in pSS

Early during the development of sialadenitis in mice, dendritic cells (DCs) infiltrate the salivary gland [112]. In patients with pSS, the number of circulating DCs is reduced, while increased in the salivary glands, especially in the early phase of the disease [98, 113-117]. The frequency of plasmacytoid DCs (pDCs) – that function as antigen-presenting cells but are also strong producers of type I IFN – is correlated to the number of infiltrating B cells in human pSS glands and glandular dysfunction in a mouse model [118, 119]. Circulating pDCs from pSS patients display an activated phenotype that is associated with cytokine production and antigen-presentation [120, 121]. Unlike conventional DCs and pDCs that originate from hematopoietic stem cells, follicular DCs are stromal cells originating from mesenchymal progenitors [122]. Follicular DCs are crucial for the generation of efficient germinal center responses [123]. Germinal center resembling structures have been observed in pSS salivary glands and contain follicular DCs that support B cell survival and production of high affinity antibodies by presenting immune complex- or complement-bound (auto) antigens to B cells [124].

Macrophages in pSS

Macrophages are among the first cells to infiltrate the salivary gland in the NOD mouse model for SS [125]. In another autoimmune mouse model, macrophages were essential for the development of tear gland dysfunction [126]. The primary functions of macrophages include the phagocytosis of cellular debris, the production of cytokines and the initiation

of tissue repair [127]. In pSS salivary glands, macrophages produce chemokines, cytokines and enzymes that promote ongoing processes of inflammation, tissue damage and repair [114, 118, 128, 129]. The extent of glandular macrophage infiltration in pSS associates with severity of glandular inflammation and salivary gland enlargement [98, 114]. Monocytes from pSS patients phagocytose apoptotic cells less efficiently, leading to secondary necrosis of the apoptotic cells with subsequent leakage of cellular constituents and further immune activation [130, 131].

Innate lymphoid cells in pSS

Innate lymphoid cells (ILCs), including NK cells, are also present in pSS salivary glands, but their role is less clear [132]. While the frequency of NK cells and ILC3s correlates with the extent of infiltration, they mainly reside outside the focal infiltrates [98, 133, 134]. ILC3s, or NKp44+ NK cells, produce various mediators that contribute to chemotaxis of lymphocytes and DCs, and B cell activation [132, 134]. Functional differences of circulating NK cells have been described in pSS compared with healthy individuals [135]. NK cells from pSS patients express higher levels of the activating receptor NKp30 and secrete more IFNγ [133]. Salivary gland epithelial cells express the ligand for NKp30 which could facilitate a functional interaction between NK cells and epithelial cells that would support DC maturation [133, 136]. Interestingly, the risk for pSS is lower in individuals with a genetic variant that reduces the expression of NKp30 [133].

Innate signaling pathways in pSS

Multiple innate immune molecules and signaling pathways, including PRRs, NFκB signaling, inflammasome activation and type I IFN signaling have been implicated in the pathogenesis of pSS [132, 137]. The NFκB signaling pathway is activated in both epithelial cells and infiltrating immune cells in the salivary glands and peripheral blood cells of pSS patients [138-140]. NFκB signaling in salivary glands correlates with the severity of local glandular inflammation as well as systemic disease activity in pSS [139]. In further support of NFκB pathway activity in pSS, hyperactivity of NFκB in mouse glandular epithelial cells is associated with SS-like disease [141].

Inflammasome formation leads to the secretion of IL-1β and IL-18 [142]. These cytokines and other inflammasome-related proteins are elevated in serum and salivary glands from pSS patients [143-147]. Furthermore, cell-free DNA has been shown to cause NLRP3 inflammasome formation in monocytes and gland infiltrating macrophages from pSS patients [146]. Cytoplasmic DNA depositions in ductal epithelial cells induce AIM2 inflammasome activation in pSS [148]. The expression levels of inflammasome components are predictive of lymphoma development in pSS [149]. In mice, inflammasome activation provoked epithelial cell death and salivary gland immune infiltration [150]. A genetic

variant in the P2X7 receptor – an NLRP3 inflammasome activating receptor – is associated with pSS in individuals without the HLA-DR3 risk allele [151]. Some of the innate immune pathways are already activated in salivary glands of B6.NOD-*Aec1Aec2* mice during early development of SS-like disease before glandular focal immune infiltration [152, 153]. The IFN signaling pathway in the context of pSS will be discussed in a subsequent section of the General Introduction.

Altogether, innate immune activation promotes chronic inflammation in pSS by stimulating dysbalanced tissue damage and repair, immune cell infiltration and adaptive immune responses. The local innate immune activation is supported by stromal cells and the epithelium of the pSS salivary gland [94, 154].

Interaction between immune cells and salivary gland epithelium

Inflammation in pSS is accompanied by dysfunction of the salivary gland. The production of saliva depends on the concerted action of the serous and mucous acinar cells and the ductal epithelial cells upon stimulation by the autonomic nervous system [155]. Parasympathetic stimulation of the cholinergic receptor muscarinic 3 (M3R) on acinar cells induces intracellular calcium signaling and activation of aquaporins (AQP), leading to secretion of the digestive enzyme α-amylase and fluid. The mucous acinar cells add mucins to the saliva, which is transported to the oral cavity through the ducts. Ductal cells create a proper balance of ions to support the antimicrobial activity of saliva and to protect the teeth.

Aberrancies in pSS salivary gland function

Abnormalities in saliva production have been described at multiple levels of the secretory machinery in pSS. Reduced responsiveness to M3R stimulation [156, 157], dysregulated calcium signaling and its downstream components [156, 158-161], perturbed cell polarity causing mislocalization of AQPs and secretion of mucin at the basal side [156, 162, 163] have all been reported at the level of acinar cells in pSS. Concentrations of specific ions are altered in pSS saliva, which could indicate disrupted ion exchange by ductal cells [164, 165]. The salivary gland progenitor cells responsible for the renewal of the acinar and ductal epithelium are both reduced in number and have shorter telomeres in pSS [166, 167]. These findings suggest that excessive cellular replication of gland progenitor cells that results in premature replicative senescence of these progenitor cells is associated with pSS [166, 167].

Impact of inflammation on pSS salivary gland function

Normal salivary gland function can be affected by chronic inflammation. Inflammatory mediators can impact acinar integrity, for example by modification of tight junctions [168]

or regulation of programmed cell death [169]. Both lower and higher susceptibility to apoptosis have been reported in salivary gland epithelial cells of pSS patients [170-173]. Additionally, an inflammatory environment can influence the expression and localization of AQPs affecting the function of acinar cells [174, 175]. Autoantibodies directed against salivary gland antigens could potentially alter the functionality of epithelial cells, but this has not been shown yet [176-178]. On the other hand, autoantibodies against the M3R, which are commonly present in pSS patients, have been shown to inhibit the signal transmission from the autonomic nervous system to the acinar cells [179-184]. Although inflammation could affect salivary gland function, the level of glandular inflammation does not directly correlate with the glandular dysfunction [185, 186].

Epithelial cells contribute to salivary gland inflammation in pSS

Not only do inflammatory processes affect the salivary gland function, the epithelial cells are considered to play an active role in the inflammatory and autoimmune response [94]. The salivary gland epithelium functions as a first line defense barrier against pathogens. Through the expression of a wide range of PRRs they are able to mount an innate immune response [187-191]. Salivary gland epithelial cells have the ability to produce various cytokines and express both HLA and costimulatory molecules that contribute to the attraction, activation and differentiation of immune cells [192]. Many of these cytokines are produced by salivary gland epithelial cells of pSS patients [94, 192]. Mucins produced by acinar cells are able to stimulate Toll-like receptor (TLR) 4 [193]. Consequently, the depolarization of acinar cells that causes inappropriate secretion of mucin at the basal side may aggravate the inflammatory response [94]. Additionally, salivary gland epithelial cells are a potential source of self-antigens in pSS. In this context, the SSA/Ro and SSB/La antigens are upregulated in pSS salivary glands and can be exposed to the immune system after apoptotic cell death of epithelial cells [194-196].

TYPE I IFN IN PRIMARY SJÖGREN'S SYNDROME

The most prominent sign of innate immune activation in pSS is the persistent type I IFN pathway activation, both in the circulation and salivary glands. This feature is present in the majority of pSS patients. Type I IFN pathway activation is also observed in subgroups of patients with other systemic autoimmune diseases, such as SLE and SSc.

Type I IFN biology

Type I IFNs belong to the IFN cytokine family originally recognized for their capacity to interfere with viral replication [197]. The IFN family is classified in type I, type II and

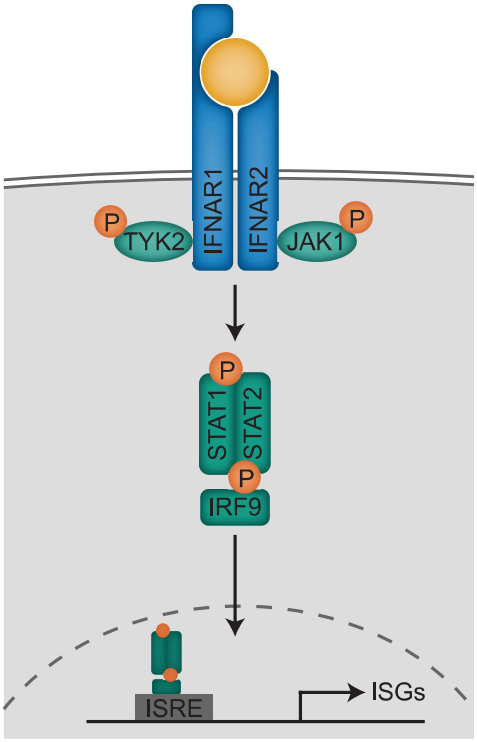


Figure 2. Type I IFN receptor signaling pathway.
Binding of type I IFN drives dimerization of the receptor subunits IFNAR1 and IFNAR2 and phosphorylation of IFNAR-associated TAK1 and TYK2. Subsequent phosphorylation of intracellular IFNAR domains enable recruitment, phosphorylation and dimerization of STAT1 and STAT2. The STAT1/2 heterodimer interacts with IFN-regulatory factor 9 (IRF9) forming the transcription factor IFN-stimulated regulatory factor 3 (ISRF3) complex. ISRF3 localizes to the nucleus, binds to IFN-stimulated response elements (ISRE) and initiates transcription of interferon-stimulated genes (ISGs).

type III IFNs, referring to the distinct receptor complexes through which they signal [198]. The human chromosome 9 (9p21) contains seventeen type I IFN genes that encode a total of 12 different IFNα proteins, IFNβ, IFNε, IFNκ and IFNω [199]. Binding of type I IFN drives the heterodimerization of the IFNα receptor (IFNAR)1 and -2 subunits, together forming a signaling competent receptor complex. The association of the IFNAR subunits brings their intracellular signaling partners JAK1 and TYK2 of the Janus tyrosine Kinases (JAK) family in close proximity. This initiates JAK1/TYK2 phosphorylation and downstream signaling through activation of signal transducer and activator of transcription (STAT)1 and STAT2 (Figure 2) [200].

The widespread expression of IFNAR and its canonical downstream mediators renders virtually every cell in the body responsive to type I IFNs [201]. By means of this signaling, type I IFNs can affect the transcription of up to ten percent of the human genes [202, 203]. Not surprisingly, this large-scale transcriptional regulation provokes broad immunomodulatory effects on both immune and non-immune cells and orchestrates basic cellular functions such as proliferation, differentiation and survival [204, 205]. In the context of an immune response, the effect of IFNAR activation depends on the activated cell type, concomitant inflammatory stimuli and the timing of activation [204].

The expression of type I IFNs is tightly regulated to provide efficient immune responses and to limit (excessive) damage to the surrounding tissues [206]. The activation of PRRs can cause rapid upregulation of type I IFNs. Among these, the nucleic acid-sensing TLRs,

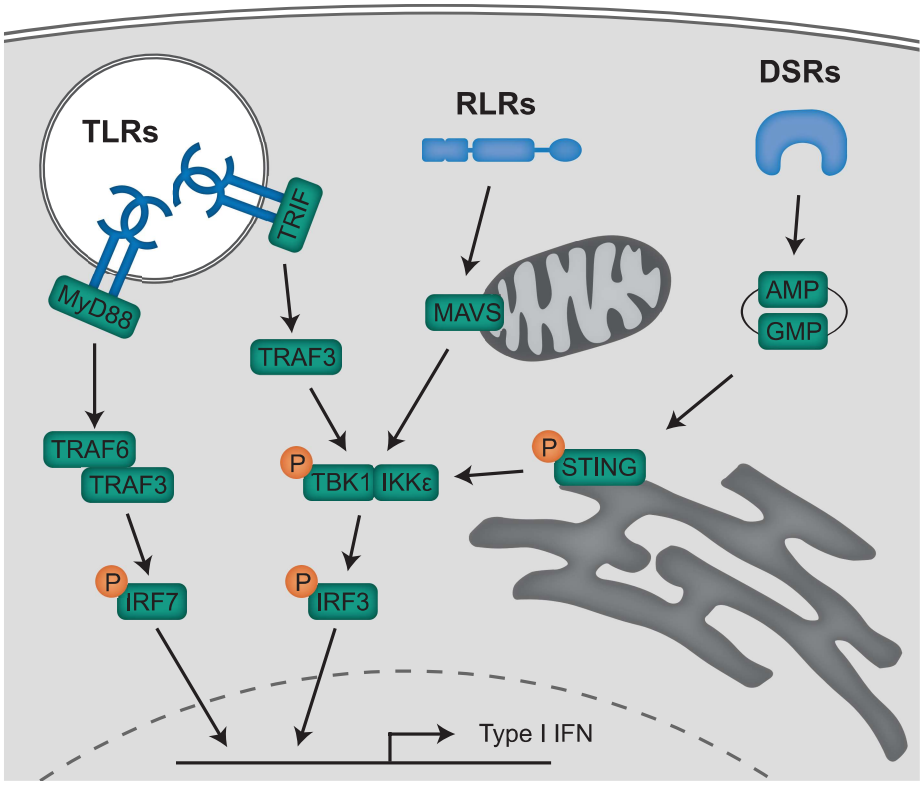


Figure 3. Nucleic acid-sensing pathway signaling leading to type I IFN production.
Endosomal Toll-like receptors (TLRs), cytosolic RIG-I-like receptors (RLRs) and cytosolic DNA-sensing receptors (DSRs) induce transcription of type I IFNs through activation of IFN-regulatory factor 7 (IRF7) or IRF3.

the cytosolic RIG-I-like receptors (RLRs) and the DNA-sensing receptors (DSRs) are fundamental to induce robust type I IFN responses (Figure 3) [206-208]. The pDC can produce large amounts of IFNα (>100 molecules/cell in 24 hours) and is therefore considered as the main type I IFN-producing cell type in the body [209]. However, almost all nucleated cells have the ability to synthesize small amounts of type I IFNs. In the context of an immune response, both the nature of the stimulus and the timing of stimulation dictate the primary cellular source of type I IFN [210-212].

In contrast to type I IFNs, the production of type II and type III IFNs is restricted to certain cell types. Activated immune cells are the primary source of type II IFN – or IFNγ – that plays a crucial role in the interaction between immune cells [213]. Production of the more recently defined type III IFNs is mainly confined to epithelial cells and specific immune cells. Type III IFNs are crucial for mucosal immunity [214-216]. Type II and type III IFNs have a low degree of homology with type I IFNs and interact with distinct receptor complexes. Yet, the downstream signaling pathways and the transcriptional responses largely overlap between the different IFN types, imposing shared functional actions [201, 213, 217].

IFN pathway activation in primary Sjögren's syndrome

Laboratory tests to detect type I IFN pathway activation

Various methods exist that measure the different elements of the type I IFN pathway reflecting type I IFN pathway activation in biological material. Direct quantification of type I IFN protein concentrations in blood or serum is challenging because these potent cytokines circulate at low levels even in some inflammatory conditions [218, 219]. The recently developed single-molecule array (Simoa) adopts the traditional solid-state immunoassay technology for the measurement of proteins at the subfemtomolar level [220]. Measurements are further complicated by the diversity in amino-acid sequence of the various type I IFN subtypes [199]. To bypass this latter challenge, *in vitro* cellular assays can be used to determine the bioactivity of IFNs in biological material, analyzing either the virus-inhibitory capacity or the induction of an IFN-induced reporter gene. These cellular assays often have fairly high lower limits of detection [221], which makes them less suitable for measurement of very low levels of IFN. Related to the cellular assays that measure a transcriptional response to IFN, the most widely applied method to assess type I IFN activation quantifies the expression of IFN-stimulated genes (ISGs) or proteins in cells or tissues isolated from patients [222].

Type I IFN activation in pSS

A remarkable and consistent upregulation of ISG transcripts – the so-called IFN signature – has been described numerous times in both pSS salivary glands and peripheral blood [120, 121, 189, 223-231], similar to the findings in blood and affected tissues from SLE patients [232, 233]. The overlap in transcriptional responses between the IFN types complicate the interpretation of the IFN signature in patients. Yet, attempts have been made to discriminate between type I and type II induced ISG expression [234, 235]. Using selected ISGs that are preferentially induced by either type I or type II IFNs, IFNγ appeared to contribute to the IFN activation in pSS salivary glands [235]. IFNγ is also implicated in the peripheral blood IFN signature of a subgroup of pSS patients with systemic IFN activation [236]. Whether an association exists between local and systemic type I IFN activation remained an open question for years. But a recent study on paired analysis of biopsies and peripheral blood from pSS patients did show a positive correlation between ISG expression in blood and major and minor salivary glands [237]. The sustained type I IFN production in pSS in the absence of evident chronic viral infections is remarkable given the various negative feedback mechanisms that normally limit excessive IFN responses.

Various associations exist between IFN activation and disease manifestations in pSS. In salivary glands, ISG expression associates with: 1) a higher focus score, 2) reduced saliva secretion, and 3) a higher risk for lymphoma, especially in glands with a predominant type

II IFN signature [235, 238]. Disease-relevant serological markers of B cell hyperactivity (e.g. autoantibody seropositivity and hypergammaglobulinemia), complement consumption (e.g. decreased C3/C4) and hematological distortion (e.g. thrombocytopenia, autoimmune anemia) are associated with both systemic and glandular IFN activation, most pronounced in the subgroup with additional systemic type II on top of type I IFN involvement. Together, these data suggest a more severe disease in patients with (type I) IFN activation.

Pathogenic role of type I IFN in primary Sjögren's syndrome

Multiple lines of evidence suggest a pathogenic role for IFNs in the development and propagation of systemic autoimmune diseases, including pSS.

Type I IFN in the initiation of autoimmune responses

Genome-wide association studies in pSS and SLE identified risk conferring genes (*IRF5* and *STAT4*) related to the IFN pathway [77, 239]. Several additional observations in humans and mouse models suggest that type I IFNs can contribute to the initiation of autoimmune responses. First, observational studies have reported incidental development of reversible systemic autoimmune features in patients that received recombinant type I IFN for the treatment of malignancies or hepatitis C infections [240-242]. Second, genetic mutations that provoke profound systemic type I IFN activation cause various autoinflammatory syndromes, also known as interferonopathies [243]. Secondary to some of these interferonopathies, autoimmune phenomena can develop later in life [244]. These observations illustrate the presumed ability of type I IFN to promote the loss of self-tolerance and induce autoimmune responses.

An indication for a role of local type I IFN in immune infiltration and salivary gland dysfunction derives from the B6.*Aec1Aec2* mouse model for SS. These mice do not display systemic type I IFN activity [245]. The glandular manifestations, but not ANA production, in this mouse model could be prevented by knockout of the IFNAR [246]. Similarly, salivary gland dysfunction induced by innate immune activation in mice was dependent on type I IFNs [57, 247].

Type I IFN in the propagation of autoimmune responses

Type I IFNs likely also contribute to the propagation of autoimmune responses in pSS. About a quarter of SLE patients have autoantibodies against IFNα. These anti-IFNα antibodies are associated with lower disease activity [248]. Autoantibodies against type I IFNs have also been described in a proportion of pSS patients. Supporting a pathogenic role for type I IFN in pSS, milder sicca symptoms and minimal glandular immune infiltration were found in a case with high titers of neutralizing anti-IFNα/ω antibodies [249].

The broad immunomodulatory effects of type I IFNs on both innate and adaptive immune cells have implications for autoimmune responses. In the context of pSS, the strong association between systemic type I IFN activation and autoantibodies has been a major focus of research. Both directly and indirectly, type I IFNs can affect the balance between effector and regulatory B cells. It has been shown that B cell-intrinsic type I IFN signaling can promote the development of autoreactive B cells, drive plasma cell differentiation and production of autoantibodies [250-252]. At the same time, the induction of regulatory B cells by pDCs is impeded by excessive type I IFN production, which has been shown to occur in SLE [253]. Importantly, both type I and type II IFNs stimulate immune cells and salivary gland epithelial cells to produce B cell activating factor (BAFF), a fundamental survival factor for B cells [254]. BAFF is elevated in pSS salivary gland tissue and in the circulation [230, 255-258]. These increased BAFF levels will likely enhance B cell activation and germinal center responses in pSS. Overexpression of BAFF in mice provokes both SS-mimicking salivary gland inflammation and dysfunction, as well as exaggerated B cell responses and autoantibody production [259, 260]. On a bigger scale, persistent type I IFN activation disturbs the normal trafficking of B cells causing disorganization of follicles in secondary lymphoid organs, shown in the context of chronic viral infections and in a murine SLE model [250, 261-263].

Type I IFNs also affect several processes at the interface of innate and adaptive immunity. The threshold for T cell activation can be influenced by type I IFNs through regulation of costimulatory molecules on antigen-presenting cells [264-267]. Indeed, autoimmune features and SS-like symptoms were reported in mice as well as cancer patients receiving checkpoint inhibition therapy (e.g. PD-L1 blockade) [268-270]. Differentiation, maturation and homing of professional antigen-presenting cells to lymphoid organs is stimulated by type I IFNs [271], thereby contributing to effective adaptive immune responses. Pretreatment of cells with type I IFNs alters their response to subsequent stimulation. Some of these effects are mediated through epigenetic regulation of inflammatory genes [272, 273]. These type I IFN-driven epigenetic mechanisms show similarities to the histone modifications that underlie trained immunity [110, 272-274].

Finally, type I IFNs could prompt the availability of self-antigens for recognition by the immune system. Self-antigens that are recognized in pSS, such as Ro52 and IFI16, are upregulated in response to type I IFNs and can be exposed to the immune system after inflammatory cell death [196, 275, 276]. Programmed cell death is influenced by type I IFNs. Together with the clearance defects resulting in secondary necrosis that have been described in pSS this could cause excessive presentation of self-antigens to the immune system and thereby contribute the self-amplifying loop of inflammation.

Integrating all of these data, the persistent production of type I IFNs in pSS, and other systemic autoimmune diseases, enhances hyperinflammation and disorganization of

immune responses resulting in tissue damage and sustainment of the autoimmune process. Inhibition of type I IFN signaling with anifrolumab, a blocking anti-IFNAR antibody, has recently provided clinical benefits in SLE and has now been FDA approved for treatment of active SLE [277]. Excitingly, clinical studies will take place in the near future to evaluate anifrolumab in active pSS.

Type I IFN-inducing pathways in primary Sjögren's syndrome

The molecular mechanisms leading to innate immune activation and type I IFN production in response to pathogen-derived nucleic acids, which are nowadays considered as textbook knowledge, have only been recognized for about 20 years. In the early 2000s, the endosomal TLR3, TLR9, TLR7 and TLR8 were among the first PRRs discovered to recognize RNA or DNA [278]. These TLRs were subsequently shown to play a crucial role in anti-viral defense. Ever since, TLRs have received much attention as potential pathogenic factors in autoimmune diseases, including pSS. Stimulation of endosomal TLRs with synthetic viral-like stimuli induce SS-like disease and autoimmunity in mice [55-57]. These observations have further fueled the long-standing hypothesis of viral etiology of pSS.

TLRs have also been the main focus for research into the origin of the type I IFN signature in systemic autoimmunity. Influential publications in this field showed that serum from pSS patients – when mixed with dead cells – could induce IFNα production through TLR7 stimulation in pDCs [219, 279, 280]. The interferogenic capacity of pSS serum correlated with the presence of anti-SSA and anti-SSB autoantibodies [219]. Anti-SSA antibodies are directed against the proteins Ro60 and/or Ro52 and anti-SSB antibodies are directed against the La protein. These three proteins exist together in a ribonucleoprotein complex containing non-coding RNA [281]. The presence of RNA in the pSS-derived *in vitro* formed immune complexes was crucial for their capacity to induce IFNα in pDCs [219]. These immune complexes are thought to be also formed from autoantibodies against RNA-binding proteins *in vivo*, which will stimulate type I IFN production by pDCs. The type I IFNs then further promote B cell hyperactivation, plasma cell formation and autoantibody production, forming an ongoing loop of inflammation (discussed in section above).

Despite the discovery of additional type I IFN-inducing nucleic acid receptors, the immune-complex-mediated TLR7 stimulation in pDCs has been the dominating hypothesis for type I IFN induction in pSS for the last 15 years. However recently, the endogenous retro-element-derived *LINE-1* RNA has been indicated as a first alternative stimulus for type I IFN via both TLRs and cytosolic RIG-I-like receptors in pSS salivary glands [282, 283]. Multiple alternative type I IFN-inducing stimuli and pathways have already been identified in SLE [284-291]. Considering the important pathogenic involvement of type I IFN activation in systemic autoimmune disease, a better understanding of the diversity of triggers

and receptors that control this type I IFN activation will assist the future development of treatment strategies targeting the specific pathways.

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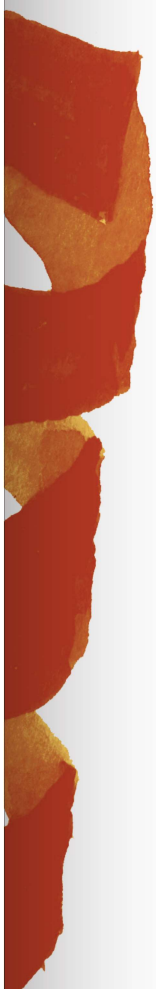
CHAPTER 1.2

Scope of this thesis



Persistent production of type I IFNs contributes to an ongoing autoimmune process in different systemic autoimmune diseases, including primary Sjogren's syndrome (pSS), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). However, the involved triggers and signaling pathways as well as the consequences of type I IFN pathway activation in these diseases are incompletely understood. The overall aim of this thesis is to generate better insight into the pathophysiological mechanisms that trigger and regulate type I IFN pathway activation in systemic autoimmune diseases – with a focus on pSS – as well as the clinical and therapeutic implications of type I IFN pathway activation.

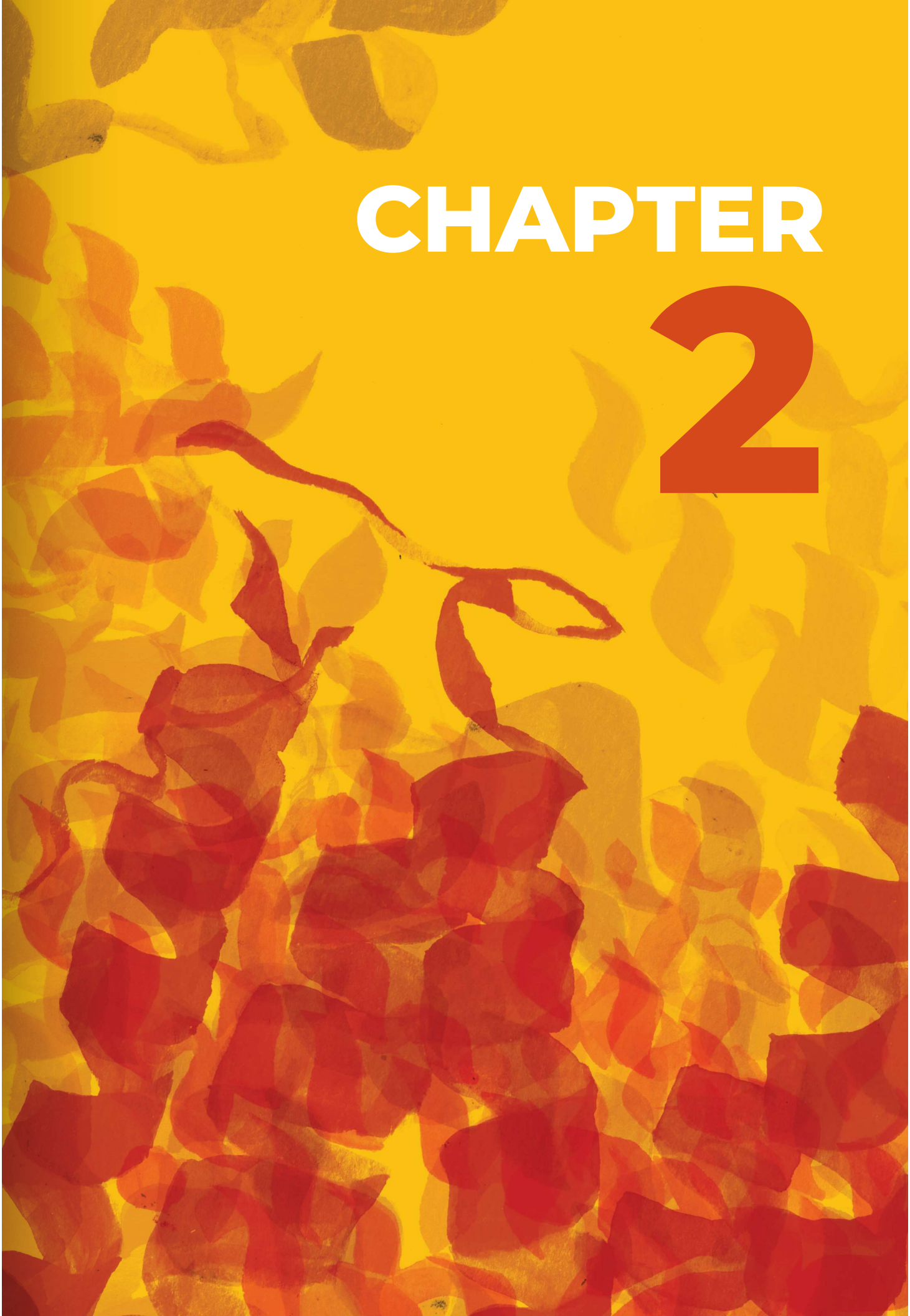
Treatments targeting the type I IFN pathway are being developed and have proven clinical benefit in SLE. Patients with aberrant production of type I IFNs are most likely to benefit from these treatments. Therefore, robust and easy-to-perform assays to detect type I IFN pathway activation are required in order to identify patients eligible for type I IFN pathway-targeting therapy. In **chapter 2** of this thesis, the performance of two immunoassays to detect systemic type I IFN pathway activation in patients with type I IFN-associated systemic autoimmune diseases was assessed. *Chapter 2.1* describes the validation of an immunoassay for quantification of type I IFN-inducible Myxovirus resistance protein 1 (MxA) in blood to detect type I IFN pathway activation in SLE and SSc. In *chapter 2.2,* the novel single-molecule array technology (Simoa) was applied to directly quantify serum IFNα2 protein concentrations in two pSS cohorts and a limited number of SLE and SSc patients. A side-by-side comparison of serum IFNα2 with whole blood ISG expression, intracellular MxA protein levels, and serum IFN-I bioactivity was performed in these cohorts.

Insight in the diverse stimuli and receptor pathways mediating the sustained type I IFN signature in patients will aid the design of new targeted treatments. Endosomal TLRs and immune complexes have been the primary focus of research in pSS, while alternative type I IFN inducers have received little attention. **Chapter 3** of this thesis explores the potential involvement of cytosolic nucleic acid-sensing pathways in the type I IFN pathway activation in pSS. *Chapter 3.1* summarizes and reviews the current evidence for a role of cytosolic RNA- and DNA-sensing pathways and their potential ligands in type I IFN activation in pSS. In *chapter 3.2,* levels of phosphorylated TANK-binding kinase 1 (TBK1) – the nucleic acid-sensing signalling hub – were analysed in plasmacytoid dendritic cells (pDCs) from IFN-high pSS, SLE and SSc. Additionally, TBK1 inhibition as a strategy to downregulate IFN-stimulated gene expression was studied in PBMCs. In *chapter 3.3*, the responsiveness of pSS monocytes and pDCs to stimulation of the cytosolic DNA-sensing STING pathway was evaluated, compared to SLE and related to systemic type I IFN pathway activation.

Type I IFNs have broad immunomodulatory effects. Priming with type I IFNs can alter the functional state of cells. A long-lasting form of cellular adaptation that persists even in the absence of the initial stimulus is termed trained immunity. This recently defined

immunological concept describes the ability of innate immune cells to develop antigen non-specific memory characterized by elevated cytokine responses. This memory-like phenotype is mediated by changes in cellular metabolism and epigenetic reprogramming. Although type I IFNs have been described to induce epigenetic changes, they have so far not been linked to trained immunity. Several observations indicate hyperresponsiveness of innate immune cells in pSS. **Chapter 4** studies the link between type I IFN and trained immunity in an *in vitro* monocytic cell model and PBMCs from pSS as well as SLE patients.

Finally, **chapter 5** summarizes the overall findings of this thesis, discusses the pathophysiological, clinical and therapeutic implications of these findings and provides an outlook on future studies.


CHAPTER
2

CHAPTER 2.1

MxA is a clinically applicable biomarker for type I interferon activation in systemic lupus erythematosus and systemic sclerosis

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Rheumatology (Oxford) 2019;58(7):1302-3

Sir, Aberrant activation of the type I interferon (IFN) system has been implicated in the pathogenesis of systemic autoimmune diseases and is a potential treatment target that is currently under clinical investigation [1]. Laborious and expensive tests for type I IFN activity limit the implementation in routine diagnostics and clinical decision making. Previously, we identified whole blood levels of intracellular Myxovirus resistance protein 1 (MxA), measured by an enzyme immunoassay, as a clinically relevant biomarker for type I IFN activity in patients with Sjögren's syndrome [2]. Here, we assessed the applicability of this assay to detect systemic type I IFN activation in patients with systemic lupus erythematosus (SLE) and systemic sclerosis (SSc).

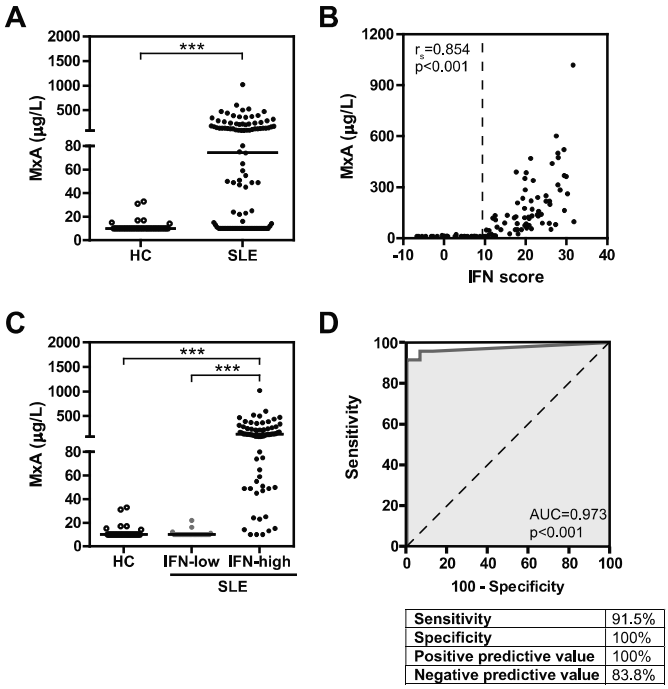
Whole blood intracellular MxA protein levels were measured in SLE patients (discovery cohort: n=25; replication cohort retrieved from the CHILL-NL cohort [3]: n=102), SSc patients (n=28) and healthy controls (HC) (Supplementary Table 1) using the MxA enzyme immunoassay (MxA-EIA) [2, 4]. IFN scores were determined from whole blood gene expression of interferon-inducible genes IFI44, IFI44L, IFIT1, IFIT3, and MxA as previously described [5]. The Medical Ethics Review Committee of the Erasmus MC Rotterdam has approved this study and written informed consent was obtained from all participants, in compliance with the Helsinki Declaration. Further details are provided in the Supplementary Methods.

MxA levels were significantly elevated in both cohorts of patients with SLE, as well as SSc, compared to HC and were highly correlated to IFN scores (r_s=0.735 to r_s=0.854, p≤0.003; Figure 1A, B and Supplementary Figure 1A, B). MxA-EIA robustly discriminated (AUC=0.938 to AUC=0.991, p≤0.007) between low and high type I IFN activity in patients with SLE, as well as SSc, with a specificity of 100% and a sensitivity of 87.5 to 94.7% at a cut-off level of 22 µg/L (Figure 1C, D and Supplementary Figure 1C). MxA is an important mediator in IFN-induced anti-viral immunity which is exclusively regulated by type I and III IFNs [6]. This is reflected in the excellent positive predictive value of the MxA-EIA.

As expected, SLE patients with autoantibodies against Smith (Sm), RNP, Ro/SSA or La/SSB antigens showed higher MxA levels and IFN scores compared to patients without these antibodies (Supplementary Figure 2A and Supplementary Table 2). A positive trend was observed between MxA levels and the number of autoantibodies present in each patient (Supplementary Figure 2B). Patients in both the discovery and the replication SLE cohorts had relatively low disease activity, with a SLEDAI ≥8 in only 13% of all SLE patients. Therefore, this cross-sectional study lacks statistical power to determine any association between type I IFN activity and disease activity.

In conclusion, MxA-EIA is a cheap, easy-to-measure, and highly specific biomarker that accurately reflects type I IFN activity in patients with SLE and SSc. This assay enables selection of patients for IFN-targeting treatments and monitoring of the efficacy of these treatments in downregulating type I IFN activity in routine diagnostics and in the context of clinical trials. Future studies should evaluate the potential applicability of MxA-EIA for

prediction of disease manifestations and monitoring of treatment responses in patient cohorts followed over time.



Sensitivity	91.5%
Specificity	100%
Positive predictive value	100%
Negative predictive value	83.8%

Figure 1. MxA-EIA identifies SLE patients with systemic type I IFN activation.

(A) MxA-EIA levels of SLE (replication cohort) and HC. **(B)** Correlation between IFN score and MxA-EIA levels in SLE. Dashed line: IFN score threshold value. **(C)** MxA-EIA levels in HC and SLE, stratified based on IFN score. **(D)** Receiver operating characteristic curve of MxA-EIA levels to discriminate between IFN-low and IFN-high in SLE. AUC, area under the curve; HC, healthy controls; MxA, Myxovirus resistance protein 1. Symbols represent individual samples, horizontal lines indicate medians. Statistics: Mann Whitney U test (A), Kruskal-Wallis test (C), Spearman's correlation test (B). ***p<0.001.

FUNDING

This work was supported by the Dutch Arthritis Foundation (Reumafonds) [14-3-404, BP12-1-261]; the National Association for LUPUS, APS, Scleroderma and MCTD (NVLE); MAV received financial support from Domainex.

ACKNOWLEDGMENTS

The research for this manuscript was (partly) performed within the framework of the Academic Centers for Rare Immunological Diseases and Inflammunity, and the Erasmus Postgraduate School Molecular Medicine. The authors acknowledge Kimberly Scheurwater for performing the serum analysis.

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SUPPLEMENTARY METHODS

Patients and controls

Twenty-five adult patients classified as SLE according to the 1997 ACR revised criteria for SLE and 28 patients classified as SSc according to the 2013 ACR/EULAR classification criteria for SSc were recruited at the Erasmus MC, Rotterdam University Medical Centre, Rotterdam, The Netherlands [1, 2]. A replication cohort of 102 adults with childhood-onset or adult-onset SLE was retrieved from the CHILL-NL cohort [3]. Information on disease duration, use of medication and serological parameters were retrieved from patient records. Age- and gender- matched healthy controls (HC) were included. Patient characteristics are summarized in Supplementary Table S1. Blood samples were collected in PAXgene RNA tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) for whole blood RNA analysis, clotting tubes for serum isolation and sodium-heparin tubes (Greiner Bio-One, Kremsmünster, Austria).

Serum analysis

Antibodies to extractable nuclear antigens (ENA) SSA/Ro52, SSA/Ro60, SSB/La, U1-RNP (RNP-70, A, C) and Smith (D) from patients of whom a recent autoantibody status was missing were detected by a combination of line immunoassay using Euroline ANA profile 3 immunoblot (Euroimmun, Lübeck, Germany) and fluorescence enzyme immunoassay on the Phadia® 250 system using EliA™ (Thermo Fisher Scientific, Freiburg, Germany). The assays were performed according to the manufacturer's instructions. Anti-ENA were considered positive only when both assays showed a positive result.

MxA enzyme immunoassay

Heparinized blood was lysed 1:20 and stored at -80°C until assayed. MxA enzyme immunoassay was performed as previously described [4, 5].

Real-time PCR

Whole blood RNA was isolated from PAXgene tubes according to manufacturer's protocol and reverse transcribed to cDNA using High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, USA). RT-PCR was performed on a Quantstudio™ 5 Real-Time PCR System using predesigned primer/probe sets (Applied Biosystems). Data were normalized to the expression of the housekeeping gene Abl. Relative gene expression was calculated using the $2^{-\Delta Ct}$ method.

Calculation of IFN score

The IFN score as measure for type I IFN activity was defined by the relative expression of 5 interferon-inducible genes: IFI44, IFI44L, IFIT1, IFIT3 and MxA. Expression levels were standardized to the $Mean_{HC}$ and $S.D._{HC}$ of each gene in the HC cohort. IFN scores per subject represent the sum of standardized scores, calculated as previously described [6]. IFN scores of SSc patients were calculated using a separate cohort of age- and gender-matched HC. Patients were classified as either IFN-low or IFN-high using a threshold of $Mean_{HC} + 2*S.D._{HC}$ [7].

Statistical analysis

Statistical analysis was performed as indicated in the figure legends. Values of $p< 0.05$ were considered statistically significant. Graphs were designed using Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA) and IBM SPSS Statistics 24.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis.

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SUPPLEMENTARY DATA

Supplementary Table 1. Patient characteristics.

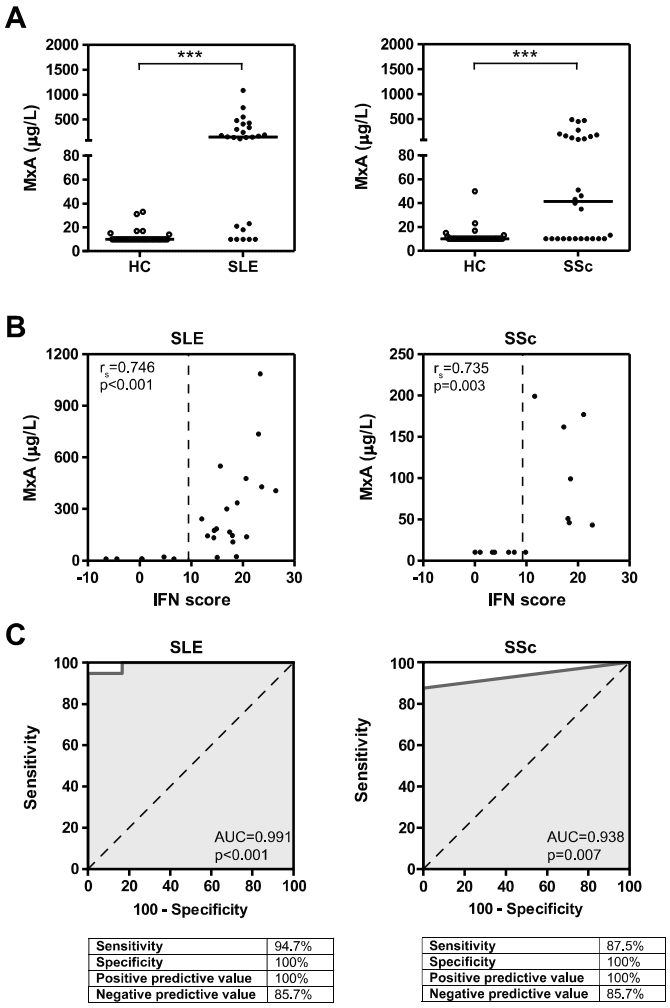
	HC	SLE		HC	SSc
	(n=39)	Discovery cohort (n=25)	Replication cohort (n=102)	(n=46)	(n=28)
Demographics					
Female (%)[a]	35/39 (90)	24/25 (96)	93/102 (91)	43/46 (94)	23/28 (82)
Age (years)[b,c]	35 (28)[b]	39 (19)[b]	34 (19)[b]	52.5 (8)[c]	55.8 (13)[c]
Patient characteristics					
Disease duration (years)[b]	NA	14 (16)	16 (16)	NA	9 (15)
SLEDAI-2K[b]	NA	2 (3)	4 (4)	NA	NA
Laboratory parameters[a]					
ANA	NA	23/25 (92)	102/102 (100)	NA	27/28 (96)
Anti-dsDNA	NA	14/25 (56)	95/101 (94)	NA	2/28 (7)
Anti-Sm	NA	9/25 (36)	30/100 (30)	NA	1/26 (4)
Anti-RNP	NA	11/25 (44)	41/100 (41)	NA	1/27 (4)
Anti-Ro/SSA	NA	12/25 (48)	34/100 (34)	NA	10/28 (36)
Anti-La/SSB	NA	6/25 (24)	15/100 (15)	NA	3/28 (11)
Current medication[a]					
Corticosteroids/DMARDs	NA	14/21 (67)	69/102 (68)	NA	9/26 (35)
Corticosteroids + DMARDs	NA	7/14 (50)	39/69 (57)	NA	0/9 (0)
Corticosteroids only	NA	5/14 (36)	11/69 (16)	NA	4/9 (44)
DMARDs only	NA	2/14 (14)	19/69 (28)	NA	5/9 (56)
HCQ	NA	16/21 (76)	78/102 (76)	NA	2/26 (8)
HCQ + corticosteroids/DMARDs	NA	12/16 (75)	56/78 (72)	NA	1/2 (50)
HCQ monotherapy	NA	4/16 (25)	22/78 (28)	NA	1/2 (50)

Data are presented as number of patients (%)[a], median (IQR)[b] or mean (S.D.)[c] according to the data distribution. Abbreviations: anti-Sm, anti-Smith; HC, healthy controls; IQR, interquartile range; NA, not applicable.

Supplementary Table 2. Association of autoantibodies with type I IFN activity.

		IFN score		
Variable	n	Variable present	Variable not present	P value
Autoantibodies				
Anti-dsDNA	109/126	17.23 (6.75 to 22.36)	17.92 (12.99 to 21.85)	0.805
Anti-Sm	39/125	19.73 (12.11 to 23.67)	15.27 (5.57 to 20.73)	0.055
Anti-RNP	52/125	19.84 (12.58 to 26.49)	13.13 (5.02 to 20.22)	0.001
Anti-Ro/SSA	46/125	18.94 (14.33 to 21.67)	12.60 (4.70 to 22.88)	0.039
Anti-La/SSB	21/125	20.16 (15.35 to 24.44)	15.18 (5.22 to 21.60)	0.012

Data are presented as median (25th to 75th percentile). Mann-Whitney U test was used to compare medians between two groups. Abbreviations: anti-Sm, anti-Smith.

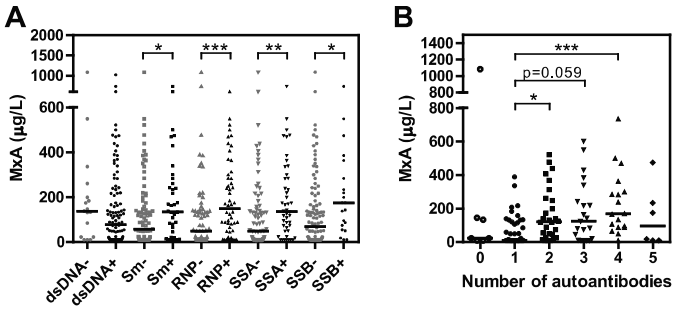


Sensitivity	94.7%
Specificity	100%
Positive predictive value	100%
Negative predictive value	85.7%

Sensitivity	87.5%
Specificity	100%
Positive predictive value	100%
Negative predictive value	85.7%

Supplementary Figure 1. MxA-EIA identifies systemic type I IFN activation in SLE and SSc.

(A) MxA-EIA levels of SLE (left, discovery cohort) and SSc (right) compared to age- and gender-matched HC. **(B)** Correlation between IFN score and MxA-EIA levels in SLE (left) and SSc (right). Dashed lines: IFN score threshold value. **(C)** Receiver operating characteristic curves of MxA-EIA levels to discriminate between IFN-low and IFN-high in SLE (left) and SSc (right). AUC, area under the curve; MxA, Myxovirus resistance protein 1. Symbols represent individual samples, horizontal lines indicate medians. Statistics: Mann Whitney U test (A) and Spearman's correlation test (B). ***p<0.001.



Supplementary Figure 2. MxA-EIA levels are related to autoantibodies in SLE.

(A) MxA-EIA levels in SLE patients without (-) or with (+) autoantibodies against dsDNA, Sm, RNP, Ro/SSA and La/SSB. **(B)** MxA-EIA levels of SLE patients stratified based on the number of the following autoantibodies present in each patient: dsDNA, Sm, RNP, Ro/SSA and La/SSB. MxA, Myxovirus resistance protein 1; Sm, Smith. Symbols represent individual samples, horizontal lines indicate medians. Statistics: Mann Whitney U test (A) and Kruskal-Wallis test (B). *p<0.05, **p<0.01, ***p<0.001.

CHAPTER 2.2

Serum IFNα2 measured by single-molecule array associates with systemic disease manifestations in Sjögren's syndrome

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Rheumatology (Oxford) 2021; online ahead of print

ABSTRACT

Objectives: Type I interferon (IFN-I) activation is a prominent feature of primary Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc). Ultrasensitive single-molecule array (Simoa) technology has facilitated the measurement of subfemtomolar concentrations of IFNs. Here, we aimed to measure IFNα2 in serum from pSS, SLE, and SSc using a Simoa immunoassay and correlate these levels to blood IFN-stimulated gene (ISG) expression and disease activity.

Methods: Serum IFNα2 was measured in patients with pSS (n=85; n=110), SLE (n=24), and SSc (n=23), and healthy controls (HC; n=68) using an IFNα Simoa assay on a HD-X analyzer. IFN-I pathway activation was additionally determined from serum by an IFN-I reporter assay and paired samples of whole blood ISG expression of *IFI44*, *IFI44L*, *IFIT1*, *IFIT3*, and *MxA* by RT-PCR or MxA-ELISA.

Results: Serum IFNα2 levels were elevated in pSS (median=61.3 fg/mL) compared to HC (median ≤5 fg/mL; p<0.001) and SSc (median=11.6 fg/mL; p=0.043), lower compared to SLE (median=313.5 fg/mL; p=0.068), and positively correlated with blood ISG expression (r=0.66-0.94; p<0.001). Comparable to MxA-ELISA (AUC=0.93), IFNα2 measurement using Simoa identified pSS with high ISG expression (AUC=0.90) with 80-93% specificity and 71-84% sensitivity. Blinded validation in an independent pSS cohort yielded a comparable accuracy. Multiple regression indicated independent associations of autoantibodies, IgG, hydroxychloroquine (HCQ) treatment, cutaneous disease and history of extraglandular manifestations with serum IFNα2 concentrations in pSS.

Conclusion: Thus, Simoa serum IFNα2 reflects blood ISG expression in pSS, SLE, and SSc. In light of IFN-targeting treatments, Simoa could potentially be applied for patient stratification or retrospective analysis of historical cohorts.

INTRODUCTION

Sustained systemic activation of the type I interferon (IFN-I) pathway is a well-known pathophysiological feature of several systemic autoimmune diseases (SADs), such as systemic lupus erythematosus (SLE), primary Sjögren's syndrome (pSS), and systemic sclerosis (SSc) [1]. Therapeutic strategies targeting IFN-I are under clinical evaluation. Anifrolumab, a blocking antibody against the IFN-I receptor (IFNAR), has recently been found to induce clinically relevant responses in patients with active SLE [2-4]. Measurement of IFN-I will likely assist in the selection of candidates for these targeted treatments, urging the development and validation of robust and easy-to-perform assays.

The IFN-I family comprises 16 protein subtypes that have important antiviral and immunomodulatory properties [5-7]. These functions are largely effectuated by signalling through the IFNAR complex. The diversity of subtypes and the low circulating levels complicate quantification of IFN-I protein in biological samples by routinely used laboratory techniques such as ELISA.

Various methods measuring different elements of the IFN pathway are being used to evaluate IFN-I pathway activation. The majority of these assays exploit downstream cellular responses to IFN by quantification of expression of either IFN-stimulated genes (ISGs) or proteins. The development of ultrasensitive single-molecule array (Simoa) digital ELISA technology has facilitated the direct measurement of IFNα protein levels [8, 9].

At present, a handful of studies have been published employing this ultrasensitive technique in patients with monogenic interferonopathies or SLE, while application in other SADs has been limited [8, 10-13]. Most of these studies use custom homebrew assays developed with autoantibodies specific for all IFNα subtypes cloned from patients with autoimmune polyglandular syndrome type 1 (APS1) [8, 10, 13].

Here, we tested the performance of a commercially available ready-to-use Simoa immunoassay kit for quantification of IFNα2 protein concentrations in serum from two independent and clinically well characterized pSS cohorts and compared the IFNα2 protein levels to those in SLE and SSc patients. Additionally, a side-by-side comparison of serum IFNα2 with whole blood ISG expression, intracellular MxA protein levels, and serum IFN-I bioactivity was performed in these cohorts.

METHODS

Patients and healthy controls

Patients with pSS (n=85), SLE (n=24), SSc (n=23), and healthy controls (HC; n=40) recruited at the Erasmus MC, University Medical Center Rotterdam, the Netherlands from

which at least paired samples of serum and PAXgene material were stored, were included in this study (Rotterdam cohort). An established cohort of pSS patients (n=110) recruited at the department of Rheumatology in Malmö, Sweden, Lund University [14], and additional HCs (n=28) recruited at the Erasmus MC were used as a validation cohort (Malmö cohort). This study has been approved by the Medical Ethics Review Committees of the Erasmus MC (MEC-2011-116; MEC-2016-202) and Lund University (2015/311; 2017/94). In accordance with the declaration of Helsinki, written informed consent was obtained from all participants. Further details are provided in Supplementary Methods. Demographic and clinical characteristics of the study cohorts are summarized in Table 1 and Supplementary Table 1.

Ultrasensitive IFNα single-molecule array

IFNα2 was measured in duplicates from serum samples (diluted two-fold in sample diluent) using the Simoa® IFN-α Advantage Kit (number 100860, Quanterix, Billerica, USA) following the instructions of the supplied manual. Sample processing and analysis was done using a HD-X analyzer (software version 1.6.1905.300; Quanterix). Lower limit of detection was 5 fg/mL.

Recombinant IFN-I subtypes

To verify the analytical specificity of the Simoa® IFN-α Advantage kit and HEK293-3C11-ISRE reporter cells, serum from a HC or special stripped serum (Valley Biomedical, Winchester, USA) were spiked with recombinant human IFNα-1(D), -D(1), -A(2a), -2(2b), -4a(M1), -4b(4), -G(5), -K(6), -J1(7), -B2(8), -H2(14), -WA(16), -I(17), -F(21), IFN-β1a (all from PBL Assay Science, Tebu-bio, Heerhugowaard, The Netherlands) or IFN-γ (PeproTech, Cranbury, USA) and snap frozen or assayed directly.

Real-time PCR IFN-stimulated genes, MxA-immunoassay, and IFN-I-reporter assay

Whole blood expression of ISGs *MxA, IFI44, IFI44L, IFIT1,* and *IFIT3* was quantified from PAXgene Blood RNA tubes (PreAnalytiX GmbH, Becton Dickinson, Vianen, The Netherlands) by RT-PCR, and an IFN-I score was calculated as previously described [15]. The threshold value for the IFN-I score was set to the 97.5th percentile of IFN-I scores in HCs, consistent with the intercept of fitted Gaussian density components in a finite mixture model and the local minimum of a nonparametric density estimate of IFN-I score data in autoimmune patients (Supplementary Figure 1). Intracellular MxA protein was measured by an immunoassay (lower limit of detection 10 µg/L) as previously described [16-18]. IFN-I activity in serum was measured using pGreenFire-ISRE reporter construct-transduced HEK293-3C11

Table 1. Demographic and clinical characteristics.

Cohort	Rotterdam				Malmö	
	HC n = 68	SLE n = 24	SSc n = 23	pSS n = 85	pSS n = 110	P value
Demographics						
Female[a]	62/68 (91.2)	22/24 (91.7)	20/23 (87.0)	77/85 (90.6)	102/110 (92.7)	0.782
Age [years][b]	51 (32.8-57)	49 (37-55)	61 (53-67.5)	62 (53-68)	64.5 (47-72)	0.737
Patient characteristics						
Disease duration [years][b]	-	15 (7.5-19.3)	11 (8-20.5)	12 (6-20.3)	12 (5-21)	0.354
Disease activity[b] (1)	-	2 (0-4)	-	2 (0-7.25)	3 (1-7.75)	0.290
Clinical disease activity[b] (2)	-	0 (0-1)	-	2 (0-9.25)	2 (0-8)	0.763
Laboratory parameters						
ANA[a]	-	24/24 (100)	18/21 (91.3)	68/85 (80)	89/110 (80.9)	1.000
Anti-SSA[a]	-	12/24 (50)	-	72/85 (84.7)	95/110 (86.4)	0.903
Anti-Ro52[a]	-	-	-	64/72 (88.9)	70/82 (85.4)	0.939
Anti-Ro60[a]	-	-	-	66/72 (91.7)	78/82 (95.1)	0.234
Anti-SSB[a]	-	7/24 (29.2)	-	48/85 (56.5)	63/110 (57.3)	1.000
Anti-dsDNA [IU/mL][b]	-	14.5 (1.63-39.5)	-	-	-	
Anti-Sm[a]	-	5/24 (20.8)	-	-	-	
Anti-RNP[a]	-	9/24 (37.5)	-	-	-	
IgG [g/L][b]	-	-	-	13.1 (10.2-16.7)	14.6 (11.3-17.3)	0.219
C3 [g/L][b]	-	1.08 (0.89-1.17)	-	1.16 (1.03-1.30)	0.93 (0.81-1.07)	**< 0.001**
C4 [g/L][b]	-	0.19 (0.16-0.22)	-	0.19 (0.14-0.23)	0.17 (0.13-0.22)	0.137
Current medication[a]						
HCQ	-	17/24 (70.8)	2/23 (8.7)	42/85 (49.4)	35/110 (31.8)	**0.019**
HCQ monotherapy	-	4/17 (23.5)	1/2 (50)	35/42 (83.3)	17/35 (48.6)	**0.003**
HCQ + corticosteroids / DMARDs / biologics	-	13/17 (76.5)	1/2 (50)	7/42 (16.7)	18/35 (51.4)	
Corticosteroids/DMARDs	-	18/24 (75)	13/23 (56.5)	12/85 (14.1)	29/110 (26.4)	0.057
Corticosteroids + DMARDs	-	2/18 (11.1)	1/13 (7.7)	2/12 (16.7)	6/29 (20.7)	-
Corticosteroids only	-	10/18 (55.6)	5/13 (38.5)	9/12 (75)	22/29 (75.9)	-
DMARDs only	-	6/18 (33.3)	7/13 (53.8)	1/12 (8.3)	1/29 (3.5)	-
Rituximab	-	0/24 (0)	0/23 (0)	0/85 (0)	8/110 (7.3)	-
Belimumab	-	2/24 (8.3)	0/23 (0)	1/85 (1.2)	0/110 (0)	-

Data are presented as number of patients (%)[a] or median (Q1-Q3)[b]. (1) Disease activity: EULAR Sjögren's syndrome disease activity index (ESSDAI) for pSS and SLEDAI-2K for SLE. (2) Clinical disease activity: ClinESSDAI for pSS and ClinSLEDAI for SLE. Mann-Whitney U test or Pearson's Chi-square test were used to compare medians or frequencies of the Rotterdam and Malmö pSS cohorts. Abbreviations: anti-Sm, anti-Smith; ns, not significant.

cells (kindly provided by Jan Rehwinkel, University of Oxford, UK)[19] and HC PBMCs. Assay details are provided in Supplementary Data 1.

Statistical analysis

Statistical analyses were performed in R (version 3.6.3) [20] using the clikcorr (Censoring Data and Likelihood-Based Correlation Estimation) package (version 1.0) for correlation analysis [21], the pROC package (version 1.16.2) for receiver operating characteristic (ROC)

analysis [22], and the CensReg (Censored Regression Tobit Models) package (version 0.5-30) for bivariate and multivariable regression analysis [23].

RESULTS

Simoa IFN-α Advantage Kit primarily detects IFNα2

To assess the analytical specificity of the Simoa® IFN-α Advantage Kit, serum from a HC was spiked with one of 15 recombinant IFNα subtypes, IFNβ or IFNγ. Quantification indicated efficient detection of IFNα2 subvariants in contrast to detection of IFNα4a, IFNα6 and IFNα10 only at the highest concentration tested (> 1500 fg/mL) (Supplementary Figure 2).

Serum IFNα2 concentrations are elevated in pSS, SLE and SSc

Detectable concentrations of IFNα2 were present in 75.3% of serum samples from pSS (Rotterdam cohort), 75% from SLE, 56.5% from SSc, and 45% from HCs. Serum IFNα2 concentrations were elevated in pSS (Rotterdam cohort, median=61.3 fg/mL) compared to HCs (median≤5 fg/mL, $p<0.001$) and SSc (median=11.6 fg/mL, $p=0.043$) (Figure 1A). The highest IFNα2 concentrations were observed in serum from SLE patients (median=313.5 fg/mL). SLE patients in remission (according to the DORIS classification [24]) showed significantly lower ($p=0.015$) serum IFNα2 concentrations compared to patients not in remission (Supplementary Figure 3), which is in line with previous observations [8, 11, 12].

Serum IFNα2 yields lower accuracy in discriminating pSS from HCs than ISG expression

Next, we compared serum IFNα2 with a whole blood 5 ISG based transcript score (IFN-I score) reflecting *in vivo* IFN-I bioactivity. In the same cohort, we also assessed whole blood intracellular MxA protein concentrations by an immunoassay that we previously described to be highly correlated with the IFN-I score in both pSS and SLE patients [16, 17]. The IFN-I score and intracellular MxA concentrations in pSS, SLE, and SSc followed a similar pattern as serum IFNα2 (Figure 1B,C). The diagnostic accuracy of serum IFNα2 to discriminate between pSS and HC was evaluated by ROC analysis. The area under the curve (AUC) was lower for serum IFNα2 (AUC=0.77) compared to both IFN-I score (AUC=0.86, $p=0.019$) and intracellular MxA (AUC=0.85, $p=0.036$) (Supplementary Figure 4A). The Youden's J index summarizes the overall diagnostic performance of a test in a value between 0 (no diagnostic value) and 1 (perfect test) by integrating sensitivity and specificity with equal weight and can be used as a criterion for selection of optimal cutoff values. A maximum Youden's J index of 0.54 was reached at a threshold of 19.3 fg/mL serum IFNα2 (Supplementary Figure 4B), yielding 62% sensitivity and 90% specificity (Supplementary Table 2).

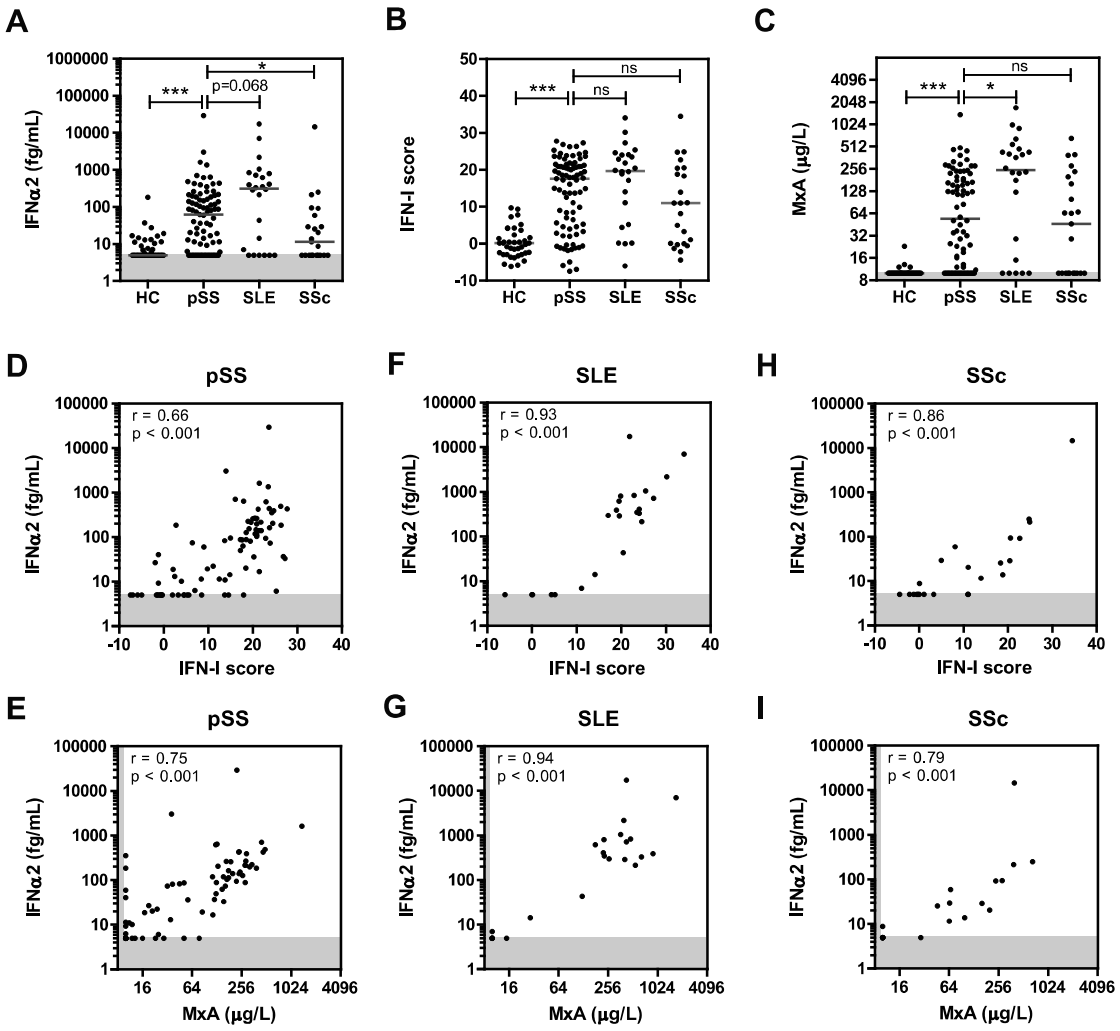

Figure 1. Serum IFNα2 concentrations positively correlated with blood ISG expression in pSS, SLE and SSc. **(A)** Serum IFNα2, **(B)** blood IFN-I score, and **(C)** blood intracellular MxA protein in healthy controls (HCs;n=40) and patients with pSS (n=85), SLE (n=24), and SSc (n=23). Correlation between serum IFNα2 and **(D,F,H)** IFN-I score or **(E,G,I)** intracellular MxA protein concentrations in **(D,E)** pSS, **(F,G)** SLE, and **(H,I)** SSc. Horizontal lines represent medians and shaded regions indicate values below lower limit of detection. Statistics: **(A,C)** Censored Regression analysis, **(B)** Kruskal-Wallis H test, **(D-I)** Likelihood-based correlation coefficient estimation. * $p < 0.05$; ***$p < 0.001$, ns = not significant.

Serum IFNα2 is positively correlated with whole blood ISG expression and serum IFN-I bioactivity

Serum IFNα2 concentrations were positively correlated with IFN-I score (r=0.66, p<0.001), intracellular MxA protein levels (r=0.75, p<0.001) and expression of individual ISG transcripts in pSS (Figure 1D,E**;** Supplementary Figure 5A). In SLE and SSc patients, similar

correlations between the IFN-I score and serum IFNα2 were observed, with the highest correlation coefficients in SLE (Figure 1F-I**;** Supplementary Figure 5B,C). A different method to assess bioactive IFN-I in serum is an IFNα/β-luciferase reporter assay (Supplementary Figure 6A). In this assay, ISG-inducing capacity of patients' sera was observed only for a minority of samples, mostly derived from SLE patients (Supplementary Figure 6B). Taking an alternative approach using HC-PBMCs, SLE sera induced higher levels of IFNAR-dependent ISG expression compared to pSS sera (Supplementary Figure 6C). The IFN-I bioactivity in SLE sera positively correlated with serum IFNα2 ($r=0.62$, $p<0.001$), IFN-I score ($r=0.61$, $p<0.001$), and MxA levels ($r=0.51$, $p=0.009$) (Supplementary Figure 6D).

Serum IFNα2 identifies pSS with high ISG expression

The IFN-I scores in pSS patients follow a bimodal distribution on the basis of which patients were classified as IFN-low or IFN-high. As expected, IFNα2 concentrations were significantly higher in IFN-high compared to IFN-low pSS (Figure 2A). ROC analysis indicated comparable discriminative ability of serum IFNα2 (AUC=0.9) and intracellular MxA (AUC=0.93) to identify IFN-high pSS patients (Figure 2B). The maximum accuracy of 83.7% and maximum Youden's J index of 0.67 were obtained for serum IFNα2 at a threshold of 19.8 fg/mL and only changed marginally when increasing the threshold to 28 or 66 fg/mL (Supplementary Figure 7; Table 2). Using these thresholds, sensitivity varied from 71-84% and specificity from 80-93%. Blinded validation in the second, independently collected pSS cohort (Malmö cohort) expanded with 28 additional HCs confirmed the measures of discriminative property and predictive ability of serum IFNα2 (Table 2; Supplementary Table 2). Notably, serum IFNα2 data, IFN-I scores, and the correlation between them were replicable in two sets of HCs and pSS patients from both cohorts despite independent

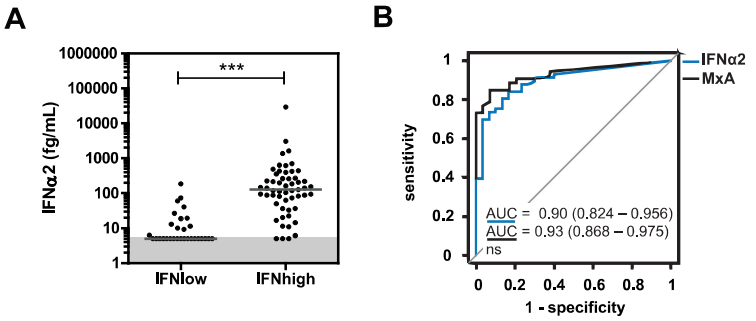

Figure 2. Serum IFNα2 identifies pSS with high ISG expression.

(A) Serum IFNα2 in pSS patients stratified according to IFN-I score. IFN-I score threshold: 97.5th percentile of HCs. Horizontal lines represent medians and shaded region indicate values below lower limit of detection. **(B)** ROC curves of serum IFNα2 and MxA for discrimination of IFN-low and IFN-high pSS (n=85). Statistics: **(A)** Censored Regression analysis and **(B)** Bootstrap test for two correlated ROC curves to compare AUCs. ***p<0.001, ns = not significant. Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic.

Table 2. Measures of accuracy of serum IFNα2 to identify IFN-high pSS.

Cohort	Rotterdam			Validation in Malmö cohort		
Threshold	19 fg/mL	28 fg/mL	66 fg/mL	19 fg/mL	28 fg/mL	66 fg/mL
Accuracy	0.83 (0.74-0.90)	0.83 (0.74-0.91)	0.79 (0.70-0.87)	0.89	0.89	0.76
Sensitivity	0.84 (0.75-0.93)	0.80 (0.70-0.91)	0.71 (0.59-0.82)	0.92	0.91	0.73
Specificity	0.80 (0.63-0.93)	0.87 (0.73-0.97)	0.93 (0.83-1)	0.81	0.85	0.89
NPV	0.73 (0.61-0.86)	0.70 (0.60-0.83)	0.63 (0.54-0.74)	0.75	0.73	0.50
PPV	0.89 (0.81-0.96)	0.92 (0.85-0.98)	0.95 (0.88-1)	0.94	0.95	0.95

Data represent measures of accuracy (95% confidence interval) calculated from ROC analysis of the Rotterdam cohort (pSS: n=85, HC: n=40) and blinded validation of the presented threshold values in the Malmö cohort (pSS: n=110) extended with 28 additional HCs. Abbreviations: NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

sample collection, processing, and measurement (Supplementary Figure 8). A total of 15 pSS patients from both cohorts that were classified as IFN-high based on their IFN-I score exhibited serum IFNα2 concentrations <19 fg/mL. In comparison to IFN-high pSS with IFNα2 >19 fg/mL, these patients had lower IFN-I scores, intracellular MxA and total IgG and were more frequently negative for antinuclear and anti-SSB autoantibodies (Supplementary Table 3). No differences between these groups were observed in use of medication, complement factors, disease activity and age.

Serum IFNα2 is associated with serological disease and HCQ treatment in pSS

Bivariate analyses indicated higher serum IFNα2 concentrations in pSS patients with autoantibodies and lower IFNα2 in patients currently treated with hydroxychloroquine (HCQ; Table 3). Serum IFNα2 was positively correlated with total IgG levels in both cohorts and negatively correlated with complement components C3 and C4 in the Rotterdam cohort (Table 3). Neither total ESSDAI score [25] nor the number of active ESSDAI domains were significantly correlated with serum IFNα2 (Table 3; Supplementary Table 4). Considering each ESSDAI domain separately, active involvement of haematological or cutaneous domains were associated with higher IFNα2 (Supplementary Table 4). No differences in serum IFNα2 were observed between patients with active cutaneous vasculitis/purpura (n=4) and patients with subacute cutaneous lupus erythematosus (n=5) (data not shown). On the other hand, patients with active articular involvement had lower IFNα2 levels (Supplementary Table 4). Multivariable regression analysis was performed on the pooled data from both cohorts to increase statistical power. Multiple regression revealed independent associations of total IgG levels, autoantibodies, use of HCQ and activity in articular and cutaneous ESSDAI domains, but not total ESSDAI score or number of active ESSDAI domains with serum IFNα2 in pSS (Table 3; Supplementary Table 4).

Table 3. Clinical characteristics associated with serum IFNα2 in pSS.

Cohort	Bivariate analysis				Multivariable analysis		
	Rotterdam		Malmö				
Variables	Serum IFNα2	P value	Serum IFNα2	P value	Estimate	Standard error	P value
(Intercept)					1.647	0.909	0.070
ESSDAI score	0.166[a]	0.139	0.014[a]	0.889	-0.017	0.024	0.482
Age	-0.158[a]	0.156	-0.122[a]	0.199	-0.015	0.009	0.116
Gender		0.349		0.933	0.332	0.469	0.479
Female	49.9 (≤5-185.8)[b]		80.9 (15.3-308.3)[b]				
Male	88.9 (49.3-188.9)[b]		102.3 (28.2-265.5)[b]				
IgG	0.552[a]	**<0.001**	0.367[a]	**<0.001**	0.092	0.027	**<0.001**
C3	-0.249[a]	**0.026**	-0.046[a]	0.629	-0.050	0.656	0.939
C4	-0.317[a]	**0.004**	-0.089[a]	0.351	-0.887	2.434	0.716
HCQ		0.056		**0.038**	-1.068	0.270	**<0.001**
No	88 (11.4-243.5)[b]		90.5 (37.8-330.5)[b]				
Yes	30 (≤5-124)[b]		47 (7-110.4)[b]				
Corticosteroids		0.777		0.676	0.355	0.350	0.311
No	61.3 (5.1-179.9)[b]		84.5 (13.2-259.3)[b]				
Yes	95.2 (9.5-192)[b]		64.9 (41.7-506.7)[b]				
DMARDs		0.722		0.485	0.444	0.636	0.486
No	67.9 (6.2-185.9)[b]		79.5 (13.3-286.1)[b]				
Yes	19.3 (12-221.2)[b]		118.8 (64.9-467.8)[b]				
ANA		**<0.001**		**<0.001**	1.040	0.407	**0.011**
Neg	≤5 (≤5-11.4)[b]		7.2 (≤5-73.9)[b]				
Pos	91 (16.1-218.9)[b]		95.5 (42.7-405.5)[b]				
SSA		**<0.001**		**<0.001**	1.412	0.450	**0.002**
Neg	≤5 (≤5-10.1)[b]		≤5 (≤5-10.5)[b]				
Pos	87.9 (12.6-214.5)[b]		86.5 (40.6-330.5)[b]				
SSB		**<0.001**		**<0.001**	0.765	0.295	**0.010**
Neg	6.1 (≤5-26.8)[b]		37.5 (6.2-148.5)[b]				
Pos	122 (57.6-259.7)[b]		90.5 (51.1-427.4)[b]				
Storage years	-0.188[a]	0.283	-	-	-0.145	0.108	0.179

Bivariate analysis: Data represent correlation coefficients[a] or medians (Q1-Q3)[b] of IFNα2 concentrations (fg/mL) in pSS patients from the Rotterdam and Malmö cohort. Correlations between log(IFNα2) and indicated variables were assessed by likelihood-based correlation coefficient estimation. Multivariable analysis: independent associations of indicated variables with log(IFNα2) in the pooled cohort were evaluated in a censored regression (Tobit) model. For both cohorts, absolute C3 levels were centralized before inclusion in multivariable analysis. Abbreviations: ESSDAI, EULAR Sjögren's syndrome disease activity index.

Higher serum IFNα2 in pSS with extraepithelial extraglandular disease manifestations

Patients with extraglandular manifestations (EGM) are at higher risk for morbidity and mortality [26-28]. We evaluated the association between serum IFNα2 and EGM defined as current or past pSS-related systemic involvement in any of the organ-specific ESSDAI domains. Although a trend for higher IFNα2 was observed in pSS with EGM compared to patients with exclusive glandular disease, this difference did not reach statistical

significance (Supplementary Table 5). Based on the underlying pathophysiological mechanisms, EGM were further classified into periepithelial (pulmonary, central nervous system, muscular, renal:tubulointerstitial nephritis), extraepithelial (cutaneous, peripheral nervous system, lymphadenopathy, haematological, renal:glomerulonephritis) and non-specific (constitutional, articular) EGM [29, 30]. Current or past extraepithelial EGM, but not periepithelial or non-specific EGM, was associated with higher IFNα2 concentrations and a positive correlation was observed between the number of extraepithelial EGM domains and serum IFNα2 (Supplementary Table 5). This association remained significant in a multivariable regression analysis adjusting for periepithelial and non-specific EGM, current use of medication, age, gender and serological parameters (Supplementary Table 6). Considering the individual extraepithelial EGM domains, median IFNα2 levels were higher in patients with a history in either one or a combination of cutaneous (69% cutaneous vasculitis), haematological or lymphadenopathy (including lymphoma) domains, but not the PNS domain, compared with patients without a history in any of the extraepithelial domains (Supplementary Figure 9).

DISCUSSION

IFN-I pathway activation is a well-known feature in multiple SADs, but direct ultrasensitive measurement of IFNα protein has been very limited in patients with SADs other than SLE. Using a commercially available Simoa IFNα immunoassay kit, we here found elevated levels of circulating IFNα2 protein in two independent cohorts of pSS patients and patients with SSc and SLE. In pSS, SLE, and SSc, these IFNα2 protein levels were positively correlated with blood ISG transcript and MxA protein expression, commonly used as surrogate markers for IFN-I pathway activation. These correlations were strongest in SLE patients. In SLE, we have found higher IFNα2 levels in patients not in remission and additionally observed high IFNα2 levels in a proportion of patients in remission. These data confirm previous observations that linked high levels of serum IFNα to active disease and risk of relapse [8, 11, 12]. The results obtained from two independent pSS cohorts were highly comparable, indicating good replicability of IFNα2 measurements between cohorts. Regression analysis did not show a significant negative association between IFNα2 and duration of serum storage in this cross-sectional cohort. This indicates that IFNα2 protein levels in serum were relatively stable for up to 6 years (-80°C) as occurred in our study. Hence, this technique is applicable for retrospective analysis of historical cohorts from which only serum is stored.

Ultrasensitive IFNα protein measurement has well-defined specificity. In this study, we used the sole commercially available IFNα Simoa kit that we verified to primarily detect IFNα2, one of the most potent inducers of ISG expression of the IFNα subtypes [31].

Others have used homebrew Simoa assays with high affinity antibodies, cloned from APS1 patients, that recognize all IFNα subtypes [8, 10, 13]. However, these antibodies are currently not commercially available. Using these antibodies, the authors reported a positive correlation between serum IFNα concentrations and ISG expression score in SLE patients [8]. Here, we show similar correlations between blood ISG score and serum IFNα2. Although the timing and magnitude of expression may vary between the different human *IFN-A* genes, IFNα2 seems to be expressed exclusively in parallel with other IFNα subtypes [32, 33]. The analogous results obtained by two Simoa assays with distinct specificities and the current knowledge on human IFNα biology suggest co-expression of multiple IFNα subtypes contributing to ISG expression in patients with SADs. A "gold standard" capturing the entirety of the concept IFN-I pathway activation does not exist. Measurement of ISGs is the most frequently used method but interpretation of this method for IFN-I specificity has been complicated by the significant overlap between gene expression modules that are activated by distinct IFN receptor complexes and even IFN-independent signalling [34-38]. Nevertheless, attempts have been made to define gene modules preferentially regulated by type I or type II IFNs [39, 40]. The five genes of the composite ISG score that we used here belong to an IFN module induced *in vivo* upon treatment with recombinant IFNα or IFNβ and upregulated *in vitro* preferentially by IFN-I over type II IFN [39]. This ISG score showed an excellent correlation with circulating IFNα2 protein.

A small subset of pSS patients, mainly characterized by overall milder serological disease activity, exhibited a discrepancy between ISG expression score and IFNα2 levels. Therefore, in accordance with studies in SLE, whole blood ISG expression seems to be a more sensitive method for IFN-I activation in patients with low levels of IFNα [8, 11]. It should however be noted that altered distribution of leukocyte subsets, a known phenomenon in autoimmune diseases, can cause variation in whole blood ISG expression [41]. The absence of elevated IFNα2 in serum while ISG expression was positive might be explained by high-affinity binding of low levels of IFNα to the membrane-bound IFNAR inducing transcriptional responses *in vivo* [42]. Alternatively, these patients may have increased concentrations of IFNα subtypes or other type I IFNs not quantified by this Simoa assay. Conversely, apparent discordant measurements of high IFNα2 and a low IFN-I score were found in only a few samples with similar frequency in pSS and HC. As this might be caused by autoantibodies against IFNα that could potentially have neutralizing capacity, we analysed sera for the presence of anti-cytokine antibodies using a bead-based method described previously [43, 44]. No autoantibodies against IFNα2 could be detected in these samples, while these were readily detected in serum from an APS1 patient (Supplementary Figure 10).

In comparison to both ISG expression and direct IFNα2 measurement, the IFN-I reporter assay is far less sensitive in our cohort as was also reported by others [11]. Accounting for

potential modulating factors in serum upstream of the IFNAR affecting cellular responses to IFN-I, reporter assays measure the bioactivity of IFN-I present in samples, rather than the concentration of protein. These assays are however limited by a poor sensitivity to detect low levels of IFN-I in patient samples and are hard to standardize for use in clinical practice.

In the two pSS cohorts, serum IFNα2 concentrations were mainly associated with disease-relevant serological parameters IgG, autoantibodies, and complement components. These results are consistent with previous literature describing associations between IFN-I pathway activation and haematological or serological parameters in pSS and other SADs [10, 15, 45-47]. In line with our findings from a placebo-controlled trial of HCQ in pSS and several cross-sectional cohorts [15, 17, 48, 49], we here found an independent negative association of HCQ treatment with serum IFNα2 protein concentrations in pSS. This indicates a persistent effect of HCQ on IFN-I pathway activation after long-term treatment. In addition, we found higher IFNα2 levels in patients with active cutaneous disease and patients with a history of extraepithelial manifestations. Extraepithelial manifestations, in particular cutaneous vasculitis, and several specific serological parameters are well established risk factors for lymphoma in pSS [50]. A limitation of our study is that we were not able to study IFN-I in salivary gland biopsies and correlate these to peripheral IFN-I pathway activation and clinical manifestations in our patient cohorts. Considering our results and the link between IFN-I pathway activation and biological markers of disease activity in pSS, future studies should evaluate potential additional prognostic value of IFN-I measurement for risk stratification.

The current development and clinical evaluation of treatments directed against IFN-I pathway and related signalling components urges identification of biomarkers for patient stratification. These markers for selection of treatment candidates should accurately reflect the pathway of interest, be useful for prediction of treatment responses and be feasible for application in clinical practice using robust methods that can be standardized across laboratories. A hypothetical marker suitable for monitoring of treatment responses or clinical disease activity should additionally be susceptible to change over time. Direct quantification of serum IFNα by Simoa has a well-defined specificity for the pathway of interest and can be easily standardized considering the automated analytical procedure. The potential clinical relevance of serum IFNα for use in risk stratification, prediction of treatment responses or monitoring in individual patients of distinct SAD entities remains to be elucidated.

In conclusion, the whole blood ISG expression in pSS, SLE, and SSc is closely linked to elevated concentrations of circulating IFNα2. Direct measurement of IFNα protein at low concentrations in biological samples has been a considerable step forward in understanding IFN-I pathway activation in SADs and may be relevant in light of patient stratification and targeted treatment.

ACKNOWLEDGMENTS

The authors would like to thank Jan Rehwinkel for providing the HEK293-3C11 cells Luciferase-reporter cells, Matti Waris for providing the antibodies for the MxA immunoassay, Chris Ramakers for performing C3/C4 and IgG measurements, Angelique van Rijswijk for performing anti-cytokine autoantibody measurements, and the department of Biostatistics, Erasmus MC for advice on statistical analysis.

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SUPPLEMENTARY METHODS

Patient characteristics

Patients with pSS (n=85), SLE (n=24) and SSc (n=23) fulfilled the 2016 ACR-EULAR Classification Criteria for primary Sjögren's Syndrome [1], 2019 ACR-EULAR Classification Criteria for SLE [2], or 2013 ACR/EULAR Classification Criteria for SSc [3], Patients were recruited at the outpatient clinic of the Erasmus MC, Rotterdam University Medical Center, Rotterdam, The Netherlands between 2013 and 2020. Age and gender matched HCs (n=40) from which serum was stored were added to this cohort (Rotterdam cohort). As a validation cohort, serum samples and clinical characteristics were retrieved from an established cohort of pSS patients (n=110) recruited at the Dep. of Rheumatology, Malmö, Lund University, Sweden [4]. Additional healthy controls (n=28) recruited at the Erasmus MC were analysed in parallel to this validation cohort (Malmö cohort). Demographic and clinical characteristics, use of medication and routine hematological and serological parameters of the Rotterdam cohort were retrieved from patient records. Sera from pSS and SLE patients with missing serological data were subjected to routine diagnostic procedures. Disease activity at the time of blood drawing was assessed using the EULAR Sjögren's syndrome disease activity index (ESSDAI) [5] or SLEDAI-2K [6]. Extraglandular involvement in pSS patients (Rotterdam cohort) was defined as current or past systemic manifestations according to the various ESSDAI domains (excluding biological domain) reported in patient records before or at time of blood drawing. Extraglandular manifestations were categorized as non-specific (constitutional; articular), periepithelial (central nervous system; pulmonary; muscular; renal: tubulointerstitial nephritis) or extraepithelial (cutaneous; peripheral nervous system; lymphadenopathy; haematological; renal: glomerulonephritis) [7, 8]. Remission in SLE was defined in accordance with the DORIS classification [9]. For remission, patients were required to have a clinical SLEDAI [10] score of 0 and to be on no other treatments than maintenance antimalarials, maintenance immunosuppressives or stable, low-dose corticosteroids (≤5 mg/day). Considering the small SLE cohort, no distinction was made between clinical or complete remission based on serology and remission off or on therapy.

Blood sampling

Blood samples from the Rotterdam cohort were collected in BD Vacutainer™ SST™ II Advance Tubes for serum isolation, PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Becton Dickinson, Vianen, The Netherlands) for whole blood RNA analysis, and NH Sodium Heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) for MxA immunoassay. Blood processing was performed within two hours and serum was stored at -80 °C.

IFN-I reporter assay

HEK293-3C11 cells were maintained in DMEM + GlutaMAX (Gibco, Thermo Fisher Scientific, Tilburg, The Netherlands), supplemented with 10% heat-inactivated fetal calf serum + 2 mM Ultraglutamine (Lonza, Maastricht, The Netherlands) + P/S (Gibco) and cultured at 37°C with 5% CO2. For the assay, 50.000 cells/well were seeded in 96-well Flat Clear Bottom TC-treated Microplates (Corning, Amsterdam, The Netherlands) that were precoated with 50 µg/mL poly-L-lysine hydrobromide (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 30 µg/mL fibronectin bovine plasma (Sigma-Aldrich). The following day, the medium was replaced by serum from patients or healthy controls diluted 1:1 in medium. After overnight incubation, luciferase expression was quantified using the ONE-Glo™ Luciferase Assay System (Promega, Leiden, The Netherlands) on a GloMax Explorer (Promega) according to manufacturer's instructions.

PBMC stimulation

Cryopreserved peripheral blood mononuclear cells (PBMCs, Ficoll separated from Sodium-Heparin tubes) from a healthy donor were seeded at a density of 4.10e5 cells/well in 96-well round bottom Nunclon Delta plates (Thermo Fisher Scientific, Tilburg, The Netherlands) in RPMI 1640 (Gibco) + 10% heat-inactivated fetal calf serum + P/S (Gibco). Cells were pre-incubated with medium, IgG2a isotype control (Invitrogen, Thermo Fisher Scientific, Tilburg, The Netherlands), or anti-IFNAR clone MMHAR-2 (Merck, Schiphol-Rijk, The Netherlands) for 30 minutes and thereafter stimulated with 50% serum from patients or healthy controls for 5 hours. RNA was isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and reverse transcribed to cDNA using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Bleiswijk, The Netherlands). RT-PCR was performed on a QuantstudioTM 5 Real-Time PCR System using predesigned primer/probe sets (Applied Biosystems). Data were normalized to the expression of the housekeeping gene Abl. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

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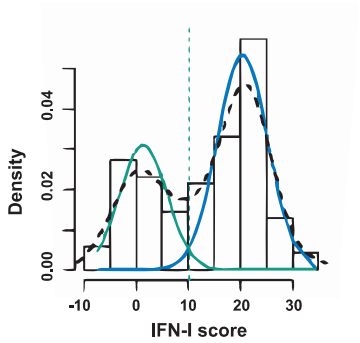
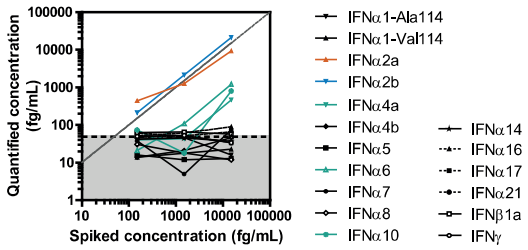
SUPPLEMENTARY DATA



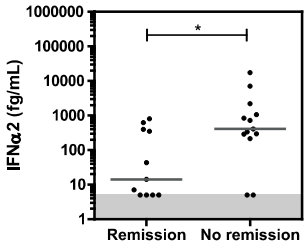
Supplementary Figure 1. Finite mixture model of IFN-I score.

Shown are data distribution (histogram), fitted Gaussian density components (solid lines) and nonparametric density estimate (black dashed line) of IFN-I score data in autoimmune patients of the Rotterdam cohort. Vertical dotted line represents 97.5th percentile of IFN-I scores in healthy controls.

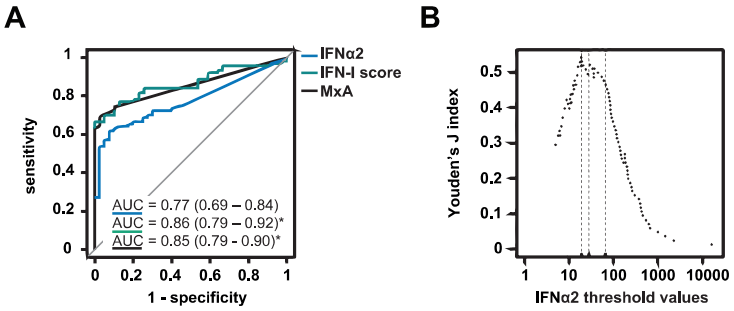


Supplementary Figure 2. Simoa® IFN-α Advantage Kit primarily detects IFNα2.

Quantified IFNα concentrations (y-axis) in serum from a healthy control spiked with low (150 fg/mL), medium (1500 fg/mL) or high (15000 fg/mL) concentrations (x-axis) of recombinant IFNα subtypes, IFNβ or IFNγ (snap frozen) measured using the Simoa IFN-α Advantage Kit. Diagonal line represents the theoretical correlation between spiked and quantified concentrations. Shaded area represents values below the blank measurement of unspiked serum. Each data point represents the average of duplicate measurements.

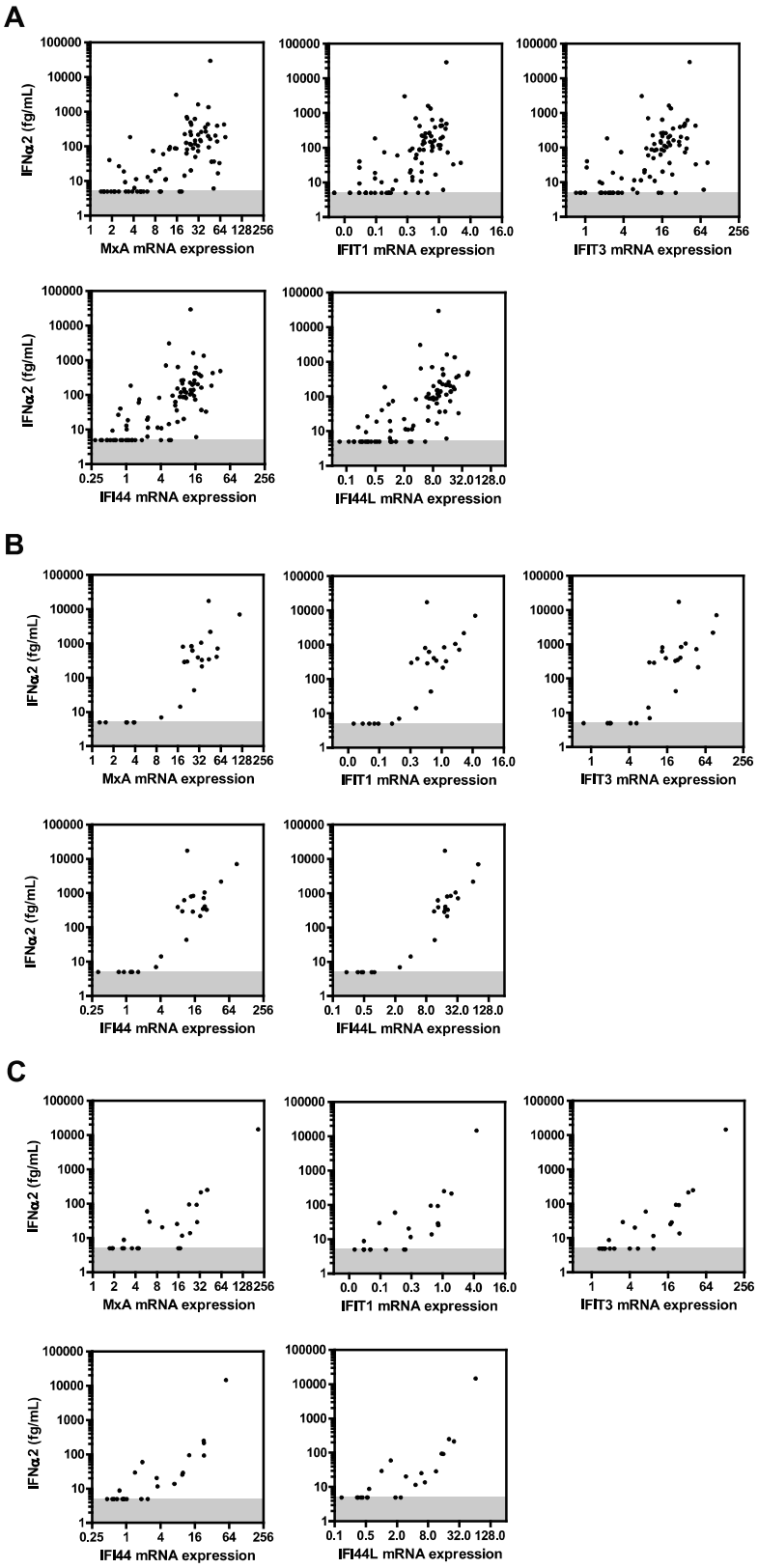


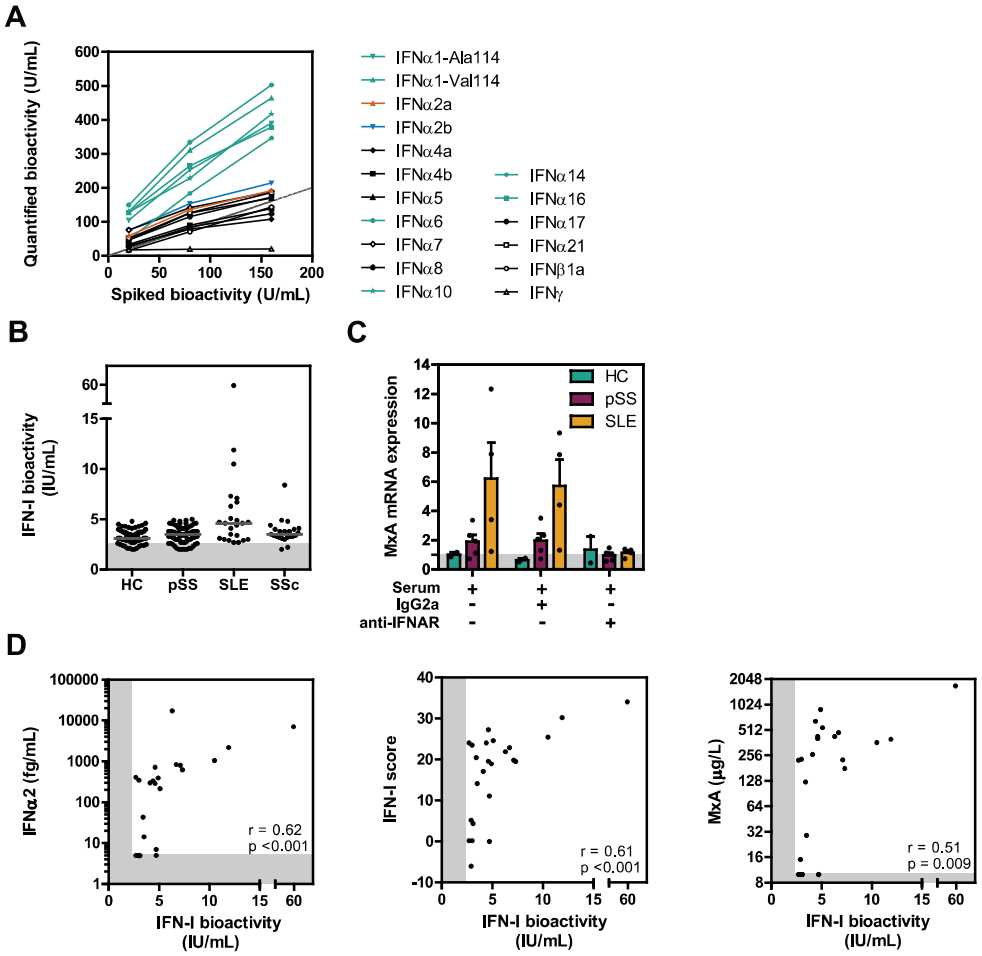
Supplementary Figure 3. Serum IFNα2 concentrations are lower in SLE patients in remission.
Serum IFNα2 concentrations in SLE patients (n=24) in remission or not in remission according to the DORIS classification. Censored Regression analysis was performed to compare log(IFNα2) between groups.



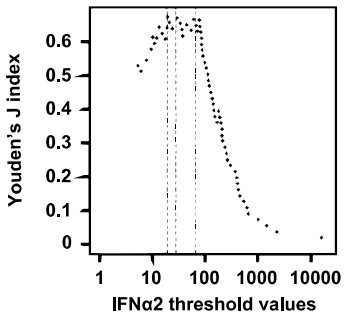
Supplementary Figure 4. Diagnostic accuracy of serum IFNα2, IFN-I score and MxA to discriminate pSS and HC.
(A) ROC curves of serum IFNα2, IFN-I score and MxA for discrimination of pSS (n=85) and HC (n=40). **(B)** Youden's J index across potential threshold values of serum IFNα2. Dotted vertical lines represent threshold at maximum Youden's J index (left; 19 fg/mL), 95th percentile of HCs (middle: 28 fg/mL) or lowest Simoa calibrator (right; 66 fg/mL). AUCs were compared with Bootstrap test for two correlated ROC curves. * $p < 0.05$. Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic.

Supplementary Figure 5 (next page). Serum IFNα2 concentrations are positively correlated to expression of individual IFN-stimulated gene transcripts.
Correlation between serum IFNα2 and whole blood relative gene expression of *MxA*, *IFIT1*, *IFIT3*, *IFI44* and *IFI44L* in **(A)** primary SS, **(B)** SLE, **(C)** and SSc from the Rotterdam cohort.


A
IFNα2 (fg/mL)
MxA mRNA expression
IFIT1 mRNA expression
IFIT3 mRNA expression
IFI44 mRNA expression
IFI44L mRNA expression
B
IFNα2 (fg/mL)
MxA mRNA expression
IFIT1 mRNA expression
IFIT3 mRNA expression
IFI44 mRNA expression
IFI44L mRNA expression
C
IFNα2 (fg/mL)
MxA mRNA expression
IFIT1 mRNA expression
IFIT3 mRNA expression
IFI44 mRNA expression
IFI44L mRNA expression

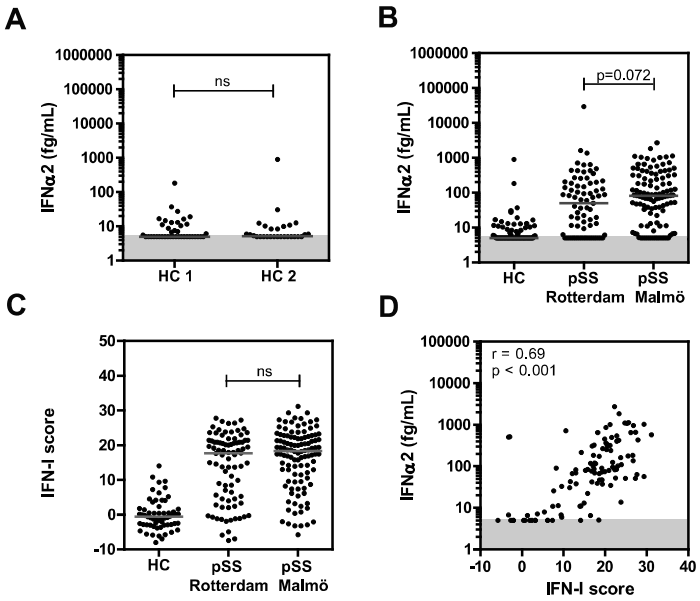


Supplementary Figure 6. IFN-I bioactivity of SLE sera correlated to serum IFNα2 and IFN-I score.
(A) Quantified IFN-I bioactivity (y-axis) in special stripped serum spiked with recombinant IFNα subtypes, IFNβ or IFNγ assessed in HEK-3C11 cells. Activity (units/mL) of recombinant proteins were matched based on cytopathic effect in A549/EMCV inhibition assay provided by datasheets. **(B)** IFN-I bioactivity in serum from HC, pSS, SLE, and SSc measured in HEK-3C11 cells. **(C)** MxA mRNA expression in HC PBMCs stimulated with 50% serum from HC, SLE or pSS ± anti-IFN-I receptor (IFNAR) or isotype control. **(D)** Correlation between serum IFN-I-luciferase bioactivity and serum IFNα2, IFN-I score, or MxA protein levels in SLE. Correlations were assessed by likelihood-based correlation coefficient estimation.



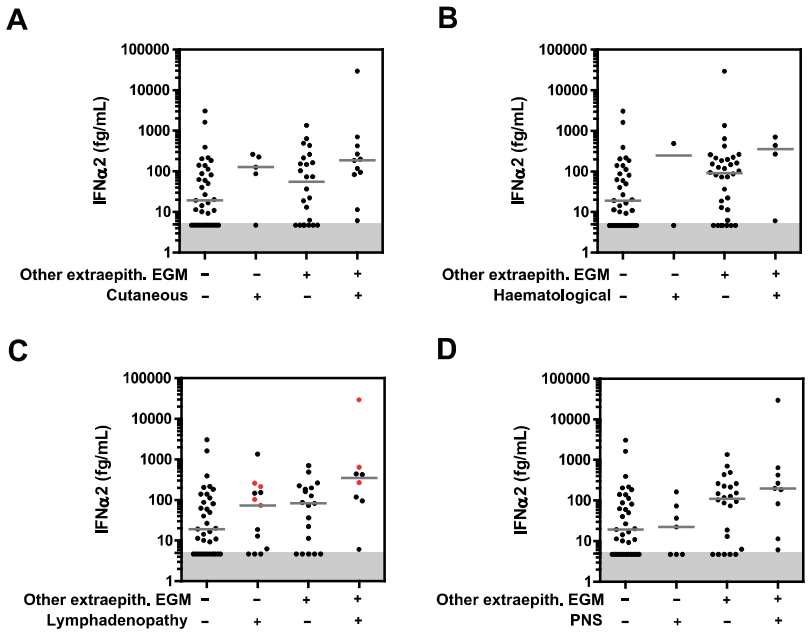
Supplementary Figure 7. Youden's J index across potential threshold values of serum IFNα2 to identify IFN-high primary SS.

Dotted vertical lines represent threshold at maximum Youden's J index of ROC analysis (left; 19 fg/mL), 95th percentile of healthy controls (middle: 28 fg/mL) or lowest Simoa calibrator (right; 66 fg/mL).



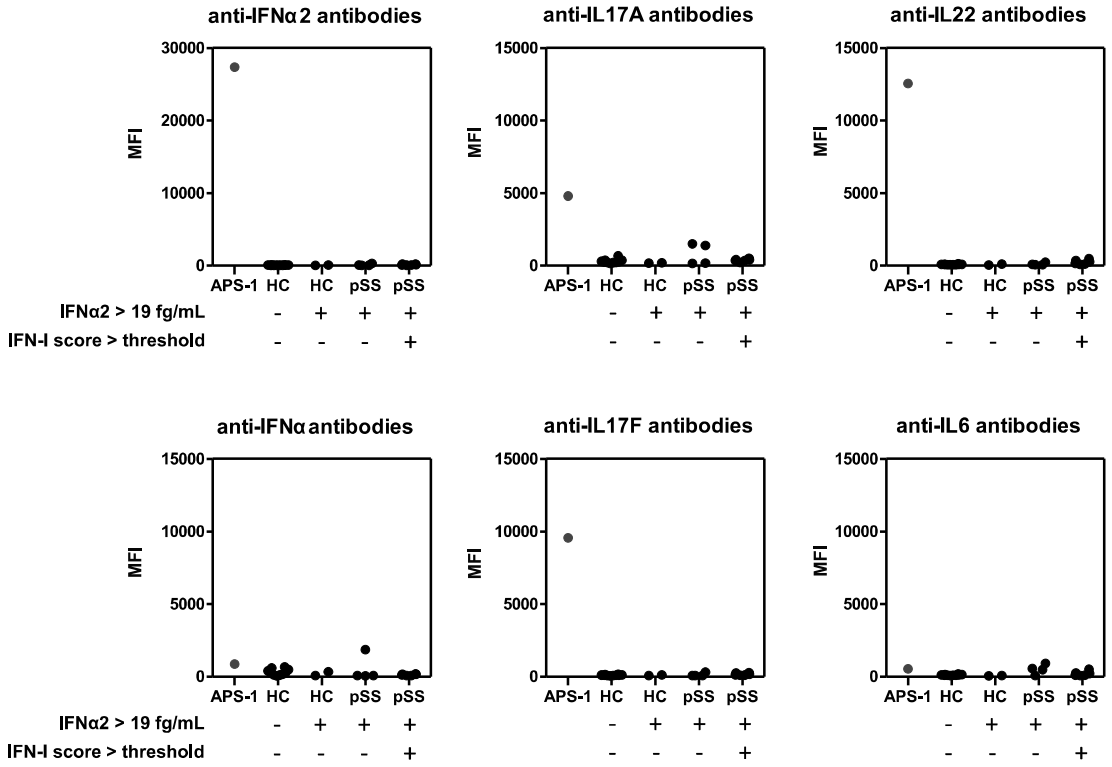
Supplementary Figure 8. pSS patients from Rotterdam and Malmö cohort show comparable systemic IFN-I activity.

(A) Quantified IFNα2 concentrations in serum samples from two independently measured cohorts of healthy controls (HC). **(B)** Serum IFNα2 and **(C)** IFN-I score in pSS patients from Malmö and Rotterdam cohorts. **(D)** Correlation between serum IFNα2 and IFN-I score in Malmö pSS cohort. Horizontal lines represent medians and shaded region indicate values below lower limit of detection. Censored Regression analysis (A, B) and Mann-Whitney U test **(C)** were performed to compare log(IFNα2) **(A, B)** and log(IFN-I score + 10) between groups. Correlation between log(IFNα2) and IFN-I score was assessed by likelihood-based correlation coefficient estimation.



Supplementary Figure 9. Higher serum IFNα2 in pSS with history of extraepithelial manifestations in cutaneous, haematological and lymphadenopathy domains.

Quantified IFNα2 concentrations in serum samples from pSS patients from the Rotterdam cohort stratified based on the occurrence of past or current extraepithelial extraglandular manifestations (EGM). Data for each patient are visualized in 4 graphs; each highlighting one of the extraepithelial EGM domains either alone or in combination with the other 3 extraepithelial EGM domains. **(A)** cutaneous involvement of which 69% cutaneous vasculitis/purpura, **(B)** haematological involvement, **(C)** lymphadenopathy including non-Hodgkin B cell lymphoma (red samples), and **(D)** peripheral nervous system involvement. Horizontal lines represent medians and shaded region indicate values below lower limit of detection.



Supplementary Figure 10. No anti-IFNα2 autoantibodies detected in sera from pSS.

Anti-cytokine autoantibodies against IFNα2, IFNγ, IL-17A, IL-17F, IL-22 and IL-6 were measured in 1:100 serum dilutions using cytokine-coupled beads. Coupling of recombinant human IFNα2 (PBL Assay Science), IL-17A, IL-17F, IL-22, IFN-γ, and IL-6 (all from R&D Systems) to their respective beads was verified with mouse monoclonal antibodies against IFNα (PBL Assay Science), IL-17A, IL-17F, IL-22, IFNγ, and IL-6 (all from R&D Systems). Beads were incubated with patient or healthy control serum. Anti-cytokine antibodies bound to the beads were subsequently stained with goat anti-human IgG-Fc-PE antibody (Bioconnect). Mean fluorescence intensities (MFI) were analyzed using a Bio-Plex Magpix 50 system (Luminex) and Manager 3.0 software (Bio-Rad Laboratories). Sera from 4 pSS patients and 2 HC with IFNα2 >19 fg/mL + low IFN-I score, 6 pSS patients with IFNα2 >19 fg/mL + high IFN-I score, and 10 HC with IFNα2 <19 fg/mL + low IFN-I score were tested. In parallel, serum from an autoimmune polyglandular syndrome type I (APS-1) patient was included as a positive control.

Supplementary Table 1. Active ESSDAI domains.

	Rotterdam	Malmö	P value
	pSS n = 85	pSS n = 110	
Active ESSDAI domains[a]			
Constitutional	6/76 (7.9%)	19/110 (17.3%)	0.065
Lymphadenopathy	2/76 (2.6%)	4/110 (3.6%)	1.000
Glandular	10/76 (13.2%)	11/110 (10%)	0.504
Articular	17/76 (22.4%)	14/110 (12.7%)	0.083
Cutaneous	3/76 (3.9%)	6/110 (5.5%)	0.740
Pulmonary	8/76 (10.5%)	12/110 (10.9%)	0.934
Renal	4/76 (5.3%)	8/110 (7.3%)	0.764
Muscular	0/76 (0%)	1/110 (0.9%)	1.000
PNS	8/76 (10.5%)	6/110 (5.5%)	0.094
CNS	0/76 (0%)	2/110 (1.8%)	0.514
Haematological	6/76 (7.9%)	22/110 (20%)	**0.023**
Biological	28/76 (36.8%)	56/110 (50.9%)	0.058

Data are presented as number of patients (%)[a]. Pearson's Chi-square Test or Fisher's exact test were performed to compare frequencies between cohorts. Abbreviations: CNS, central nervous system; ESSDAI, EULAR Sjögren's syndrome disease activity index; PNS, peripheral nervous system.

Supplementary Table 2. Diagnostic accuracy of serum IFNα2 to discriminate pSS and HC.

Cohort	Rotterdam			Validation in Malmö cohort		
Threshold	19 fg/mL	28 fg/mL	66 fg/mL	19 fg/mL	28 fg/mL	66 fg/mL
Accuracy	0.71 (0.63-0.79)	0.69 (0.62-0.76)	0.64 (0.57-0.72)	0.78	0.77	0.66
Sensitivity	0.62 (0.51-0.71)	0.57 (0.46-0.67)	0.49 (0.38-0.59)	0.75	0.73	0.58
Specificity	0.90 (0.80-0.98)	0.95 (0.88-1)	0.98 (0.93-1)	0.93	0.93	0.96
NPV	0.52 (0.45-0.6)	0.51 (0.45-0.57)	0.47 (0.42-0.53)	0.48	0.46	0.37
PPV	0.93 (0.87-0.98)	0.96 (0.91-1)	0.98 (0.93-1)	0.98	0.98	0.99

Data represent measures of accuracy (95% confidence interval) calculated from receiver operating characteristic (ROC) analysis of the Rotterdam cohort (pSS: n=85, HC: n=40) and blinded validation of the presented threshold values in the Malmö cohort (pSS: n=110) extended with 28 additional HC. Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

Supplementary Table 3. Clinical characteristics of IFN-high pSS with serum IFNα2 below 19 fg/mL.

Cohort	Rotterdam			Malmö			Pooled
Variable	Serum IFNα2 < 19 IFN-I score +	Serum IFNα2 > 19 IFN-I score +	P	Serum IFNα2 < 19 IFN-I score +	Serum IFNα2 > 19 IFN-I score +	P	P
Age[a]	71.1 (57.3-75.3)	61 (53-66)	0.138	66 (56.5-71.5)	62 (47-72)	0.808	0.213
ESSDAI[a]	2 (0-2.5)	3 (1-11)	0.330	9 (1-13)	3 (1-7)	0.357	0.812
MxA[a]	12 (10.5-51.5)	177.5 (124.8-282)	**<0.001**	-	-	-	-
IFN score[a]	14.7 (13.75-18.84)	20.88 (18.55-23.6)	**0.019**	14.94(14.2-17.48)	20.53 (17.47-3.3)	**0.019**	**<0.001**
IgG[a]	10.2 (9.5-11.2)	15.85 (13.1-20.1)	**<0.001**	15.1 (12.4-16.6)	15.4 (12.4-17.7)	0.739	**0.003**
C3[a]	1.18 (1.01-1.26)	1.13 (1.02-1.23)	0.869	0.95 (0.85-1.06)	0.89 (0.8-1.05)	0.481	0.346
C4[a]	0.19 (0.15-0.22)	0.18 (0.14-0.22)	0.761	0.2 (0.13-0.21)	0.17 (0.13-0.22)	0.760	0.744
ANA[b]	6/8	46/47	0.052	4/7	70/77	**0.034**	**0.006**
SSA[b]	7/8	47/47	1.000	6/7	75/77	0.232	0.058
SSB[b]	2/8	38/47	**0.003**	3/7	54/77	0.204	**0.002**
HCQ[b]	6/8	18/47	0.067	2/7	21/77	1.000	ns
DMARDs[b]	0/8	1/47	1.000	0/7	5/77	1.000	ns
CS[b]	1/8	7/47	1.000	2/7	20/77	1.000	ns

Data are presented as median (Q1-Q3)[a] or number of patients[b]. Mann Whitney U test was used to compare continuous data and Fisher's exact test was performed to compare frequencies between groups. Abbreviations: ESSDAI, EULAR Sjögren's syndrome disease activity index.

Supplementary Table 4. Clinical characteristics associated with serum IFNα2.

		Bivariate analysis		Multivariable analysis Model 2			Multivariable analysis Model 3		
Variables		**Serum IFNα2**	**P value**	**Estimate**	**Standard error**	**P value**	**Estimate**	**Standard error**	**P value**
(Intercept)				1.634	0.910	0.073	2.136	0.884	0.016
ESSDAI # domains		0.134[a]	0.161	-0.057	0.118	0.628	-	-	-
Constitutional	*No*	79.2 (10.9-213.6)[b]	0.608	-	-	-	0.370	0.358	0.301
	Yes	69.2 (11.2-424.7)[b]							
Lymphadenopathy	*No*	81.8 (11.2-242.9)[b]	**0.037**	-	-	-	-1.149	0.744	0.123
	Yes	7 (5.1-41.3)[b]							
Glandular	*No*	79.5 (10.1-217.4)[b]	0.299	-	-	-	0.364	0.396	0.358
	Yes	68.8 (16.7-223.9)[b]							
Haematological	*No*	73.1 (9.5-195)[b]	**0.046**	-	-	-	0.357	0.359	0.321
	Yes	117 (51-490.7)[b]							
Articular	*No*	84.7 (12.2-264.3)[b]	**0.006**	-	-	-	-1.190	0.345	**<0.001**
	Yes	20.3 (≤5-94.3)[b]							
Cutaneous	*No*	73.9 (10.1-217.2)[b]	**0.024**	-	-	-	1.345	0.549	**0.014**
	Yes	198 (119-4-405.5)[b]							
Pulmonary	*No*	78.3 (10.9-222.3)[b]	0.990	-	-	-	0.103	0.402	0.798
	Yes	72.4 (10.1-211.3)[b]							
Renal	*No*	74 (9.5-211.5)[b]	0.094	-	-	-	0.104	0.500	0.836
	Yes	189 (51.1-440.9)[b]							
Muscular	*No*	79.2 (10.9-223.9)[b]	0.978	-	-	-	-1.555	1.639	0.343
	Yes	47.2[b]							
PNS	*No*	75.8 (10.9-222.3)[b]	0.646	-	-	-	-0.114	0.437	0.794
	Yes	104.3 (10.2-261.3)[b]							
CNS	*No*	79.4 (10.7-227.9)[b]	0.920	-	-	-	-1.370	1.157	0.236
	Yes	58.9 (54.9-62.9)[b]							
Age		-0.135[a]	0.061	-0.015	0.009	0.096	-0.019	0.009	**0.036**
Gender	*Female*	73.9 (9.7-245.7)[b]	0.508	0.296	0.465	0.524	0.116	0.455	0.799
	Male	91.5 (28.9-188.9)[b]							
IgG		0.460[a]	**<0.001**	0.095	0.028	**<0.001**	0.064	0.026	**0.015**
C3 (centralized)		-0.150[a]	**0.040**	-0.067	0.656	0.920	0.199	0.633	0.753
C4 (absolute)		-0.212[a]	**0.003**	-0.752	2.430	0.757	-1.221	2.318	0.598
HCQ	*No*	90.2 (20.8-314.5)[b]	**0.002**	-1.067	0.271	**<0.001**	-1.047	0.258	**<0.001**
	Yes	38.8 (5.1-118.8)[b]							
CS	*No*	79.4 (11-214.5)[b]	0.456	0.328	0.347	0.345	0.388	0.344	0.260
	Yes	66.3 (24.8-423.9)[b]							
DMARDs	*No*	77.5 (11-217.2)[b]	0.672	0.423	0.637	0.506	0.271	0.611	0.658
	Yes	92.5 (30.3-424.3)[b]							
ANA	*Neg*	6.6 (≤5-24.8)[b]	**<0.001**	1.043	0.407	**0.010**	1.065	0.391	**0.006**
	Pos	94 (33.1-308.9)[b]							
SSA	*Neg*	≤5 (≤5-11)[b]	**<0.001**	1.414	0.451	**0.002**	1.710	0.439	**<0.001**
	Pos	87.8 (28.1-266.2)[b]							
SSB	*Neg*	12.2 (≤5-115.8)[b]	**<0.001**	0.759	0.296	**0.010**	0.677	0.286	**0.018**
	Pos	101.9 (51.1-378.3)[b]							
Storage years		-0.121[a]	0.099	-0.140	0.108	0.195	-0.153	0.103	0.136

Bivariate analysis: Data represent correlation coefficients[a] or medians (Q1-Q3)[b] of IFNα2 concentrations (fg/mL) in pSS patients from the pooled Rotterdam and Malmö cohort. The number of patients for each categorical variable are indicated in Table 1 and Supplementary Table 1. Correlations between log(IFNα2) and indicated variables

(continued) were assessed by likelihood-based correlation coefficient estimation. Multivariable analysis: independent associations of indicated variables with log(IFNα2) in the pooled cohort were evaluated in a censored regression (tobit) model. Abbreviations: CNS, central nervous system; ESSDAI, EULAR Sjögren's syndrome disease activity index; ns, not significant; PNS, peripheral nervous system.

Supplementary Table 5. Association between extraglandular manifestations and serum IFNα2.

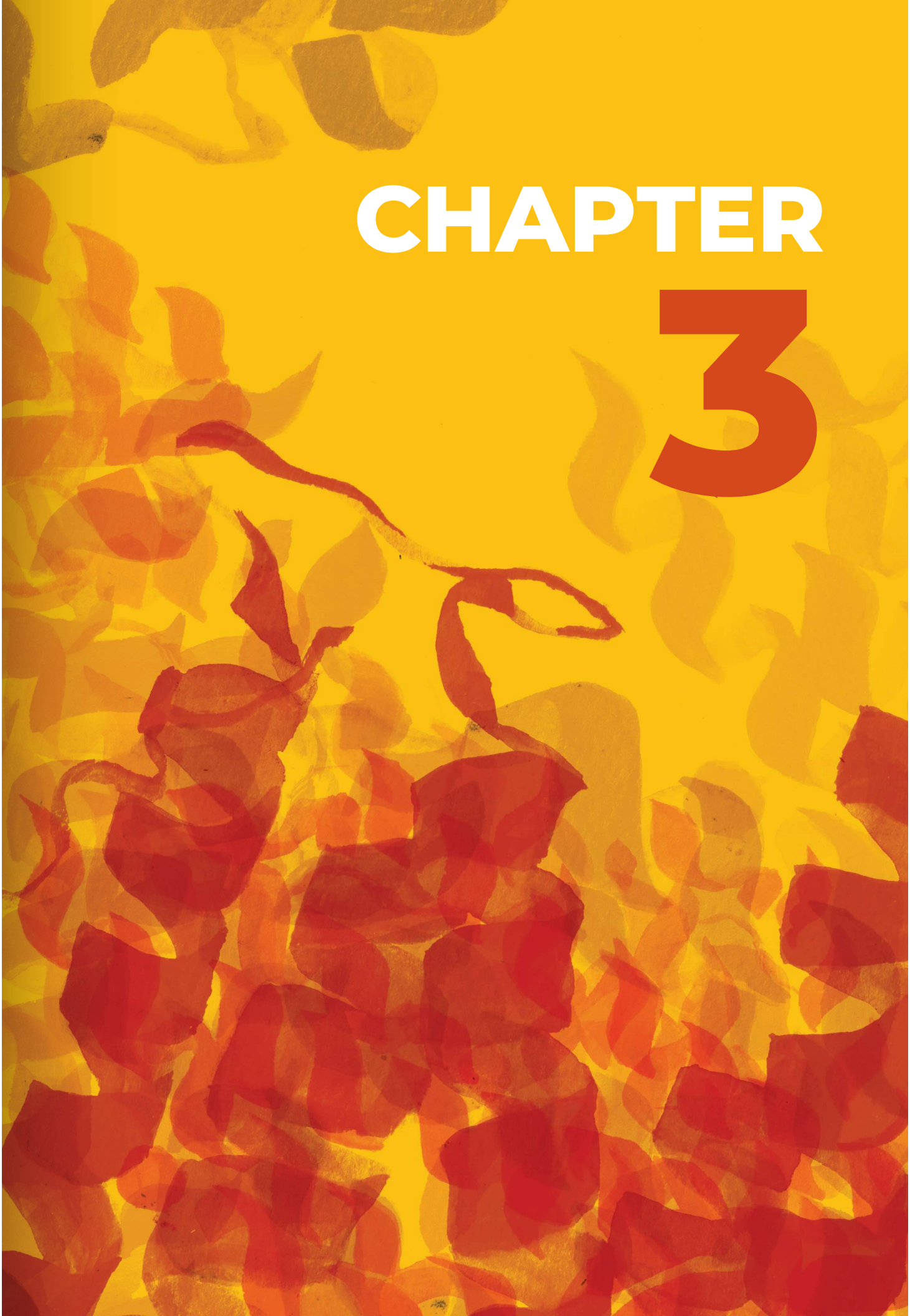
	Bivariate analysis	
Variables	**Serum IFNα2**	**P value**
EGM		0.133
No (n=17)	14.3 (≤5-40.5)[b]	
Yes (n=58)	85.1 (6.2-209.7)[b]	
EGM # domains	0.127[a]	0.281
Periepithelial EGM		0.380
No (n=49)	26.8 (≤5-153.3)[b]	
Yes (n=26)	95.8 (14.4-195)[b]	
Periepithelial EGM # domains	0.065[a]	0.581
Extraepithelial EGM		0.061
No (n=35)	19.3 (≤5-100.1)[b]	
Yes (n=40)	99.5 (10.1-232.6)[b]	
Extraepithelial EGM # domains	0.291[a]	**0.010**
Non-specific EGM		0.736
No (n=34)	70.7 (10.4-151.9)[b]	
Yes (n=41)	49.9 (≤5-213.6)[b]	
Non-specific EGM # domains	-0.097[a]	0.414

Bivariate analysis: Data represent correlation coefficients[a] or medians (Q1-Q3)[b] of IFNα2 concentrations (fg/mL) in pSS patients from the Rotterdam cohort. Correlations between log(IFNα2) and indicated variables were assessed by likelihood-based correlation coefficient estimation. Associations of indicated variables with log(IFNα2) were evaluated in a censored regression (Tobit) model. Abbreviations: EGM, extraglandular manifestations.

Supplementary Table 6. Association between extraglandular manifestations and serum IFNα2.

	Multivariable analysis 1			Multivariable analysis 2			Multivariable analysis 3		
Variables	**Estimate**	**SE**	**P value**	**Estimate**	**SE**	**P value**	**Estimate**	**SE**	**P value**
(Intercept)	3.230	0.430	<0.001	3.615	0.510	<0.001	-0.011	1.857	0.995
Periepithelial EGM # domains	-0.150	0.461	0.745	0.012	0.469	0.980	-0.171	0.382	0.654
Extraepithelial EGM # domains	0.906	0.316	**0.004**	0.903	0.337	**0.007**	0.524	0.264	**0.047**
Non-specific EGM # domains	-0.594	0.394	0.132	-0.574	0.389	0.141	-0.483	0.312	0.121
HCQ				-0.717	0.562	0.203	-0.523	0.473	0.269
DMARDs				-0.766	1.525	0.616	1.650	1.422	0.246
Corticosteroids				-0.421	0.899	0.639	0.005	0.715	0.995
IgG							0.131	0.046	**0.005**
ANA							0.326	0.848	0.700
SSA							1.291	0.850	0.129
SSB							1.382	0.570	**0.015**
Age							-0.017	0.019	0.365
Gender							0.687	0.765	0.369

Multivariable analysis: independent associations of indicated variables with log(IFNα2) in the pooled cohort were evaluated in a censored regression (tobit) model. Abbreviations: EGM, extraglandular manifestations; SE, standard error.


CHAPTER
3

CHAPTER 3.1

Making sense of intracellular nucleic acid sensing in type I interferon activation in Sjögren's syndrome

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Journal of Clinical Medicine 2021, 10(3), 532

ABSTRACT

Primary Sjögren's syndrome (pSS) is a systemic autoimmune rheumatic disease characterized by dryness of the eyes and mucous membranes, which can be accompanied by various extraglandular autoimmune manifestations. The majority of patients exhibit persistent systemic activation of the type I interferon (IFN) system, a feature that is shared with other systemic autoimmune diseases. Type I IFNs are integral to anti-viral immunity and are produced in response to stimulation of pattern recognition receptors, among which nucleic acid (NA) receptors. Dysregulated detection of endogenous NAs has been widely implicated in the pathogenesis of systemic autoimmune diseases. Stimulation of endosomal Toll-like receptors by NA-containing immune complexes are considered to contribute to the systemic type I IFN activation. Accumulating evidence suggest additional roles for cytosolic NA-sensing pathways in the pathogenesis of systemic autoimmune rheumatic diseases. In this review, we will provide an overview of the functions and signaling of intracellular RNA- and DNA-sensing receptors and summarize the evidence for a potential role of these receptors in the pathogenesis of pSS and the sustained systemic type I IFN activation.

INTRODUCTION

The immunogenicity of nucleic acids (NAs) and their shaping of the immune response has been recognized for many decades [1, 2]. The discovery of pattern recognition receptors (PRRs) has provided a molecular mechanism for these observations [3]. Although at first NA-sensing receptors were thought to mainly sense pathogen-derived NAs, currently they are widely recognized to be able to sense self-NAs as well [4-6]. Upon activation, NA-sensing receptors induce the production of pro-inflammatory cytokines and type I interferons (IFNs) [4, 7, 8].

Type I IFNs are highly potent cytokines with direct anti-viral effects and a wide range of immunomodulatory functions [9, 10]. Inappropriate amplitude and timing of type I IFN responses has detrimental effects on defense against pathogens and host tissue integrity [11]. Persistent systemic type I IFN activation occurs in primary Sjögren's syndrome (pSS) and other systemic autoimmune diseases and is considered to contribute to the ongoing loop of inflammation [12].

Lymphocytic infiltrates in exocrine glands and symptoms of dryness of eyes and mouth are typical features of pSS [13]. The majority of patients with pSS and related systemic autoimmune diseases have anti-nuclear antibodies, reflecting the exposure of nuclear components to the immune system. Internalization of immune complex-bound NAs and activation of endosomal PRRs is thought to represent a major type I IFN-stimulating mechanism in these patients [14-16]. Recent identification of additional IFN-inducing NAs and detection of activated downstream sensing pathways suggested their potential contribution to IFN activation in systemic autoimmune diseases [17-23].

Here we will outline the signaling and regulation of intracellular RNA- and DNA-sensing pathways and discuss the current knowledge on the role of these pathways in type I IFN activation in pSS.

TYPE I IFN SIGNALING IN PRIMARY SJÖGREN'S SYNDROME

IFN cytokine family and signaling

The IFN cytokine family comprises type I, type II and type III IFNs, classified based on the receptor complexes that they interact with. Type I IFNs in humans include multiple IFNα subtypes, IFNβ, IFNε, IFNκ and IFNω, which all signal through the heterodimeric IFNα receptor (IFNAR) complex [24]. The IFNAR, composed of subunits IFNAR1 and IFNAR2, and its canonical downstream mediators of the JAK and STAT families are widely expressed throughout the body [25]. IFNγ, the only member of type II IFN, is mainly produced by activated immune cells in response to cytokines or antigen-specific stimulation, and

signals through the IFNγ receptor (IFNGR) complex [26]. IFNλ1 (IL29), IFNλ2 (IL28A), IFNλ3 (IL28B) and IFNλ4 are type III IFNs which are primarily produced at mucosal sites [27, 28]. Functional IFNλ signaling seems to be confined to epithelial cells and immune cells that express both subunits of the IFNλ receptor (IFNLR) complex [29]. Despite signaling through distinct receptor complexes, downstream intracellular signaling pathways and transcriptional responses largely overlap between the types of IFN [25, 26, 30]. Cellular response to IFN receptor activation is dependent on cell type, context and timing of the immune response [25].

IFN induction and immunomodulatory functions

IFNs are named for their capacity to induce an antiviral state in responder cells, thereby interfering with viral replication [9]. In addition, IFNs have a wide range of immunomodulatory functions in orchestrating both innate and adaptive immunity and affecting cellular differentiation, proliferation and survival [10]. Under homeostatic conditions, type I IFNs are expressed at very low levels, but their expression can be rapidly induced upon signaling of PRRs [31]. Key pathways involved in induction of type I or type III IFNs are PRRs recognizing NAs. These include endosomal Toll-like receptors (TLRs), cytosolic RIG-I like receptors (RLRs) and DNA-sensing receptors (DSRs) [31-33]. Plasmacytoid dendritic cells (pDCs), the professional type I IFN-producing cells, are especially equipped to rapidly produce massive amounts of IFNα in response to TLR7 or TLR9 activation [34]. However, virtually every cell type can produce small amounts of type I IFNs and depending on the type and timing of stimulation, different cell types can be the primary source of type I IFNs [35-37]. Expression of IFNs is tightly regulated to ensure properly timed robust anti-viral responses, while avoiding excessive tissue damage [31]. Persistent IFN activation, as occurs in systemic autoimmune diseases, drives a detrimental combination of chronic inflammation and immunosuppression contributing to tissue damage and disease progression [11].

Persistent IFN activation in pSS

Multiple lines of evidence strongly suggest a pathogenic role for IFNs in systemic autoimmune diseases [38]. Anifrolumab, a blocking antibody against the IFNAR, has recently yielded promising results in patients with active systemic lupus erythematosus (SLE) [39-42]. The so-called type I IFN signature has first been described in SLE when transcriptomic analysis revealed remarkable upregulation of IFN-stimulated gene (ISG) transcripts [43, 44]. Expression of ISGs is widely used to assess the activation state of the IFN system. Direct measurement of IFN proteins in patient material is complicated by their diversity and the low circulating levels, although recent development of the single-molecule array (SiMoA) technology has advanced this field [45, 46].

Transcriptomic analyses on pSS salivary glands have revealed upregulated ISG transcripts in whole salivary gland biopsies [47-50] and epithelium-enriched fractions [51, 52]. Protein expression of type I [14, 53, 54], type II [55, 56] and type III IFNs [57, 58] has been observed in salivary glands. Immunostainings indicated pDCs as IFNα-producers, B- and T-lymphocytes as primary type II-producers and epithelial cells as type I and type III IFN-producing cells in pSS salivary glands [14, 53-55, 57-59]. IFN activity in salivary glands has been associated with higher focus score, secretory dysfunction and higher prevalence of antinuclear antibodies and hyperglobulinemia [55, 60].

Upregulated ISG expression has also consistently been found in peripheral blood and individual leukocyte subsets from pSS patients [61-66]. Serum IFNα levels in pSS are usually below detection limit of conventional enzyme-immunoassays [14]. Application of the SiMoA technology indicated elevated IFNα protein levels in serum compared to controls [67](and unpublished results). Systemic IFN activity has been primarily linked to serological markers of B cell hyperactivity, complement consumption and hematological aberrations [55, 62, 63, 67, 68]. Autoantibodies against IFNα and IFNω were recently detected in pSS patients [69]. Interestingly, a case with high titer of partially neutralizing anti-IFNα/ω antibodies experienced milder sicca symptoms and minimal focal infiltrates in salivary glands supporting the pathogenic role of IFN in pSS.

The cellular source of type I IFNs in the circulation remains elusive. Similar to SLE, IFN-producing cells have not been found in the circulation of pSS patients so far, suggesting their migration into tissues [70]. In addition to type I IFNs, also IFNγ appears to contribute to the observed IFN signature in glandular tissue [55] as well as in peripheral blood of a subgroup of patients [68]. In salivary glands, a predominant type II IFN signature is associated with a higher focus score and is commonly found in patients with lymphoma or at high risk for lymphoma [55, 60]. The subgroup of patients with additional systemic type II on top of type I IFN involvement have more pronounced serological and hematological manifestations [68]. The interconnection between IFN activation in target tissues and peripheral blood is currently unclear. Research in SLE illustrates that IFN activation does not necessarily co-occur in various tissues and peripheral blood [71, 72].

ENDOSOMAL TLR SIGNALING AND INVOLVEMENT IN PRIMARY SJÖGREN'S SYNDROME

Endosomal TLR signaling

TLRs are transmembrane proteins located on the cell surface or endosomal membranes. In humans, ten types of TLRs have been described, together recognizing a variety of pathogen- and danger- associated molecular patterns [73]. TLR3, TLR7, TLR8 and TLR9 are located on endosomal membranes and respond to various types of NAs delivered to the endosomes through receptor-mediated endocytosis or autophagic delivery [74]. Activating ligands and signaling pathways of endosomal TLRs leading to production of type I IFNs and/or pro-inflammatory cytokines have been summarized by others [7, 75, 76].

Surface receptors such as Fc receptors, complement receptors, B cell receptors, and receptor for advanced glycation end products (RAGE) facilitate efficient endocytosis of TLR ligands [74]. Distinct classes of TLR9-activating CpG oligodeoxynucleotides induce contrasting cytokine responses in pDCs. Class A CpG primary localizes to early endosomes and elicits a type I IFN response through IRF7 activation, while class B CpG preferentially locates to late endosomes resulting in NFκB-mediated production of pro-inflammatory cytokines [77]. This illustrates that differential cytokine responses to TLR activation are at least partially dependent on localization of ligands to distinct endosomal signaling compartments [74].

Endosomal TLRs in pSS

Observations from both mouse and human studies have linked aberrant expression and activation of TLRs to pSS, which has recently been reviewed [78]. Important early work in this field showed the presence of autoantibodies in the circulation of patients with pSS and SLE that have the capacity to form interferogenic RNA-containing immune complexes [14, 15]. When mixed with apoptotic or necrotic material, these in vitro formed pSS-derived immune complexes stimulated FcγRIIa (CD32)-dependent and presumably TLR7-mediated IFNα production by pDCs, which could be abrogated by RNase treatment [14, 15]. In this study, the interferogenic capacity of serum was associated with the presence of focal infiltrates in salivary glands and several extraglandular manifestations [14]. The specific RNAs causing TLR activation in these experimental conditions have not been elucidated. Later however, anti-Ro60 autoantibodies isolated from SLE sera were found to bind to Alu RNA motifs, Y-RNAs and poly G RNAs [79]. These self-RNAs have the potential to activate NA receptors and elicit cell type specific cytokine responses that are heavily influenced by the (macro)molecular structure and experimental context of RNA delivery [15, 79-83]. Similarly, apoptotic material that contains pSS-associated autoantigens and hY RNA elicits

TLR-dependent cytokine release in the presence of autoantibodies [83, 84]. Additionally, high-mobility group box 1 (HMGB1)-bound DNA, nucleosomes or immune complexes may activate TLR9 through RAGE-mediated endocytosis [85]. Interestingly, HMGB1 is elevated in serum and released in salivary glands from pSS patients [86]. Given the abundance of cell free DNA in pSS [87-89], this pathway could be relevant even in the absence of anti-dsDNA or anti-nucleosome antibodies. Research on type I IFN activation in pSS has been largely focused on TLRs while the other NA receptors have received less attention.

Monogenic interferonopathies

Rare monogenic IFN-driven autoinflammatory and autoimmune syndromes provide valuable insights in molecular pathways implicated in type I IFN activation and pathogenic consequences of chronically elevated IFN levels. The majority of mutations causing these inheritable interferonopathies are located in genes related to NA metabolism or NA-sensing receptor signaling [90]. At present, mutations in seven genes have been described to cause Aicardi-Goutières syndrome (AGS), a prototypic autoimmune syndrome [91]. Each of these genes is involved in metabolism of endogenous NAs released during processes such as DNA replication, transcription and translation. Mutations in the DSR-adaptor protein TMEM173/STING induce a prominent type I IFN signature in STING-Associated Vasculopathy with onset in Infancy (SAVI) [91]. In contrast to AGS patients, which often present with a range of autoantibodies at a young age, chronic exposure to IFNs in SAVI may lead to secondary autoimmune features later in life [92]. Therefore, monogenic interferonopathies yield important clues for the interconnection between aberrant IFN activation and autoimmune features.

CYTOSOLIC IFN-INDUCING RNA-SENSING PATHWAYS IN PRIMARY SJÖGREN'S SYNDROME

RIG-I like receptor signaling and other IFN-inducing RNA-sensors

The RLRs, an important family of RNA-sensing receptors, play an important role in the defense against viral and intracellular bacterial infections and have been shown to contribute to IFN activation in monogenic interferonopathies and SLE-like syndromes [93]. The RLRs are primarily located in the cytoplasm and expressed by most cell types [8]. The members of this family, RIG-I, MDA5 and LGP2 recognize RNA by coaction of their central helicase domain and carboxy-terminal domain [8]. RIG-I and MDA5 can confer downstream signaling leading through the CARD domain, whereas LGP2 which lacks the CARD domain is thought to regulate RLR activity. RIG-I mainly recognizes RNAs with a 5'end triphosphate

moiety, a biochemical feature that is absent in the majority of cytosolic self-RNAs. RIG-I can also be activated by long dsRNA through a lower affinity binding that is independent of 5′ triphosphate [94]. MDA5 preferentially binds long dsRNAs independent of 5′ triphosphate [95]. Stable ligand binding to RIG-I or MDA5 triggers oligomerization at the CARD domains resulting in formation of helical filaments [96, 97]. Recruitment of downstream adaptor protein MAVS and subsequent activation of TBK1, IKKε and transcription factors IRF3, IRF7 and NFκB lead to the production of type I IFNs and other cytokines [8].

Multiple other less well characterised RNA-binding proteins and RNA helicases have been described and some of these are proposed to affect IFN signaling [93]. Here we will highlight some elements of protein kinase R (PKR) and RNase L signaling. PKR, encoded by the EIF2AK2 gene can be activated by double-stranded RNA and several other stressors [98, 99]. PKR is one of the downstream mediators of IFN signaling, but is also involved in the induction of type I IFN expression both as amplifier of IFNβ production and as an essential downstream mediator in MDA5-mediated IFNβ production [100, 101]. Catalytic activity of PKR itself may even directly stimulate MAVS-dependent type I IFN production [101]. Upon activation by cytosolic dsRNA, oligoadenylate synthetases (OAS) catalyzes the synthesis of 2′-5′-oligoadenylates, which activates the endoribonuclease RNaseL [102]. In addition to its viral restriction capacity, RNaseL also potentiates immune activation by generating small RNA fragments from viral [103, 104] and endogenous RNA [105] that are able to stimulate RLRs or MAVS-dependent NLRP3 inflammasome formation.

Regulation of RNA-sensing pathway signaling

Erroreous activation of RLRs by cellular RNAs is prevented by capping, shielding and compartmentalization [4, 8]. Signaling of RLRs is regulated by posttranslational modifications, protein-protein interactions, non-coding RNAs and autophagy [4, 8]. Sensing of endogenous RNAs has in some instances been shown to potentiate immune responses in the context of infections [105-107]. On the other hand, some forms of self-RNA sensing are essential for the prevention of aberrant immune responses. Abundant prevalence of dsRNA-containing circular RNAs prevents PKR activation and subsequent IFN production in homeostatic conditions, but is disturbed in SLE [23]. Dysregulation of RLR signaling or RNA metabolism can lead to activation of RLRs in sterile conditions, as occurs in monogenic interferonopathies and cancer [107]. Anti-tumor effects of some of the classic cancer treatments rely on treatment-induced perturbations in RNA metabolism and induction of type I IFNs [107-110]. Knowledge on key regulators of RNA metabolism and epigenetic repression of endogenous retroelements acquired by cancer research, may be highly relevant for the field of autoimmunity.

RNA-sensing receptors in pSS

Salivary gland epithelium provides an important barrier function as first line defense against infectious agents. In normal salivary gland epithelial cells, RIG-I, MDA5 and PKR proteins are expressed at low levels [64, 111, 112], but their expression can be rapidly upregulated after stimulation [113]. Downstream adaptor molecule MAVS is highly expressed at protein level in both ductal and acinar epithelium of normal salivary gland [112]. The RLR-MAVS and PKR pathways have been proven functionally active in primary human salivary gland epithelial cells [111, 113]. Transcriptomic analyses of pSS-derived salivary gland have repeatedly found upregulation of multiple ISGs involved in cytosolic RNA-sensing pathways or type I IFN signaling [47-50, 114-116]. These findings were most pronounced in salivary glands with extensive mononuclear cell infiltrates [50-52, 114, 117]. Prominent staining of RIG-I and MDA5 has primarily been observed in infiltrating immune cells [64], which may be a reflection of localized IFN production.

Immune cells are especially equipped to initiate immune responses to foreign NAs. Most immune cell types express cytosolic RNA-sensing receptors and their downstream signaling molecules at steady state [112]. Considering that the RNA-sensing receptors themselves are IFN-regulated, it is not surprising to see increased expression of IFIH1/MDA5, DDX58/RIG-I, EIF2AK2/PKR and OAS genes in whole blood and specific leukocyte subsets from pSS patients [62, 64, 66, 118-121]. Studies detecting active signaling and functional properties of these pathways in pSS cells have been very limited so far. Increased levels of phosphorylated TBK1, suggesting activated signaling, have been detected in pDCs from pSS patients with high ISG expression [120]. Together, these expression patterns indicate an immunoactive status of pSS circulating immune cells which may translate to hyperresponsiveness to NA stimuli and contribute to the ongoing loop of inflammation.

Potential ligands of cytosolic RNA-sensing pathways in pSS

Various forms of cellular RNA have the potential to induce type I IFNs. Endogenous retroelements, as exemplified by patients with AGS, represent an important potential source of IFN-inducing cellular RNAs (Box A1) [122-125]. Increased levels of LINE1 RNA, that were positively correlated to type I IFN expression have been detected in pSS minor salivary glands [59]. In accordance, LINE1 promotor methylation levels were reduced in pSS salivary glands, which was most pronounced in patients with multiple risk factors for lymphoma [126]. RNA sequencing of SLE peripheral blood mononuclear cells (PBMCs) indicated higher number of Alu-derived transcripts compared to control PBMCs [79]. Hitherto, quantification of retroelement-derived transcripts in pSS has been limited to salivary gland tissue.

In addition to endogenous retroelements, other forms of (non-coding) RNAs may induce IFN expression. For example, microRNA (miR)-1248 that regulates calcium signaling

Box A1

Transposable elements

A large proportion of the human genome is composed of transposable elements which can integrate in new locations in genome. These transposable elements can be classified into those with long terminal repeats (LTRs), the endogenous retroviruses (ERVs), and those without LTRs, which include the short interspersed nuclear elements (SINE) and long interspersed nuclear elements (LINE) [209]. Retrotransposon activity occasionally leads to disease when causing frame shifts, splicing defects or deletions and seem to contribute to genetic diversity related to disease risk [210].

LINE-1 is an autonomous transposon that is currently active in human genomes [211, 212]. LINE-1 is transcribed by RNA polymerase II and encodes a RNA-binding protein, a reverse transcriptase and an endonuclease [209]. A large proportion of SINE are Alu retroelements that require LINE1 activity for transposition [213]. Alu RNAs are heavily involved in regulation of transcription, splicing, RNA stability, and translation [214]. The majority of expressed Alu RNAs are located within polymerase II-transcribed mRNAs [214]. Alus located outside coding regions can be transcribed by polymerase III, a process that occurs at low levels in healthy cells, but can be induced by various stressors [213-216].

Endogenous retroelement-derived RNA transcripts contain biochemical structures or form secondary and tertiary structures that – in case of improper processing - can activate TLRs, RLRs and PKR and induce type I IFN production [59, 106, 123-125, 213, 217-219]. Reverse transcribed retroelemental cDNA and DNA fragments from transposon-induced DNA damage are potential triggers of cGAS-STING pathway [59, 123].

has been reported to induce IFNβ by direct RIG-I binding [111]. Overexpression of miR-1248 has been correlated to ISG expression in pSS salivary gland [111]. Mitochondrial RNA leaking into the cytoplasm or released by dying cells could theoretically induce type I IFNs [127], but this mechanism has not been explored in pSS yet. Transfection of total RNA from pSS PBMCs in p125-HEK293 IFNβ reporter cells [128] failed to induce detectable responses, indicating that PBMCs from pSS patients do not contain large quantities of MDA5 or RIG-I stimulating RNAs (unpublished data).

Viral etiology of pSS has been a longstanding hypothesis and multiple viruses can cause pSS-like sicca symptoms [129-133]. Important immunological and histological differences exist between pSS and infection-associated sicca [130, 133]. Even in the absence of active viral infection, traces of viral RNA may remain present after spontaneous resolution or in seronegative occult localized infections [134-137]. Interestingly, hepatitis D virus (HDV) antigen and viral RNA have been detected in minor salivary glands from a large proportion of HBV-seronegative pSS patients and non-pSS sicca [138]. The cell types infected

with HDV have not been identified and it remains unknown whether HDV RNA can be detected systemically. In mice, HDV cannulation induced sialoadenitis and anti-SSA/SSB antibodies [138]. Although HDV induces strong acute type I IFN expression through MDA5, its replication was not effected [139], providing an explanation for its persistence in pSS salivary glands. A risk conferring variant in the gene encoding OAS1 further relates inefficient viral clearance and sustained type IFN production to pSS [140, 141]. Functionally, this variant shifts favored splicing sites, producing alternative isoforms with reduced enzymatic activity and/or lower expression levels [140-142]. Overall, it cannot be excluded that hidden viral infections may be a source of type I IFN-inducing NAs in some individuals. In summary, several potential activators of cytosolic RNA-sensing pathways and type I IFNs have been linked to pSS. Studies have been mainly focused on salivary glands and less on circulating immune cells. Therefore, future studies should aim to reproduce these findings, identify the cellular sources of potential ligands and characterize both tissue and circulating compartments.

CYTOSOLIC IFN-INDUCING DNA-SENSING PATHWAYS IN PRIMARY SJÖGREN'S SYNDROME

3.1

Signaling and regulation of cytosolic DNA-sensing pathways

Cytosolic dsDNA from both microbial and endogenous origin can be recognized by DNA-sensing receptors mediating protection against pathogens and antitumor immunity [143-145]. cGAS is the primary cytosolic DNA sensor [143]. This sensor efficiently recognizes the sugar-backbone of dsDNAs longer than 45 bp with its two main DNA-binding domains in a sequence–independent manner [146-148]. Within liquid-phase separated foci, activated cGAS catalyzes the conversion of GTP and ATP to 2'3'cGAMP [149, 150]. This second messenger prompts translocation of the adaptor protein STING from the endoplasmic reticulum (ER) into the perinuclear ER intermediate Golgi complex (ERGIC) [151, 152]. There, STING recruits and activates downstream kinases IKK and TBK1, which in turn phosphorylate transcription factors IRF3/IRF7 and stimulate type I IFN production [153]. STING can also be activated independent of cGAS by several cyclic dinucleotides [144].

Since the discovery of the first cytosolic putative DNA/RNA-sensing receptor DAI/ZBP-1 [154, 155], multiple other putative cytosolic DNA sensors have been proposed to induce type I IFN, NFκB-dependent cytokines or inflammasome activation [3, 156]. In this context, IFI16 is known to induce ASC-inflammasome formation [157-159]. Moreover, IFI16 is an integral part of cGAS-STING signaling pathway ensuring both optimal cGAS-mediated cGAMP production and acting as a crucial mediator for downstream recruitment of TBK1

[160, 161]. Other putative DNA-sensing receptors are considered less important than cGAS, but their activation and signaling are still poorly characterized.

In homeostatic conditions, inappropriate sensing of self-DNA is limited by compartmentalization of cellular DNA and sensors, and metabolization of DNA by endogenous nucleases [144, 148]. Signaling of the cGAS-STING pathway is regulated by various epigenetic, transcriptional and post-translational mechanisms [148]. Defects in these mechanisms can lead to STING-dependent type I IFN production and disease [144, 162-164].

DNA-sensing receptors in pSS

Findings from several studies have provided some indications for involvement of cytosolic DNA-sensing pathways in pSS and their potential contribution to type I IFN activation. Protein expression of the DNA sensor cGAS and downstream mediator STING can be readily detected in salivary gland epithelial cells [112, 165]. The STING pathway was shown to be functionally active in primary murine salivary gland cells, resulting in the production of type I IFN [165]. In contrast, IFI16 is not constitutively expressed in normal salivary gland epithelium [112, 166].

Transcriptomic analysis of salivary gland biopsies and epithelium-enriched fractions revealed increased expression levels of IFI16, but not cGAS or TMEM173/STING, in pSS compared to controls [50, 51, 166, 167]. Immunohistochemical staining demonstrated prominent nuclear and cytoplasmic IFI16 expression in pSS salivary gland epithelium [166, 168]. In ductal epithelial cells, the cytoplasmic IFI16 was organized in filamentous structures [169], suggesting activation by dsDNA or by spontaneous assembly of high concentrated monomers [169-171]. Although not investigated in this study, the activated IFI16 may induce type I IFN production [169]. Notably, the filamentous IFI16 can be effectively bound by anti-IFI16 autoantibodies from pSS patients, which have previously been associated with a more severe disease phenotype [167, 169].

Cytoplasmic IFI16 staining has also been shown for infiltrating immune cells [166], but the macromolecular organization of IFI16 has not been reported. Transcript levels of IFI16, but not TMEM173/STING, are elevated in peripheral blood [62, 119, 121] and circulating pDCs [64, 66] from pSS patients. Although pDCs are primarily known for their responsiveness to TLR7 and TLR9 ligands, they also possess a functional cGAS-STING pathway. Stimulation of this pathway in pDCs inhibits TLR9 activity [172]. In this context, it is worth noting that pDCs from both pSS and SLE patients tend to produce less type I IFN in response to TLR9 stimulation [66, 173]. Interestingly, increased levels of cGAMP, indicative for active STING signaling, have been reported in PBMCs from SLE patients [20, 174]. Indications for activation of cGAS-STING pathway in pSS are still lacking.

Summarizing, current literature suggests activated IFI16 signaling in pSS salivary glands which may potentially contribute to type I IFN production.

Potential ligands of cytosolic DNA-sensing pathways in pSS

Cytoplasmic deposition of genomic or mitochondrial DNA caused by perturbations in DNA metabolism may elicit DNA-sensing pathway activation and type I IFN production. pSS-derived salivary gland epithelial cell lines and salivary gland biopsies contain cytosolic dsDNA depositions that have been linked to reduced DNase I activity and AIM2 inflammasome activation [175]. The authors did not detect strong expression of ISGs and hence consider type I IFN production induction by dsDNA depositions in these salivary gland epithelial cell lines unlikely [117]. Similar cytoplasmic DNA depositions have been identified in salivary gland infiltrating macrophages in pSS that show signs of NLRP3 inflammasome activation and pyroptotic death [89]. In accordance with this inflammatory type of cell death, dsDNA strands were observed in the extracellular space around ductal structures.

The ATP-gating ion channel and pore-forming P2X7 receptor (P2X7R) that regulates inflammasome activation, has recently also been implicated in ATP-gated cGAMP transport from the extracellular space mediating STING-dependent type I IFN production [176, 177]. P2X7R is abundantly present on immune cells and salivary gland epithelium [112, 178]. A genetic variant in P2X7R has been linked to seropositive pSS in individuals not carrying HLA-DR3 risk alleles [179]. Additionally, P2X7R is expressed at higher levels in salivary glands of pSS and its expression is correlated to focus score and lymphoma development [180, 181]. These data indicate that P2X7R might play a role in autoinflammation in pSS salivary glands by impacting various innate immune functions.

Reduced DNAse II activity and increased amounts of short-fragmented cytosolic DNA have been reported in PBMCs from pSS patients, most notably in patients with a high-risk phenotype for lymphoma development [89]. DNase II is highly expressed in lysosomes of macrophages and degrades DNA internalized by endocytosis or through phagocytosis of apoptotic bodies [182]. Mice lacking DNase II develop an autoimmune-like phenotype and anti-nuclear antibodies [164, 183]. In the absence of DNase II, insufficient elimination of DNA from the lysosomes results in leakage of undigested DNA into the cytoplasm [89] inducing STING-dependent type I IFN production [184].

Excessive DNA damage may disturb the balance between supply and digestion of DNA fragments causing cytosolic DNA accumulation and thereby providing ligands for the cytosolic DNA-sensing receptors. Several studies describe excessive DNA damage in pSS salivary glands, specifically in the lymphoepithelial lesions [175, 185]. The endonuclease TREX1 is one of the crucial mediators in cytosolic DNA degradation. In pSS, several genetic variants in TREX1 have been described [186]. Interestingly, one TREX1-variant has been negatively associated with pSS-lymphoma development and positively associated with elevated ISGs in minor salivary glands [187].

In addition to genomic DNA, mitochondrial DNA released into the cytoplasm is able to activate DNA-sensing pathways. Leakage of mitochondrial DNA from damaged

mitochondria resulting from excessive IFNα signaling and impaired autophagy has recently been shown to induce STING signaling in SLE monocytes [21]. In pSS, disturbances in autophagy [188-190] have not been investigated in the context of type I IFN production.

In conclusion, impaired DNA clearance, leakage of DNA from intracellular compartments and excessive DNA damage contribute to accumulation of genomic DNA in pSS. How these extracellular and cytosolic DNA depositions relate to type I IFN activation in pSS patients remains to be elucidated.

CROSSTALK BETWEEN NUCLEIC ACID-SENSING PATHWAYS

The NA-induced signaling pathways can hardly be considered in isolation as significant crosstalk between these pathways occur at multiple levels [191]. First, expression levels of cytosolic and endosomal NA-sensing receptors are often coregulated [191]. Second, distinct NA-sensing receptor families may share part of the downstream signaling components [192]. Third, modification of NAs may result in activation of multiple NA sensors in parallel. For example, RNAs can be reverse transcribed into DNA creating ligands for the DNA-sensing pathway. RNA polymerase III is able to transcribe 5'-triphosphate-containing RNAs from dsDNA, creating ligands for RIG-I [193, 194]. Fourth, NA signaling pathways also negatively regulate each other, which is illustrated by the seemingly contradictory roles of STING in autoimmunity. Knockout of STING has been reported to resolve autoimmunity in some animal autoimmune models [162-164], while exacerbating autoimmunity in other models [195]. The latter observations could at least in part be explained by the negative regulatory role of STING on TLR7 and/or TLR9 signaling in certain immune cells, which is potentially mediated through SOCS1/3 [172, 195]. Similar mechanisms may explain some of the conflicting reports on other immune sensors in autoimmune models as well. Recently, SID1 transmembrane family member 2 (SIDT2) has been identified to transport dsRNA from the endolysosomal compartment to the cytoplasm where it can stimulate the cytosolic NA receptors [196], integrating NA-sensing pathways from different cellular compartments.

EXTRACELLULAR NUCLEIC ACIDS: POTENTIAL LIGANDS FOR CYTOSOLIC NUCLEIC ACIDS SENSORS?

Defects in regulation of apoptosis or clearance of cellular debris can cause rare forms of monogenic SLE or increase the risk for SLE development [90, 197]. Observations from pSS patients and mouse models suggest abnormal regulation of apoptosis, which has been

considered a key mechanism for exposure of autoantigens to the immune system [83, 198, 199].

If not cleared effectively, apoptotic cells may undergo secondary necrosis and leak their intracellular components causing immune activation [200]. Multiple studies indicated impaired clearance of apoptotic cells in pSS patients [201-203] and increased levels of circulating nucleosomes and cell free DNA [87, 88]. These levels were inversely correlated to the activity of serum DNase I which digests excessive extracellular DNA [88, 89]. Autoantibodies against DNase I were described in almost half of the studied pSS sera [204]. Although not experimentally proven yet, analogous associations of several serological markers with both anti-DNAse I autoantibodies [204] and plasma cell free DNA [87] suggest neutralizing ability of these antibodies. Similar autoantibodies in SLE have already been shown to inhibit the DNase I enzymatic activity [205].

Opsonization of insufficiently degraded apoptotic cell remnants by pSS-derived autoantibodies drive efficient phagocytosis [88]. Even intact apoptotic bodies are potentially immunogenic as illustrated by the secretion of pro-inflammatory cytokines by pDCs following internalization of these extracellular vesicles [83]. Apoptosis-derived membrane vesicles that are present in serum from SLE patients have been shown to induce cGAS-STING–driven ISG expression [174]. The pSS sera that were tested in this study did not have this STING-mediated IFN-inducing capacity.

Recently, a new type of highly immunogenic extracellular vesicle has been described that is produced by various cell types during apoptosis [206]. These apoptotic exosome-like vesicles contain non-coding RNAs with molecular structures allowing the recognition by RLRs and endosomal TLRs [95]. The same immunogenicity and type of cargo was described for exosomes released from various cancer cell types that were shown to stimulate IFN production through TLR3 and RLRs [207, 208]. Considering that these exosome-like vesicles seem to be produced by damaged or abnormal cells, these vesicles may also be released in autoimmune diseases characterized by excessive tissue damage.

In conclusion, dysregulation of apoptosis and impaired clearance of cellular debris in pSS promote exposure of autoantigens and NAs to the immune system. Depending on macromolecular presentation, efficiency of endolysosomal degradation and cell type, these internalized NAs may activate endosomal TLRs and/or cytosolic NA sensors.

CONCLUSIONS AND FUTURE DIRECTIONS

Knowledge from multiple disciplines has advanced our understanding of potential driving mechanisms of type I IFN activation in systemic autoimmune diseases. NAs when highly abundant, incorrectly processed or mislocalized may activate cytosolic PRRs or enter

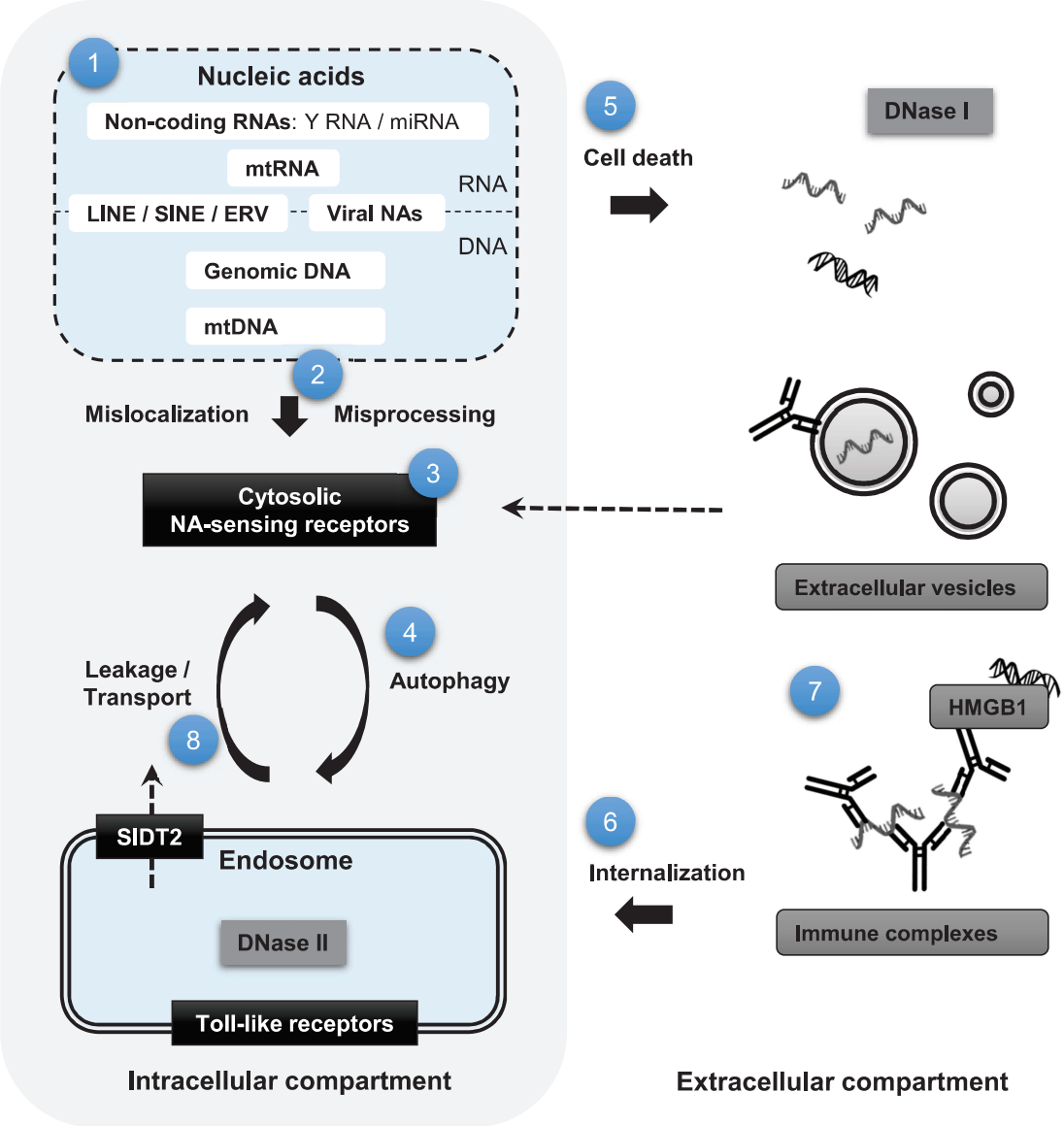

Figure 1. Graphical overview of mechanisms potentially contributing to activation of intracellular nucleic acid-sensing pathways and type I IFN production in primary Sjögren's syndrome.
Intracellular nucleic acids (NAs) from viral or endogenous origin **(1)** may, as a result of abundant expression, misprocessing or mislocalization **(2)**, be sensed by cytosolic NA-receptors **(3)**, be delivered to the endolysosomal compartment through autophagy **(4)**, or be released into the extracellular space through cell death or active secretion **(5)**. If not cleared sufficiently by nucleases or efferocytosis, internalization of extracellular NAs by immune cells **(6)** may lead to activation of endosomal Toll-like receptors. Extracellular NAs can exist in various macromolecular complexes **(7)** that impact their immunogenicity, the efficiency and mechanism of internalization, and cellular response. NAs confined to the endolysosomal compartment may escape into the cytoplasm via specialized transporters or as a result of inefficient digestion and loss of endosomal integrity **(8)**.

the extracellular compartment from which they can be internalized by immune cells and activate endosomal PRRs (Figure 1). Multiple observations support a potential role for both intra- and extracellular NAs in type I IFN activation in pSS. Dysregulation of endolysosomal digestion and autophagic trafficking under inflammatory pressure may integrate signaling via the endosomal and cytosolic NA-sensing pathways in pSS. Interestingly, hydroxychloroquine affects both endolysosomal maturation and autophagic flux. Therefore it is highly relevant to study the impact of regularly used medications on NA processing and the various sensing pathways. Future research should identify the cellular sources of IFN-inducing NAs and study negative regulatory mechanisms. Advanced techniques such as single cell sequencing and spacial transcriptomics will elucidate cell specific alterations in relation to their localization in tissue. Detailed characterization of activated NA-sensing pathways in individual patients may shed light on clinical heterogeneity and has important implications for treatment of systemic autoimmune diseases.

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CHAPTER 3.2

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

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Journal of Autoimmunity 2018;91:97-102

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.

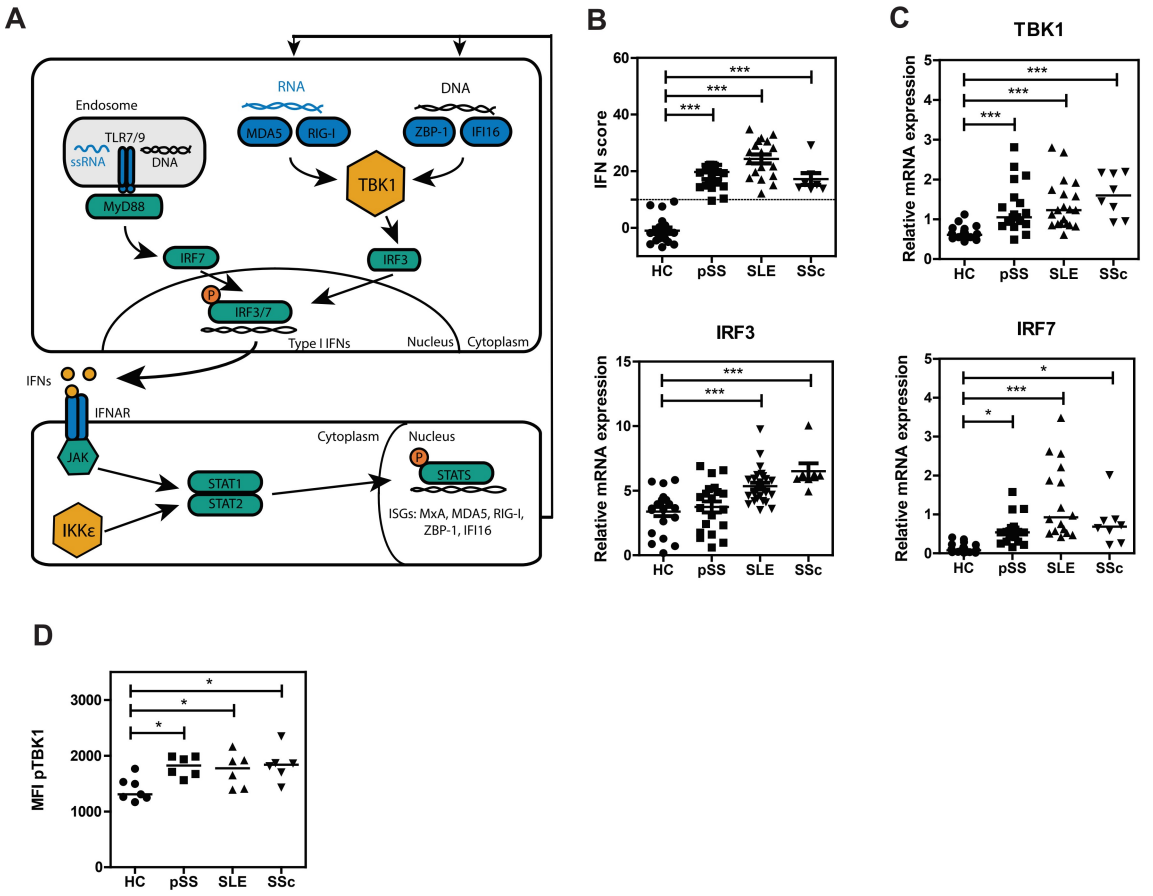

Figure 1. Systemic activation of TBK1 in IFN type I positive autoimmunity.

(A) Simplified scheme of the IFN type I inducing pathways and the signaling hub TBK1/ IKKε, which can be targeted by the inhibitor BX795. **(B)** IFN scores of IFN type I 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 IFN type I 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 1. 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 $mean_{HC} + 2 \times SD_{HC}$.

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.

ELISA and reporter assay

Type I IFN 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 1A). 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 (Ser172) (for gating strategy see Supplementary Figure 1B). 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 1C, D). These results suggest that increased phosphorylation of TBK1 plays a role in the observed IFN-I upregulation, and this i 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

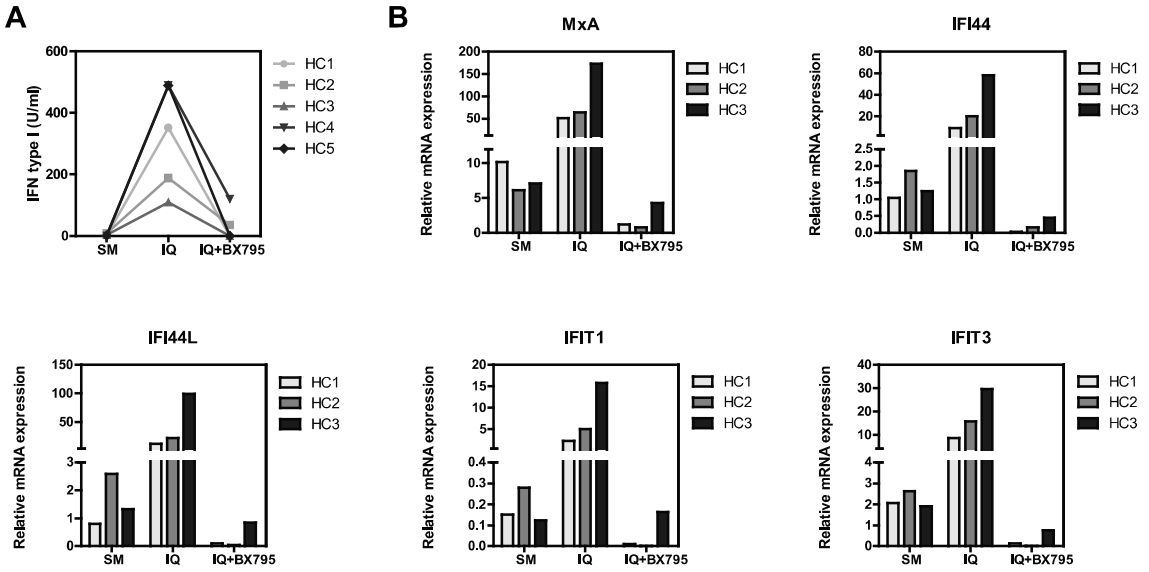

Figure 2. IFN type I inhibition by BX795 in healthy control peripheral blood mononuclear cells after Toll-like receptor 7 triggering with imiquimod.

(A) IFN type I 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).

PBMCs is shown in Supplementary Figure 2. 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).

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 3A, B).

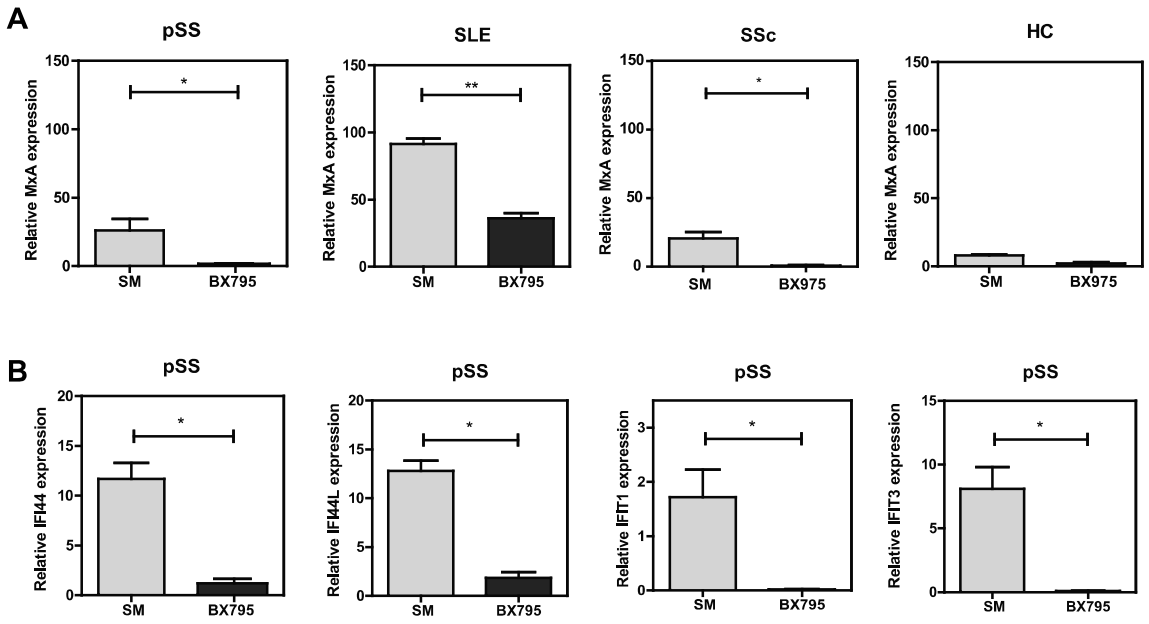

Figure 3. BX795 treatment inhibits spontaneous IFN type I activation in peripheral blood mononuclear cells of patients with systemic autoimmunity.

(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.

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 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 of 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 TLR3,4 also downstream of the IFNAR. We show that pDCs, which lack TLR3,4, 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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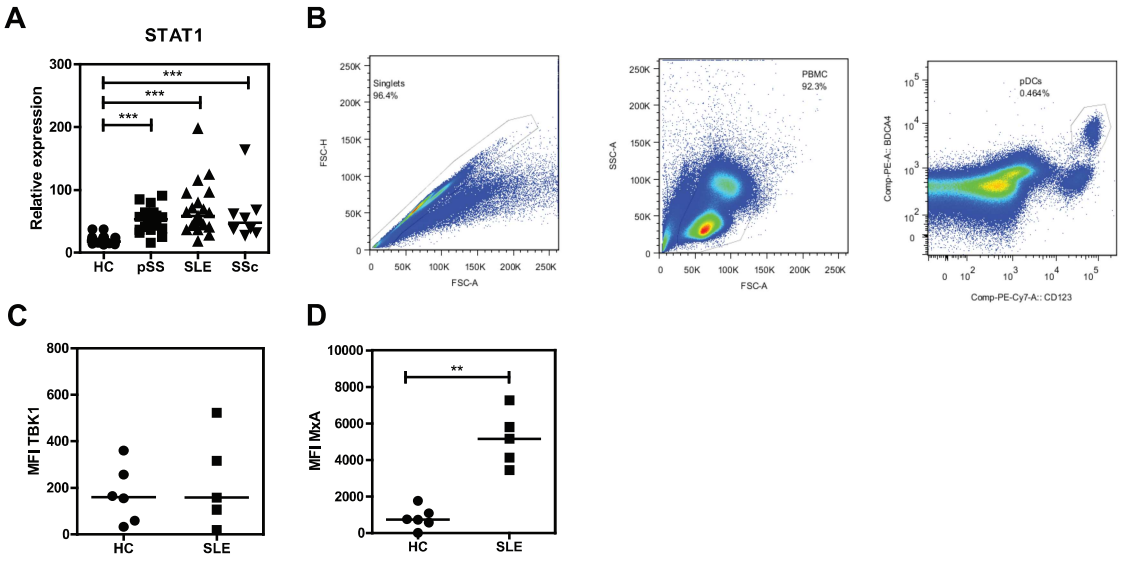
SUPPLEMENTARY DATA

Supplementary Table 1. Demographics, characteristics and medication use by participants.

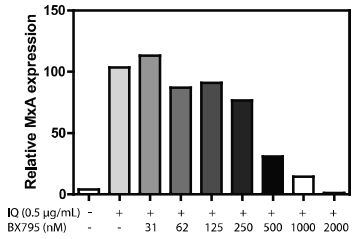
	HC (n=22)	pSS (n=22)	SLE (n=21)	SSc (n=10)
Demographics				
Female (%)[c]	21/22 (96)	20/22 (91)	16/21 (76)	8/10 (80)
Mean age (years)[a]	55.0 ± 5.9	56.1 ± 12.5	41.4 ± 16.0	53.3 ± 8.8
Patient characteristics				
Disease duration (years)[a]	-	12.7 ± 8.0	18.6 ± 14.6	9.6 ± 7.5
Disease activity[a,b]	-	9.0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



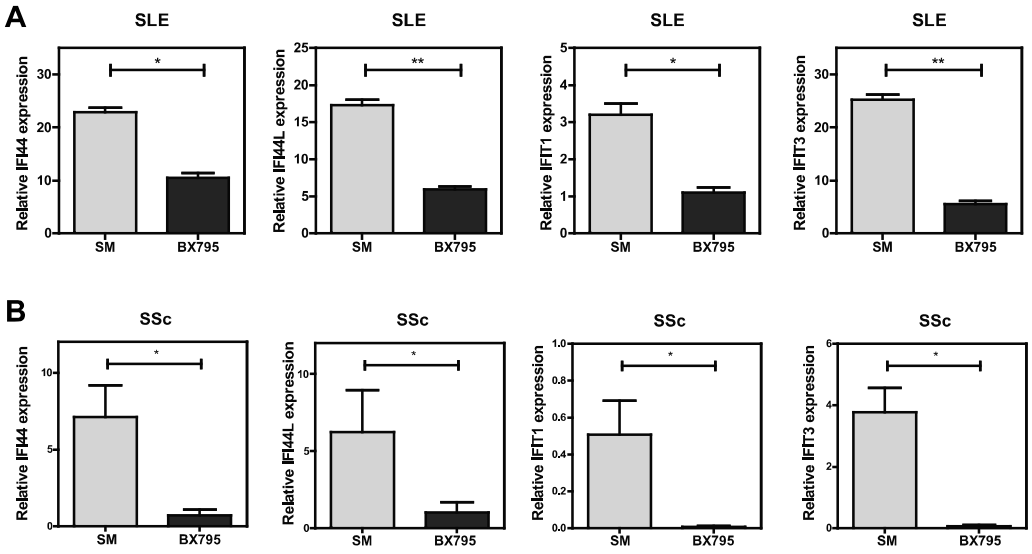
Supplementary Figure 1.

(A) Gene expression of STAT1 was determined in IFN type I 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 2.

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 3.

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 3.3

Hyperresponsive cytosolic DNA-sensing pathway in monocytes from primary Sjögren's syndrome

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Rheumatology (Oxford) 2022; online ahead of print

ABSTRACT

Objectives: Cytosolic DNA-sensing pathway stimulation prompts type I IFN (IFN-I) production, but its role in systemic IFN-I pathway activation in primary SS (pSS) is poorly studied. Here, we investigate the responsiveness of pSS monocytes and plasmacytoid dendritic cells (pDCs) to STING activation in relation to systemic IFN-I pathway activation and compare this to SLE.

Methods: Expression of DNA-sensing receptors cGAS, IFI16, ZBP-1 and DDX41, signalling molecules *STING*, *TBK1* and *IRF3*, positive and negative STING regulators, and IFN-I-stimulated genes *MxA*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* was analysed in whole blood, CD14+ monocytes, pDCs, and salivary glands by RT-PCR, monocyte RNA-sequencing data, flow cytometry and immunohistochemical staining. Peripheral blood mononuclear cells (PBMCs) from pSS, SLE and healthy controls (HC) were stimulated with STING-agonist 2′3′-cGAMP. STING phopshorylation (pSTING) and intracellular IFNα were evaluated using flow cytometry.

Results: STING activation induced a significantly higher proportion of IFNα-producing monocytes – but not pDCs – in both IFN-low and IFN-high pSS compared with HC PBMCs. Additionally, a trend towards more pSTING+ monocytes was observed in pSS and SLE, most pronounced in IFN-high patients. Positive STING regulators *TRIM38*, *TRIM56*, *USP18* and *SENP7* were significantly higher expressed in pSS than HC monocytes, while the dual-function STING regulator *RNF26* was downregulated in pSS monocytes. STING was expressed in mononuclear infiltrates and ductal epithelium in pSS salivary glands. STING stimulation induced pSTING and IFNα in pSS and SLE pDCs.

Conclusion: pSS monocytes and pDCs are hyperresponsive to stimulation of the STING pathway, which was not restricted to patients with IFN-I pathway activation.

INTRODUCTION

The majority of primary SS patients display persistent systemic type I IFN (IFN-I) pathway activation. Associations between clinical characteristics and IFN-I activation have been described in pSS [1]. Yet, the cellular source of IFN-I and the initiating triggers are still enigmatic.

IFN-I can be rapidly induced upon binding of ligands to pattern recognition receptors, in particular nucleic acid-sensing receptors. Plasmacytoid DCs (pDCs) are considered the classical IFN-I producing cells, predominantly via the TLR7/9 pathway [2]. IFN-I pathway activation in pSS frequently coincides with autoantibodies against nucleic acid-binding proteins [1]. RNA-containing immune complexes generated from pSS-derived autoantibodies are able to induce IFNα production by pDCs, presumably through TLR7 [3]. Nonetheless, the primary cellular source of IFN-I and the molecular pathways triggering IFN-I secretion is context dependent [2]. Along this line, a diverse range of cell types including monocytes express cytosolic sensors that detect dsDNA from both microbial and endogenous origin and can provoke the production of IFN-I [4].

The primary cytosolic DNA-sensing receptor is cGAS. Ligation of cGAS triggers IFN-I production by production of 2'3'-cGAMP and subsequent signalling via downstream mediators STING, TBK1 and IRF3. Multiple additional putative DNA sensors have been described to induce IFNs [5]. Recent observations, including monocyte hyperresponsiveness to STING stimulation, have provided clues for a contribution of the DNA-sensing pathway to IFN-I pathway activation in SLE [6, 7]. In pSS, peripheral blood mononuclear cells (PBMCs) have been observed to contain elevated levels of short-fragmented dsDNA along with reduced expression and activity of DNase II [8]. In this study, we aimed to investigate the functional responsiveness of pSS monocytes and pDCs to DNA-sensing pathway activation and the association with systemic IFN-I activation, and compare this to SLE.

METHODS

PBMCs were isolated from patients with pSS (n=34 IFN-high, n=27 IFN-low) or SLE (n=8) and HCs (n=32) (Supplementary Table 1). Expression of DNA-sensing receptors and downstream mediators was analysed by RT-PCR and flow cytometry in CD14+ monocytes and pDCs (Supplementary Figure 1). STING in pSS salivary glands was visualized by immunohistochemical staining. PBMCs were stimulated with 2'3'-cGAMP (STING agonist), Imiquimod-R837 (TLR7 agonist), or CpG ODN2216 (TLR9 agonist). Phosphorylation of STING and intracellular IFNα were measured by flow cytometry (Supplementary Figure 2 and 3). Secretion of IFN-I was quantified in a cellular IFN-I reporter assay. Activation of the

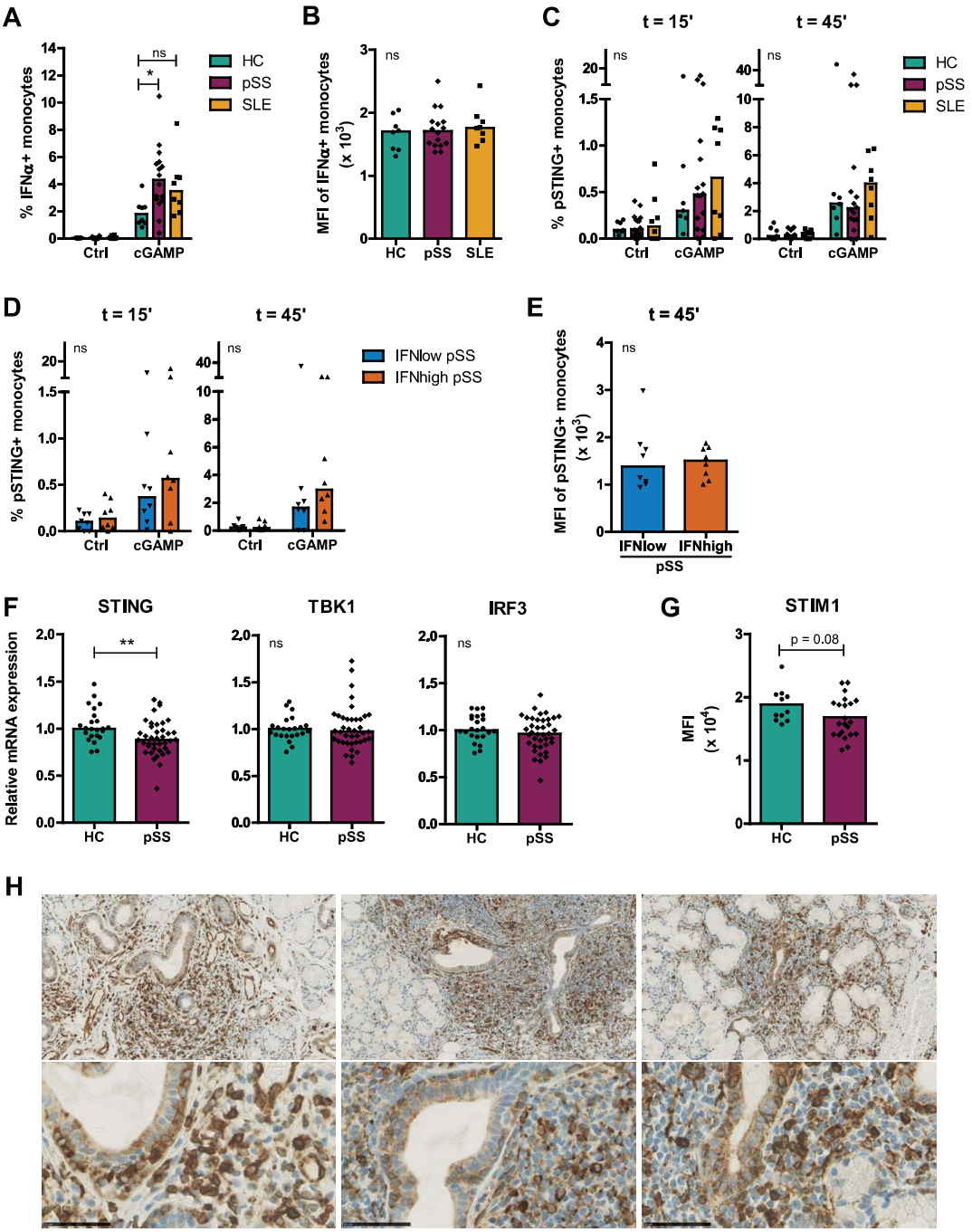

Figure 1. Increased proportions of cGAMP-inducible IFNα-producing monocytes in pSS.

(A) Frequency and **(B)** median fluorescence intensity (MFI) of IFNα+ monocytes in PBMCs stimulated with 25 µg/mL 2'3'-cGAMP for 6 hours. **(C-D)** Frequency and **(E)** MFI of pSTING+ monocytes in 2'3'-cGAMP-stimulated PBMCs from (HC, SLE) and pSS, **(D-E)** stratified by IFN-I score. **(F)** Relative mRNA expression of *STING*, *TBK1*, and *IRF3* in CD14+ monocytes. Symbols represent individual samples, bars indicate medians. **(G)** STIM1 MFI in CD14+ monocytes. **(H)** STING expression in pSS labial salivary glands (n=3; scale bar=50µm). *p<0.05, **p<0.01, ns; not significant.

IFN-I pathway was determined from a composite expression IFN-I score of ISGs *MxA*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* in whole blood. Details are provided in Supplementary Methods.

RESULTS

High IFI16 expression in monocytes and pDCs is associated with systemic IFN-I activation in pSS

The expression levels of the most well-known (putative) DNA-sensing receptors cGAS, IFI16, ZBP-1 and DDX41 were explored in peripheral blood monocytes and pDCs. The primary cytosolic DNA-sensing receptor cGAS was expressed at equal levels in monocytes and pDCs from pSS patients and HC (Supplementary Figure 4A and 5). Monocytes from IFN-high pSS patients expressed significantly higher IFI16 mRNA and protein levels compared with IFN-low pSS (Supplementary Figure 4B and 5). Despite upregulated *ZBP-1* mRNA expression in IFN-high pSS monocytes relative to HC, no significant differences in protein expression were observed between pSS and HC (Supplementary Figure 4C and 5). pDCs from IFN-high pSS showed significantly higher expression of IFI16 and DDX41 compared with IFN-low pSS (Supplementary Figure 4D).

cGAMP induces phosphorylation of STING and IFNα production in monocytes and pDCs

Next the responsiveness of the STING pathway in PBMCs was investigated. Activation of the STING pathway with cGAMP, as well as specific TLR7 or TLR9 stimulation, induced IFNα in PBMCs (Supplementary Figure 6A). The majority of IFNα-producing PBMCs upon STING stimulation were monocytes and pDCs in HC, while the contribution of monocytes to IFNα-producing PBMCs is higher in pSS and SLE (Supplementary Figure 6B). In line with this, the cGAMP-induced STING phosphorylation (pSTING) was most prominent in monocytes, peaking at 30 to 60 minutes (Supplementary Figure 7A,B and Supplementary Figure 3C). No difference was observed in the distribution of cell types among pSTING-positive PBMCs between HC and patients with pSS or SLE (Supplementary Figure 7B).

Increased proportions of cGAMP-inducible IFNα-producing monocytes in pSS

The proportion of cGAMP-induced IFNα-producing monocytes was higher in pSS than HC PBMCs, while the median fluorescence intensity (MFI) of IFNα-positive monocytes was comparable (Figure 1A,B). A similar trend was observed in PBMCs from SLE patients (Figure 1A,B). Mirroring these data, cGAMP upregulated the IFNβ mRNA expression and the secretion of IFN-I by PBMCs, which were increased in pSS compared with HC (Supplementary

Figure 8A,B). The cGAMP-induced response showed no association with HCQ treatment nor the patient's IFN score (Supplementary Figure 9).

Trend towards more pSTING+ monocytes in cGAMP-stimulated PBMCs from pSS and SLE

The frequency of pSTING+ monocytes in response to cGAMP stimulation showed considerable donor-to-donor variability. Although not reaching statistical significance, the proportion of monocytes positive for pSTING tended to be higher in SLE than HC PBMC cultures at 15 and 45 minutes and to a lesser extent in pSS after 15 minutes of cGAMP stimulation (Figure 1C). The cGAMP-induced pSTING+ monocytes tended to be more abundant in IFN-high pSS than IFN-low pSS (Figure 1D), while the MFI of pSTING+ monocytes were comparable between both groups (Figure 1E). Compared with HC, a small but significantly lower *TMEM173/STING* transcript abundance was observed in monocytes from pSS patients (Figure 1F). Downstream signalling mediators *TBK1* and *IRF3* were expressed at equal levels in pSS and HC monocytes (Figure 1F and Supplementary Figure 5). Although not statistically significant ($p = 0.08$), protein expression of STIM1, a negative regulator of STING signalling, appeared slightly reduced in pSS monocytes compared with HC monocytes (Figure 1G). STING was abundantly expressed in infiltrating mononuclear cells and ductal epithelial cells in labial salivary glands of pSS patients (Figure 1H).

Monocytes from pSS differentially express positive regulators of DNA-sensing pathway

A complex regulatory network involving post-translational modifications ensures balanced control of STING signalling. Monocytic expression of genes known to regulate the STING pathway was analysed using a publicly available RNAseq dataset of pSS monocytes [GSE173670] (Supplementary Table 2 and 3). The explored negative regulators of STING did not differ between pSS and HC (Supplementary Figure 5). Expression of positive regulators *TRIM38*, *TRIM56*, *USP18* and *SENP7*, each modulating post-translational modifications, was higher in pSS monocytes compared with HC. In contrast, RNF26 that promotes STING activity early on but suppresses in late response [9], was expressed at lower levels in pSS monocytes.

pDCs from pSS and SLE phosphorylate STING upon cGAMP stimulation

pDCs are uniquely equipped to generate robust IFN-I responses, particularly upon engagement of TLR7/9. Yet, STING activation in pDCs can also induce IFN-I production (Supplementary Figure 6B) [10]. Therefore, the responsiveness of each of these IFN-I-inducing pathways in pDCs was further explored. As expected, TLR9 stimulation strongly stimulated IFNα production by pDCs (Supplementary Figure 10A). HCQ is known to inhibit

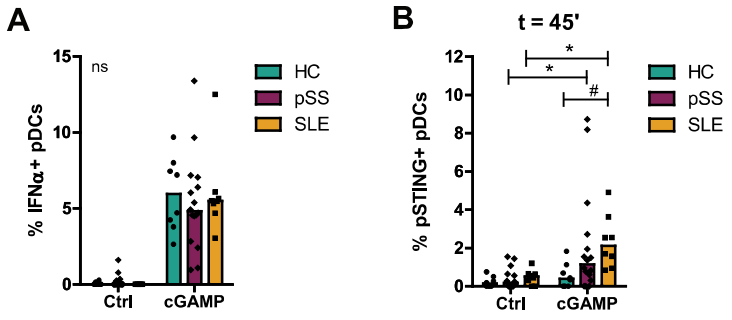

Figure 2. Plasmacytoid dendritic cells from pSS and SLE phosphorylate STING upon cGAMP stimulation. **(A)** Frequency of IFNα+ plasmacytoid dendritic cells (pDCs) and **(B)** frequency of pSTING+ pDCs of total pDCs in 25 µg/mL 2'3'-cGAMP-stimulated PBMC cultures from patients with pSS, SLE or HC. Symbols represent individual samples and bars indicate medians. *#p<0.05, ns; not significant.

endosomal TLR signalling. In line with this, the proportions of TLR7/9-induced IFNα-producing pDCs were significantly lower in PBMCs from HCQ-treated pSS (Supplementary Figure 10B). In HCQ-untreated pSS, frequency and MFI of IFNα+ pDCs were comparable with HC upon TLR9 stimulation (Supplementary Figure 10C,D). In contrast, IFNα-producing pDCs were increased in TLR7-stimulated PBMCs from IFN-high pSS (Supplementary Figure 10C,E). Opposed to monocytes, the frequency of IFNα-positive pDCs in cGAMP-stimulated PBMC cultures did not differ between HC and pSS or SLE (Figure 2A). While cGAMP did not induce pSTING in pDCs from HC, pSTING was clearly induced in pDCs from pSS and SLE patients, most notably after 45 minutes of stimulation (Figure 2B and Supplementary Figure 11). The frequency of pSTING+ pDCs was significantly increased in SLE compared with HC at 45 minutes of cGAMP stimulation and a trend for a higher frequency was also observed in pSS (Figure 2B). No association was observed between the patient's IFN score and *in vitro* cGAMP-induced pSTING in pDCs (data not shown).

DISCUSSION

Activation of the cytosolic DNA-sensing pathway induces IFN-I production, but the role of this pathway in systemic IFN-I pathway activation in pSS is poorly studied. Here, we demonstrated a hyperresponsiveness of pSS monocytes to STING stimulation, illustrated by an increased number of IFNα-producing monocytes. In accordance with literature [7], similar findings were observed in SLE monocytes.

Several positive regulators of STING pathway activity mediating post-translational modifications were upregulated in pSS monocytes relative to HC. On the other hand, STIM1 that negatively regulates STING by retaining it at the endoplasmic reticulum, was slightly reduced in pSS monocytes. Targeting STIM1 by an Influenza-A-derived peptide has been

reported to inhibit IFN-I production in an *in vitro* culture of SLE PBMCs [11]. Thus, altered balances between positive and negative regulators could potentially alter the sensitivity of the STING pathway in pSS monocytes.

In comparison to HC, monocytes from pSS displayed hyperresponsive IFNα production after STING stimulation. Yet, this did not associate with *in vivo* systemic IFN-I pathway activation. The expression of *SLC46A2*, the presumed primary cGAMP-importer in CD14+ monocytes [12], was unaltered in pSS monocytes (Supplementary Figure 5). The mechanism underlying the elevated cGAMP-stimulated IFNα production in IFN-low pSS remains to be elucidated, but might potentially be based on IFN-independent inflammatory pathways, epigenetic imprinting or STING-regulators. Notably, STIM1 was equally downregulated in IFN-low and IFN-high pSS compared with HC. Lower expression of STIM1 protein has previously been described in pSS salivary gland epithelium and linked to inhibition of STIM1 translation by an Epstein Barr virus-derived miRNA [13]. Although evidence is currently lacking for a similar mechanism in monocytes, it might be an interesting hypothesis to explore given unchanged STIM1 mRNA levels in pSS monocytes.

Although STING phosphorylation tended to be increased in IFN-high pSS, cGAMP-induced IFNα production did not differ between IFN-high and IFN-low pSS. This apparent discrepancy between the degree of STING phosphorylation (indicator of active STING signalling) and the final amount of IFN-I could have been influenced by various factors, acting at different levels of the pathway. Auto- and paracrine signalling are important in coordinating cellular responses. Not surprisingly, inhibition of the IFNAR receptor affected the number of IFNα-producing monocytes (data not shown). In this context, it is interesting to note that the positive STING-regulator USP18, which was significantly higher expressed in IFN-high pSS than IFN-low pSS, also has inhibitory activity on IFNAR signalling by interacting with IFNAR2 and STAT2 [14]. Therefore, differential regulation of auto-/paracrine IFNAR signalling in IFN-high and IFN-low pSS might impact the final IFNα response. Alternatively, methodological factors such as assay sensitivity and measurement of intracellular IFNα which is only one of the IFN-I, could have influenced these results.

The unique expression pattern of TLR7/9 and the transcription factors IRF7/IRF3 in pDCs drives robust IFN-I responses after endosomal TLR stimulation [15]. Despite this specialized function, pDCs contain a functional cGAS-STING pathway able to induce IFN-I [10]. Here, we showed that DNA-sensing pathway stimulation induced active STING signalling and IFNα production by pDCs from pSS and SLE patients. STING activation in pDCs has been demonstrated to inhibit TLR9 signalling [16]. In contrast to previous observations [17], we did not observe reduced TLR9-stimulated IFNα production in pSS pDCs. TLR7-stimulated IFNα production on the other hand was increased in pDCs from IFN-high pSS compared with IFN-low pSS, consistent with previous data [17]. While acknowledging methodological differences and the limited number of patients included in our study, our

findings contrast with the refractory state of pDCs described in SLE [18]. This suggests that functional differences exist between pDCs from SLE and pSS.

HCQ treatment of patients greatly affected the *in vitro* responsiveness of PBMCs to TLR7/TLR9 stimulation, but not STING activation. Although HCQ downregulates blood IFN scores in pSS [19], patients using HCQ in clinical practice often still display elevated IFN scores [1]. These data support involvement of IFN-I-inducing pathways beyond TLR7/9 in pSS, such as the cytosolic DNA-sensing pathway. Importantly, PBMCs from pSS patients have been reported to contain excessive cytosolic dsDNA [8]. This mislocalized dsDNA might be originating from leakage from the endosomal compartments caused by reduced DNase II activity and could potentially activate the cGAS-STING pathway and increase IFN-I production [20]. STING pathway activation has the potential to elicit pSS-like disease in mice [21]. Our study highlights the relevance of this observation for human pSS showing both functional alterations in STING pathway sensitivity in circulating immune cells as well as readily detectable STING expression in the mononuclear cell infiltrates and ductal epithelial cells in pSS salivary glands.

In conclusion, monocytes and pDCs from patients with pSS are hyperresponsive to stimulation of the STING pathway. This phenomenon was not restricted to patients with IFN-I pathway activation and was unaffected by HCQ treatment.

ACKNOWLEDGMENTS

The authors would like to thank Senada Koljenovic from the department of Pathology, Erasmus MC Rotterdam for retrieving the historical pSS biopsy specimens.

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SUPPLEMENTARY METHODS

Patients and healthy controls

Patients classified as primary SS (pSS) according to the 2016 ACR-EULAR Classification Criteria for primary Sjögren's Syndrome [1], or SLE according to the 2019 ACR-EULAR Classification Criteria for SLE [2] were recruited at the outpatient clinic of the Erasmus MC, Rotterdam University Medical Center, Rotterdam, The Netherlands. Demographic and clinical characteristics, use of medication and routine hematological and serological parameters were retrieved from patient records. Disease activity at the time of blood drawing was assessed using the EULAR Sjögren's syndrome disease activity index (ESSDAI) for pSS and Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) for SLE [3, 4]. Age and sex matched healthy controls (HC) were included. Characteristics of subjects included in this study are summarized in Supplementary Table S1. The Medical Ethics Review Committee of the Erasmus MC (MEC-2011-116;MEC-2016-202) has approved this study. Written informed consent was obtained from all participants in compliance with the declaration of Helsinki.

Blood sampling and processing

Peripheral blood samples were collected in NH Sodium Heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Becton Dickinson, Vianen, The Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation within two hours after sampling. PBMCs were cryopreserved in liquid nitrogen. Monocytes were enriched from PBMCs (n=21 IFN-high pSS, n=18 IFN-low pSS, n=23 HC) by immunomagnetic separation (autoMACS Pro, Miltenyi Biotec) using CD14 MicroBeads (Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer's instructions or by fluorescence-activated cell sorting on a FACSAria III (BD Biosciences) based on anti-CD14-APC-H7 (BD Biosciences, Vianen, The Netherlands) staining.

Real-time PCR

RNA was isolated from PAXgene Blood RNA Tubes using the PAXgene Blood RNA Kit (PreAnalytiX GmbH) or from PBMCs/monocytes using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). cDNA was synthesized using High-Capacity Reverse Transcription Kit (Applied Biosystems, Bleiswijk, The Netherlands). RT-PCR was performed on a QuantstudioTM 5 Real-Time PCR System using predesigned primer/probe sets (Applied Biosystems, Bleiswijk, The Netherlands). Relative gene expression normalized to the housekeeping gene Abl was calculated using the $2^{-\Delta\Delta Ct}$ method.

Calculation of IFN-I score

Whole blood expression of interferon-stimulated genes *MxA*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* was quantified from PAXgene Blood RNA tubes by RT-PCR, and an IFN-I score was calculated as previously described [5]. The sum of Z-scores of VST transformed counts of *MxA*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* was used to calculate the IFN-I scores from RNAseq data. The threshold for stratification of patients in IFN-low and IFN-high was determined by the $Mean_{HC} + 2*SD_{HC}$.

PBMC stimulations

Cryopreserved PBMCs were rested for 30 minutes in RPMI 1640 + GlutaMAX medium (Gibco, Thermo Fisher Scientific, Tilburg, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% P/S (Gibco) at 37°C before plating at a density of 4.10e5 cells/well in cell-repellent polystyrene round bottom 96-well microplates (Greiner Bio-one) for flow cytometry of IFNα (n=8 IFN-high pSS, n=8 IFN-low pSS, n=8 IFN-high SLE, n=8 HC), in Nunclon Delta flat bottom 96-well plates (Thermo Fisher Scientific, Tilburg, The Netherlands) for 24 hour stimulations (n=3-4 IFN-high pSS, IFN-low pSS, HC), or at a density of 4.10e6 cells/mL in 5 mL polypropylene tubes for pSTING analysis (n=8 IFN-high pSS, n=8 IFN-low pSS, n=8 IFN-high SLE, n=7 HC). PBMCs were stimulated with either 2'3'-cGAMP (InvivoGen, San Diego, USA), Imiquimod (R837; InvivoGen), CpG ODN2216 (InvivoGen) or medium in an humidified incubator at 37°C / 5% CO_2. PBMCs were stimulated for 15 or 45 minutes for pSTING analysis, 6 hours for intracellular IFNα staining, and 24 hours for gene expression analysis and IFN-I reporter assay. For intracellular IFNα staining, 1 µg/mL GolgiPlug Protein Transport Inhibitor (BD Biosciences) was added to the culture after 3 hours.

IFN-I reporter assay

IFN-I was quantified in culture supernatants using HEK-Blue IFN-α/β reporter cells (InvivoGen) according to the manufacturer's protocol. Recombinant human IFNβ1a (PBL Assay Science, Tebu-bio, Heerhugowaard, The Netherlands) was used for calibration.

Protein expression DNA-sensing receptors

For flow cytometric analysis of DNA-sensing receptor protein expression, PBMCs (n=11 IFN-high pSS, n=11 IFN-low pSS, n=11 HC) were stained with eBioscience Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific), blocked with 5% normal donkey serum (Abcam, Amsterdam, The Netherlands) in PBS/0.5% BSA/0.1% NaAz (FACS staining buffer) and stained with anti-CD56-BV421, anti-CD19-PE/Cy7, anti-CD3-APC/F750, CD14-PerCP/Cy5.5 combined with either anti-BDCA4-PE and anti-CD123-FITC (tube 1) or anti-CD16-PE and anti-HLA-DR-AF488 (tube 2) (all from Biolegend, Amsterdam, The Netherlands). Cells

were fixed and permeabilized using the eBioscience FoxP3 Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions. Intracellular staining was performed using one of the following primary antibodies: anti-cGAS (15102, Cell Signaling Technology, Leiden, The Netherlands), anti-IFI16 (ab169788, Abcam), anti-DDX41 (15076, Cell Signaling Technology), anti-ZBP1 (PA5-20455, Thermo Fisher Scientific), anti-STIM1 (5668, Cell Signaling Technology) or anti-Mx1 (137501AP, ProteinTech, Manchester, UK), and a secondary donkey anti-rabbit IgG-AF647 (Biolegend). Appropriate isotype-matched and FMO controls were included. Cells were analysed on a FACS CANTO II HTS (BD Biosciences).

Intracellular IFNα staining

Cultured PBMCs were stained with eBioscience Fixable Viability Dye eFluor 506, blocked with 10% heat-inactivated pooled human AB serum (obtained from Sanquin, Amsterdam, The Netherlands) in FACS staining buffer and stained with anti-CD56-APC/F750, anti-CD19-APC/F750, anti-CD3-APC/F750, anti-CD14-BV421, anti-CD123-PE, anti-CD64-PE/Cy7, anti-CD11c-PerCP/Cy5.5 and anti-HLA-DR-AF488 (all from Biolegend). Cells were fixed and permeabilized using the eBioscience Intracellular Fixation and Permeabilization Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions. Permeabilization was performed overnight at 4°C. Subsequently, cells were stained with anti-IFNα-APC (Miltenyi Biotec) and analysed on a FACS CANTO II HTS.

Flow cytometry phosphorylated STING

Prior to stimulation, PBMCs were stained with eBioscience Fixable Viability Dye eFluor 506, blocked with 10% heat-inactivated pooled human AB serum in FACS staining buffer and stained with anti-CD56-APC/F750, anti-CD19-APC/F750, anti-CD3-APC/F750 and anti-CD14-BV421 (all from Biolegend). Cells were resuspended in heat-inactivated FCS, rested for 30 minutes at 37°C and subsequently stimulated with 2'3'-cGAMP or FCS only. Cells were fixed and stained with anti-CD123-PE, anti-CD64-PE/Cy6, anti-CD11c-PerCP/Cy5.5, anti-HLA-DR-AF488 (all from Biolegend), and anti-pSTING-AF647 (Cell Signaling Technology) using the PerFix EXPOSE kit (Beckman Coulter, Woerden, The Netherlands) following the instructions provided by the manufacturer. Appropriate isotype-matched and FMO controls were included. Cells were analysed on a FACS CANTO II HTS.

Gating strategy

Analysis was performed on collected events after exclusion of doublets and dead cells. Gating strategies for the analysis of DNA-sensing receptor expression, intracellular IFNα and pSTING in monocytes and plasmacytoid dendritic cells are provided in Supplementary Figure 1-3. The frequency of pSTING-positive monocytes increased over the course of 2 hours after 2'3'-cGAMP stimulation, with the maximum median fluorescence intensity

reached after 30-60 minutes (Supplementary Figure 3B,C). In parallel, the expression of STING (anti-STING-AF647, BD Biosciences) was downregulated upon 2'3'-cGAMP stimulation (Supplementary Figure 3D).

Immunohistochemical staining STING on labial salivary gland biopsies

Historical formalin-fixed paraffin-embedded labial salivary gland biopsies from three pSS patients were obtained from the department of Pathology, Erasmus MC, Rotterdam, the Netherlands. Immunohistochemistry was performed on 4 µm tissue slices with an automated, validated and accredited staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucsen, AZ, USA) using optiview universal DAB detection Kit. In brief, following deparaffinization and heat-induced antigen retrieval the tissue samples were incubated with polyclonal rabbit anti-STING antibody (1000x dilution; SAB1306550; Thermo Fisher Scientific) for 32 minutes applying the Optiview CC1 32' procedure. Incubation was followed by hematoxylin II counter stain for 12 minutes and a blue colouring reagent for 8 minutes according to the manufactures instructions (Ventana). Images were acquired using a Hamamatsu Nanozoomer 2.0 HT digital slide scanner.

RNA Sequence Analysis

Paired-end raw FASTQ files were downloaded from the GEO database using GEO Series accession number GSE173670 [6], and were analysed with the nf-core/RNA-seq pipeline (v3.1) using Nextflow (21.05.0.edge) and its default settings [7, 8]. Quality of the sequencing was reported with FastQC (v11.9). Subsequently, bases with low Phred scores (≤ 30) were either trimmed or the complete reads were removed using Trim Galore! (v6.6). Trimmed FASTQ reads were mapped to the human reference genome version GRCh38 with our in-house GRCh38 gencode 37 gene annotation file using RSEM (v1.3.1), which umbrellas STAR (v2.7.6a) as read aligner. Next, SAMtools (v 1.10) processed the alignment files and extracted mapping statistics of the post-alignment [9-11]. Quality of each sample alignment was visually inspected using reports derived from RSeQC (v3.0.1), Qualimap (v2.2.2-dev) and Preseq (v3.1.1), including read inner distance plots, splice junction annotations, the genomic origin of the mapped reads, and the estimated complexity of the sequencing library [12-14]. RSEM estimated transcript counts were imported into R (v4.1.0), transformed to gene counts using tximport (v1.20) and analyzed with DESeq2 (v1.32.0) [15, 16]. Only protein-coding genes were kept for subsequent analyses. Gene counts were transformed using the "varianceStabilizingTransformation" (VST) function of DESeq2.

Differential expression analysis

Differentially expressed genes were calculated using DESeq2. p-values were calculated using Wald statistical test and corrected with the Benjamini-Hochberg multiple hypothesis

testing method for all protein-coding genes. For the comparison of pSS patients against HC, the HC were set as base reference. For the analysis between the IFN-high and IFN-low pSS patients the IFN-low subgroup was set as base reference. Fold Changes were shrunk with the DESeq2 function "lfcshrink" using method "apeglm" [17]. Heatmaps were made using the R package heatmap (v1.0.12), Z-scores were calculated per gene on the VST transformed counts.

Statistical analysis

Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA) was used for graph design and statistical analyses. Depending on the data distribution, independent-samples t-test or Mann-Whitney U test was used to compare means/medians of two groups. One-way ANOVA followed by Tukey's HSD test or Kruskal-Wallis H test followed by Dunn's Multiple Comparison test was used to compare means/medians of three groups.

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SUPPLEMENTARY DATA

Supplementary Table 1. Demographic and clinical characteristics.

	HC n = 32	pSS n = 61	SLE n = 8
Demographics			
Female[a]	30/32 (93.8)	55/61 (90.1)	8/8 (100)
Age [years][b]	51 (44.5-56)	61 (51-67)	42 (24-53.8)
Patient characteristics			
Disease duration [years][b]	-	11.5 (6-21)	21 (3.8-31.3)
Disease activity[b] (1)	-	3.5 (1.3-9)	4 (2.8-4.3)
Laboratory parameters			
ANA[a]	-	44/55 (80)*	8/8 (100)
Anti-SSA[a]	-	53/61 (86.9)	7/8 (87.5)
Anti-Ro52[a]	-	47/60 (78.3)*	6/8 (75)
Anti-Ro60[a]	-	48/60 (80)*	7/8 (87.5)
Anti-SSB[a]	-	34/61 (55.7)	4/8 (50)
Anti-dsDNA [IU/mL][b]	-	-	25.5 (13.1-64.5)
Anti-Sm[a]	-	-	2/8 (25)
Anti-RNP[a]	-	-	3/8 (37.5)
C3 [g/L][b]	-	1.19 (1.05-1.32)	0.97 (0.85-1.15)
C4 [g/L][b]	-	0.20 (0.15-0.24)	0.18 (0.12-0.21)
Current medication[a]			
HCQ	-	38/61 (62.3)	7/8 (87.5)
HCQ monotherapy	-	30/38 (78.9)	4/7 (57.1)
HCQ + corticosteroids / DMARDs	-	8/38 (7.9)	3/7 (42.9)
Corticosteroids/DMARDs	-	10/61 (16.4)	4/8 (50)
Corticosteroids + DMARDs	-	2/10 (20)	1/4 (25)
Corticosteroids only	-	7/10 (70)	2/4 (50)
DMARDs only	-	1/10 (10)	1/4 (25)

Data are presented as number of patients (%)[a] or median (Q1-Q3)[b]. (1) Disease activity: EULAR Sjögren's syndrome disease activity index (ESSDAI) for pSS and SLEDAI-2K for SLE. *Missing values for some of the patients. Abbreviations: anti-Sm, anti-Smith; HC, healthy controls; pSS, primary SS.

Supplementary Table 2. Differential Gene Expression pSS vs HC.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000020922.13	chr11	94415570	94493885	-	protein_coding	MRE11	562,5776582	1,80126E-05	0,001443	0,0645	0,224008
ENSG00000079246.16	chr2	2,16E+08	2,16E+08	+	protein_coding	XRCC5	6328,410162	-0,00033917	0,001463	0,520222	0,724143
ENSG00000124256.15	chr20	57603846	57620576	-	protein_coding	ZBP1	299,6110756	1,261867265	0,279314	2,4E-07	5,79E-05
ENSG00000124831.19	chr2	2,38E+08	2,38E+08	+	protein_coding	LRRFIP1	8583,331727	7,89961E-06	0,001443	0,425241	0,650795
ENSG00000163565.19	chr1	1,59E+08	1,59E+08	+	protein_coding	IFI16	4031,630689	0,754759738	0,25467	0,000107	0,004809
ENSG00000163568.15	chr1	1,59E+08	1,59E+08	-	protein_coding	AIM2	99,90963479	1,00769E-05	0,001443	0,006218	0,058115
ENSG00000164430.17	chr6	73413515	73452297	-	protein_coding	CGAS	484,0715884	1,49816E-05	0,001443	0,018251	0,111817
ENSG00000183258.12	chr5	1,78E+08	1,78E+08	-	protein_coding	DDX41	1739,131544	-2,63464E-05	0,001443	0,035373	0,160538
ENSG00000196419.13	chr22	41621163	41664048	+	protein_coding	XRCC6	6470,124729	-2,72187E-05	0,001443	0,023853	0,130647
ENSG00000253729.8	chr8	47773111	47960178	-	protein_coding	PRKDC	1715,073353	-1,84165E-05	0,001443	0,998523	0,999143
ENSG00000006062.18	chr17	45263119	45317029	-	protein_coding	MAP3K14	1297,368349	-1,89106E-05	0,001443	0,091944	0,276378
ENSG00000077150.20	chr10	1,02E+08	1,02E+08	+	protein_coding	NFKB2	2188,738241	-5,74245E-08	0,001443	0,63381	0,801123
ENSG00000100906.11	chr14	35401513	35404749	-	protein_coding	NFKBIA	16208,23049	1,07019E-05	0,001443	0,294895	0,535513
ENSG00000104365.16	chr8	42271302	42332460	+	protein_coding	IKBKB	1809,609189	0,372578798	0,132203	0,000153	0,006117
ENSG00000104825.17	chr19	38899700	38908893	+	protein_coding	NFKBIB	590,462625	-6,34771E-05	0,001443	0,193847	0,423516
ENSG00000104856.14	chr19	45001449	45038198	+	protein_coding	RELB	1284,988939	-1,27278E-05	0,001443	0,012241	0,088112
ENSG00000109320.13	chr4	1,03E+08	1,03E+08	+	protein_coding	NFKB1	1915,449148	2,61177E-05	0,001443	0,47093	0,685384
ENSG00000126456.16	chr19	49659569	49665875	-	protein_coding	IRF3	1163,873769	1,5276E-05	0,001443	0,1733	0,398018
ENSG00000146232.17	chr6	44258166	44265788	-	protein_coding	NFKBIE	1317,863443	-7,56187E-06	0,001443	0,020742	0,120884
ENSG00000162924.15	chr2	60881521	60931612	+	protein_coding	REL	3031,500953	2,46729E-05	0,001443	0,256305	0,495774
ENSG00000168036.18	chr3	41194741	41260096	+	protein_coding	CTNNB1	5177,427772	-5,1068E-06	0,001443	0,230733	0,468551
ENSG00000173039.19	chr11	65653597	65663090	-	protein_coding	RELA	2284,57391	-8,62225E-06	0,001443	0,370764	0,60457
ENSG00000183735.11	chr12	64452090	64502114	+	protein_coding	TBK1	947,6734532	-4,54346E-06	0,001443	0,672545	0,826874
ENSG00000184584.13	chr5	1,39E+08	1,39E+08	-	protein_coding	STING1	2921,65171	6,11676E-06	0,001443	0,522489	0,725813
ENSG00000213341.11	chr10	1E+08	1E+08	-	protein_coding	CHUK	759,4545886	0,236005611	0,12418	0,001333	0,023225
ENSG00000263528.8	chr1	2,06E+08	2,06E+08	+	protein_coding	IKBKE	1054,010265	-7,1935E-06	0,001443	0,462307	0,678936
ENSG00000269335.6	chrX	1,55E+08	1,55E+08	+	protein_coding	IKBKG	1081,935088	8,9776E-06	0,001442	0,280046	0,520722
ENSG00000084693.16	chr2	27042364	27070622	+	protein_coding	AGBL5	155,5433091	-5,52651E-06	0,001443	0,283688	0,525131

Supplementary Table 2 (continued). Differential Gene Expression pSS vs HC.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000090432.7	chr1	20499448	20508151	-	protein_coding	MUL1	594,4805953	-1,91594E-05	0,001443	0,043153	0,180357
ENSG00000108443.14	chr17	59893046	59950574	+	protein_coding	RPS6KB1	699,0111487	-3,17825E-06	0,001443	0,783276	0,889577
ENSG00000112343.11	chr6	25962802	25991231	+	protein_coding	TRIM38	2119,798342	0,362506806	0,108151	2,77E-05	0,001939
ENSG00000129667.13	chr17	76470891	76501790	-	protein_coding	RHBDF2	2282,040695	-4,95294E-05	0,001443	0,032622	0,154036
ENSG00000136878.14	chr9	1,3E+08	1,3E+08	+	protein_coding	USP20	502,1980883	7,63464E-06	0,001443	0,546809	0,744848
ENSG00000138468.16	chr3	1,01E+08	1,02E+08	-	protein_coding	SENP7	657,8221615	0,288387439	0,104673	0,000186	0,006904
ENSG00000138942.16	chr22	31160182	31207019	+	protein_coding	RNF185	799,8755905	-8,45156E-07	0,001443	0,927189	0,965198
ENSG00000159461.15	chr16	56361452	56425545	-	protein_coding	AMFR	2336,194129	-1,24171E-05	0,001443	0,079676	0,254649
ENSG00000169871.13	chr7	1,01E+08	1,01E+08	+	protein_coding	TRIM56	824,7413353	0,816637213	0,210664	3,72E-06	0,000451
ENSG00000173456.5	chr11	1,19E+08	1,19E+08	+	protein_coding	RNF26	614,7368597	-0,178527243	0,116463	0,002383	0,032812
ENSG00000184979.10	chr22	18150170	18177397	+	protein_coding	USP18	286,0740911	2,426160172	0,583075	1,12E-06	0,000196
ENSG00000186094.17	chr1	48532854	50023954	-	protein_coding	AGBL4	0,225017328	-5,78796E-08	0,001443	0,923799	
ENSG00000186480.13	chr7	1,55E+08	1,55E+08	+	protein_coding	INSIG1	1635,702857	6,72749E-05	0,001443	0,505788	0,712473
ENSG00000083290.20	chr17	19770829	19867936	-	protein_coding	ULK2	1170,989476	4,93621E-05	0,001443	0,825348	0,913169
ENSG00000105612.9	chr19	12875209	12881466	-	protein_coding	DNASE2	1308,64197	0,000148333	0,001446	0,04934	0,195484
ENSG00000119401.11	chr9	1,17E+08	1,17E+08	+	protein_coding	TRIM32	25,07970683	4,31036E-06	0,001443	0,065123	0,224708
ENSG00000135912.11	chr2	2,19E+08	2,19E+08	+	protein_coding	TTLL4	1004,14573	2,4424E-05	0,001443	0,016072	0,103624
ENSG00000137976.8	chr1	84398484	84415018	+	protein_coding	DNASE2B	0,043542273	1,61646E-07	0,001443	0,948983	
ENSG00000142208.17	chr14	1,05E+08	1,05E+08	-	protein_coding	AKT1	4074,607666	-8,50689E-05	0,001444	0,061234	0,218808
ENSG00000163904.13	chr3	1,86E+08	1,86E+08	+	protein_coding	SENP2	610,250186	-7,43427E-06	0,001443	0,839165	0,920711
ENSG00000167323.12	chr11	3854527	4093210	+	protein_coding	STIM1	2042,106922	-1,38892E-05	0,001442	0,372539	0,606556
ENSG00000170703.15	chr17	48762235	48817214	-	protein_coding	TTLL6	0				
ENSG00000177169.10	chr12	1,32E+08	1,32E+08	+	protein_coding	ULK1	2126,026209	6,73759E-06	0,001443	0,626681	0,796776
ENSG00000197594.13	chr6	1,32E+08	1,32E+08	+	protein_coding	ENPP1	0,047822805	1,79808E-07	0,001443	0,946431	
ENSG00000198925.12	chr2	2,19E+08	2,19E+08	-	protein_coding	ATG9A	996,254262	6,31166E-06	0,001443	0,377652	0,610013
ENSG00000204308.8	chr6	32178405	32180793	+	protein_coding	RNF5	609,784736	5,68091E-06	0,001443	0,506051	0,712647
ENSG00000213689.14	chr3	48465811	48467645	+	protein_coding	TREX1	1018,300166	1,18872E-05	0,001443	0,238279	0,476511
ENSG00000119457.8	chr9	1,13E+08	1,13E+08	-	protein_coding	SLC46A2	850,7676547	6,21316E-06	0,001443	0,261304	0,50192

Supplementary Table 2 (continued). Differential Gene Expression pSS vs HC.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000136802.12	chr9	1,29E+08	1,29E+08	+	protein_coding	LRRC8A	613,0314603	-0,42117982	0,156133	0,000199	0,00717
ENSG00000171488.15	chr1	89633072	89769903	+	protein_coding	LRRC8C	1207,39908	2,07283E-05	0,001443	0,009184	0,074996
ENSG00000173638.19	chr21	45493572	45573365	-	protein_coding	SLC19A1	949,6401683	-1,24286E-05	0,001442	0,590701	0,774813

Supplementary Table 3. Differential Gene Expression IFN-high vs IFN-low pSS.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000020922.13	chr11	94415570	94493885	-	protein_coding	MRE11	647,7922925	7,5863E-06	0,001443	0,341899	0,756836
ENSG00000079246.16	chr2	2,16E+08	2,16E+08	+	protein_coding	XRCC5	6624,317833	-4,60125E-06	0,001443	0,624937	0,895053
ENSG00000124256.15	chr20	57603846	57620576	-	protein_coding	ZBP1	422,4800067	1,194527557	0,375784	3,62E-05	0,009951
ENSG00000124831.19	chr2	2,38E+08	2,38E+08	+	protein_coding	LRRFIP1	9011,060755	7,06219E-06	0,001443	0,201574	0,638644
ENSG00000163565.19	chr1	1,59E+08	1,59E+08	+	protein_coding	IFI16	5251,122761	-1,42787E-06	0,001443	0,131614	0,544
ENSG00000163568.15	chr1	1,59E+08	1,59E+08	-	protein_coding	AIM2	136,4031334	5,73737E-06	0,001443	0,735068	0,930387
ENSG00000164430.17	chr6	73413515	73452297	-	protein_coding	CGAS	564,7144171	7,16097E-07	0,001443	0,814029	0,952788
ENSG00000183258.12	chr5	1,78E+08	1,78E+08	-	protein_coding	DDX41	1703,838245	-1,61703E-05	0,001443	0,190759	0,628669
ENSG00000196419.13	chr22	41621163	41664048	+	protein_coding	XRCC6	6199,261627	9,91458E-05	0,001444	0,206023	0,643223
ENSG00000253729.8	chr8	47773111	47960178	-	protein_coding	PRKDC	1779,918785	0,467346348	0,209804	0,000599	0,066136
ENSG00000006062.18	chr17	45263119	45317029	-	protein_coding	MAP3K14	1252,202074	4,02589E-06	0,001443	0,609985	0,890086
ENSG00000077150.20	chr10	1,02E+08	1,02E+08	+	protein_coding	NFKB2	2338,307286	-5,59014E-07	0,001443	0,380013	0,773908
ENSG00000100906.11	chr14	35401513	35404749	-	protein_coding	NFKBIA	14225,51471	-0,000122585	0,001445	0,986516	0,997432
ENSG00000104365.16	chr8	42271302	42332460	+	protein_coding	IKBKB	2094,675289	-1,84357E-05	0,001443	0,362289	0,766022
ENSG00000104825.17	chr19	38899700	38908893	+	protein_coding	NFKBIB	561,0282989	1,35505E-07	0,001443	0,796939	0,947178
ENSG00000104856.14	chr19	45001449	45038198	+	protein_coding	RELB	1112,761632	-2,65847E-07	0,001443	0,879509	0,970347
ENSG00000109320.13	chr4	1,03E+08	1,03E+08	+	protein_coding	NFKB1	2032,290991	8,15031E-06	0,001443	0,151433	0,577339
ENSG00000126456.16	chr19	49659569	49665875	-	protein_coding	IRF3	1266,542784	-4,99101E-06	0,001443	0,530007	0,854194
ENSG00000146232.17	chr6	44258166	44265788	-	protein_coding	NFKBIE	1121,674219	3,36979E-06	0,001443	0,314334	0,73814
ENSG00000162924.15	chr2	60881521	60931612	+	protein_coding	REL	2855,22721	5,17161E-06	0,001443	0,107316	0,511603
ENSG00000168036.18	chr3	41194741	41260096	+	protein_coding	CTNNB1	4770,18477	-2,35126E-05	0,001443	0,614922	0,891537
ENSG00000173039.19	chr11	65653597	65663090	-	protein_coding	RELA	2277,493685	2,59537E-06	0,001443	0,847652	0,96188
ENSG00000183735.11	chr12	64452090	64502114	+	protein_coding	TBK1	986,5973276	-1,55062E-05	0,001443	0,143507	0,563038
ENSG00000184584.13	chr5	1,39E+08	1,39E+08	-	protein_coding	STING1	3066,662531	8,35592E-06	0,001443	0,480888	0,833209
ENSG00000213341.11	chr10	1E+08	1E+08	-	protein_coding	CHUK	857,70421	9,3458E-07	0,001443	0,558268	0,867924
ENSG00000263528.8	chr1	2,06E+08	2,06E+08	+	protein_coding	IKBKE	1130,729454	1,07816E-05	0,001443	0,055316	0,409813
ENSG00000269335.6	chrX	1,55E+08	1,55E+08	+	protein_coding	IKBKG	1135,462348	-3,25889E-06	0,001443	0,876514	0,97012
ENSG00000084693.16	chr2	27042364	27070622	+	protein_coding	AGBL5	144,7839793	-6,28937E-06	0,001443	0,105656	0,508693

Supplementary Table 3 (continued). Differential Gene Expression IFN-high vs IFN-low pSS.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000090432.7	chr1	20499448	20508151	-	protein_coding	MUL1	563,0620265	-8,60325E-06	0,001443	0,223585	0,66087
ENSG00000108443.14	chr17	59893046	59950574	+	protein_coding	RPS6KB1	732,3753237	3,29551E-06	0,001443	0,296862	0,72613
ENSG00000112343.11	chr6	25962802	25991231	+	protein_coding	TRIM38	2465,830495	1,15981E-05	0,001443	0,014728	0,282809
ENSG00000129667.13	chr17	76470891	76501790	-	protein_coding	RHBDF2	2531,729929	-8,10259E-06	0,001443	0,511911	0,847301
ENSG00000136878.14	chr9	1,3E+08	1,3E+08	+	protein_coding	USP20	521,6823428	5,15689E-06	0,001443	0,522149	0,851889
ENSG00000138468.16	chr3	1,01E+08	1,02E+08	-	protein_coding	SENP7	752,8875525	1,04172E-05	0,001443	0,163337	0,592753
ENSG00000138942.16	chr22	31160182	31207019	+	protein_coding	RNF185	814,2707127	3,64052E-05	0,001443	0,704691	0,922634
ENSG00000159461.15	chr16	56361452	56425545	-	protein_coding	AMFR	2191,579917	3,10472E-06	0,001443	0,585068	0,880293
ENSG00000169871.13	chr7	1,01E+08	1,01E+08	+	protein_coding	TRIM56	1073,843174	5,18611E-06	0,001443	0,178137	0,611455
ENSG00000173456.5	chr11	1,19E+08	1,19E+08	+	protein_coding	RNF26	574,3410978	7,12754E-06	0,001443	0,493255	0,838709
ENSG00000184979.10	chr22	18150170	18177397	+	protein_coding	USP18	489,685306	3,328193387	0,455354	5,86E-15	8,04E-11
ENSG00000186094.17	chr1	48532854	50023954	-	protein_coding	AGBL4	0,200164491	1,43194E-07	0,001443	0,885675	
ENSG00000186480.13	chr7	1,55E+08	1,55E+08	+	protein_coding	INSIG1	1777,945213	5,25598E-06	0,001443	0,043294	0,380434
ENSG00000083290.20	chr17	19770829	19867936	-	protein_coding	ULK2	1178,393794	1,24132E-05	0,001443	0,017005	0,298413
ENSG00000105612.9	chr19	12875209	12881466	-	protein_coding	DNASE2	1237,574072	8,3184E-06	0,001443	0,265551	0,701685
ENSG00000119401.11	chr9	1,17E+08	1,17E+08	+	protein_coding	TRIM32	31,25405291	-1,52717E-07	0,001443	0,931353	0,981098
ENSG00000135912.11	chr2	2,19E+08	2,19E+08	+	protein_coding	TTLL4	1087,511359	-4,59167E-05	0,001443	0,08843	0,480771
ENSG00000137976.8	chr1	84398484	84415018	+	protein_coding	DNASE2B	0,084404628	-2,45475E-07	0,001443	0,80794	
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ENSG00000163904.13	chr3	1,86E+08	1,86E+08	+	protein_coding	SENP2	623,7988976	8,62519E-06	0,001443	0,054227	0,407029
ENSG00000167323.12	chr11	3854527	4093210	+	protein_coding	STIM1	2026,437765	-2,49894E-05	0,001443	0,068296	0,440541
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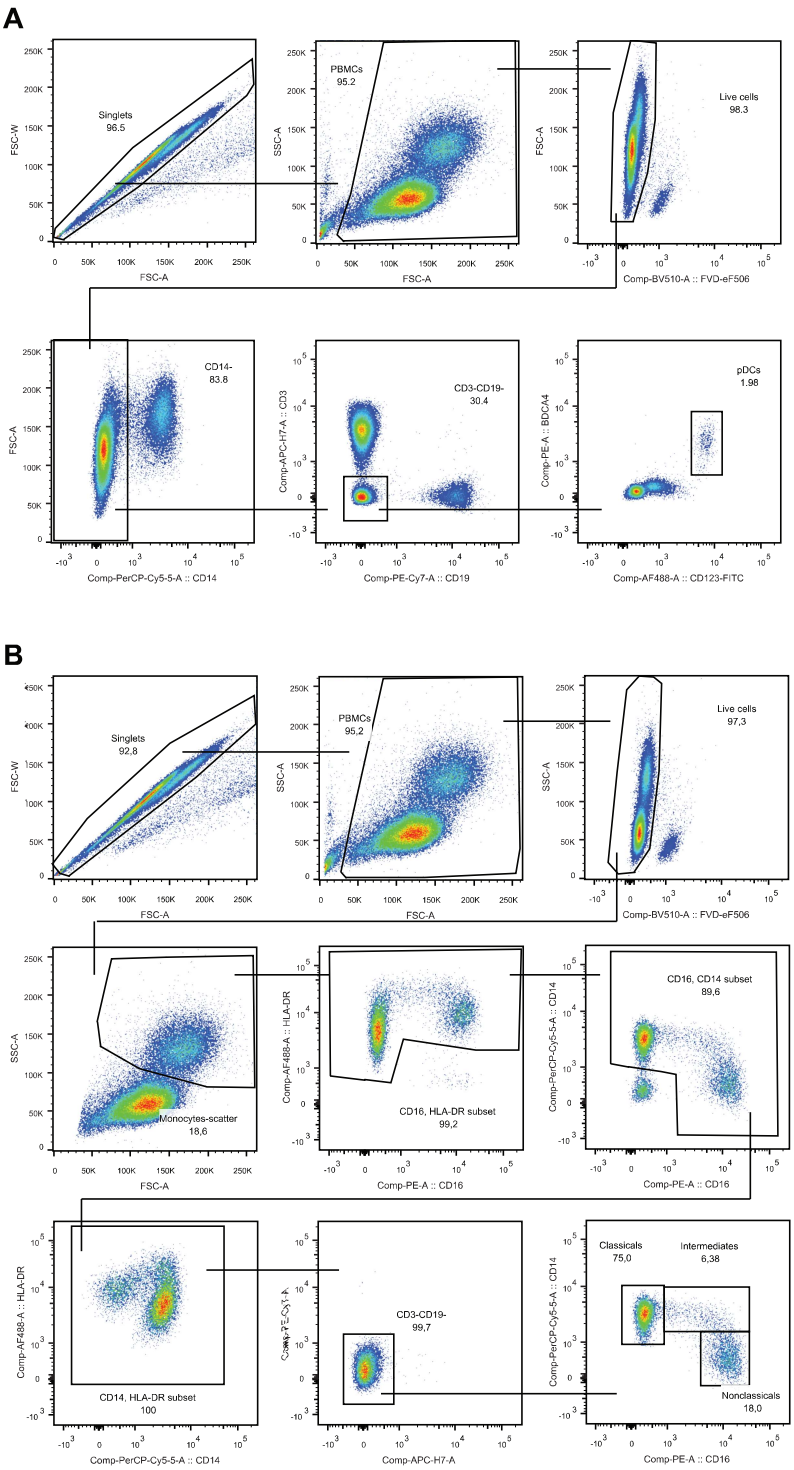
ENSG00000197594.13	chr6	1,32E+08	1,32E+08	+	protein_coding	ENPP1	0,100138686	-1,4018E-07	0,001443	0,868642	
ENSG00000198925.12	chr2	2,19E+08	2,19E+08	-	protein_coding	ATG9A	1059,236289	2,00863E-06	0,001443	0,686995	0,919196
ENSG00000204308.8	chr6	32178405	32180793	+	protein_coding	RNF5	637,6450744	-6,93916E-06	0,001443	0,207639	0,644468
ENSG00000213689.14	chr3	48465811	48467645	+	protein_coding	TREX1	1083,072638	2,28653E-06	0,001443	0,679817	0,916002
ENSG00000119457.8	chr9	1,13E+08	1,13E+08	-	protein_coding	SLC46A2	952,7055824	-1,4914E-05	0,001443	0,067361	0,43917

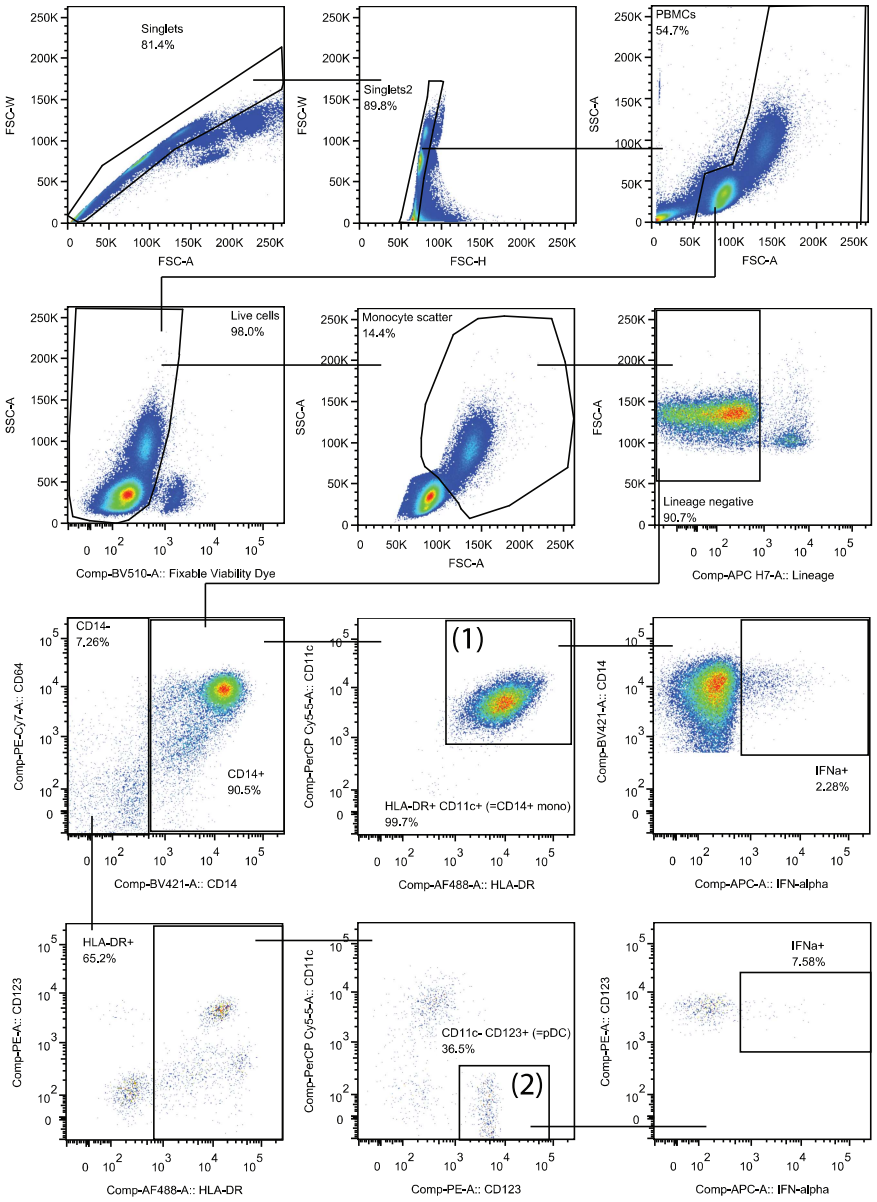
Supplementary Table 3 (continued). Differential Gene Expression IFN-high vs IFN-low pSS.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000136802.12	chr9	1,29E+08	1,29E+08	+	protein_coding	LRRC8A	529,2369245	-5,94498E-06	0,001443	0,744631	0,933401
ENSG00000171488.15	chr1	89633072	89769903	+	protein_coding	LRRC8C	1388,317044	4,97672E-06	0,001443	0,23985	0,676276
ENSG00000173638.19	chr21	45493572	45573365	-	protein_coding	SLC19A1	982,5479066	-0,000136658	0,001446	0,818831	0,954203



Supplementary Figure 1. Gating strategy for analysis of protein expression in monocytes and plasmacytoid dendritic cells.

Gating strategy for identification of live (A) $CD3^-CD19^-CD14^-CD123^+$ $BDCA4^+$ pDCs (tube 1) and (B) live $CD3^-$ $CD19^-$ $HLA\text{-}DR^+$ $CD16^-CD14^+$ classical monocytes (tube 2) in *ex vivo* PBMCs.

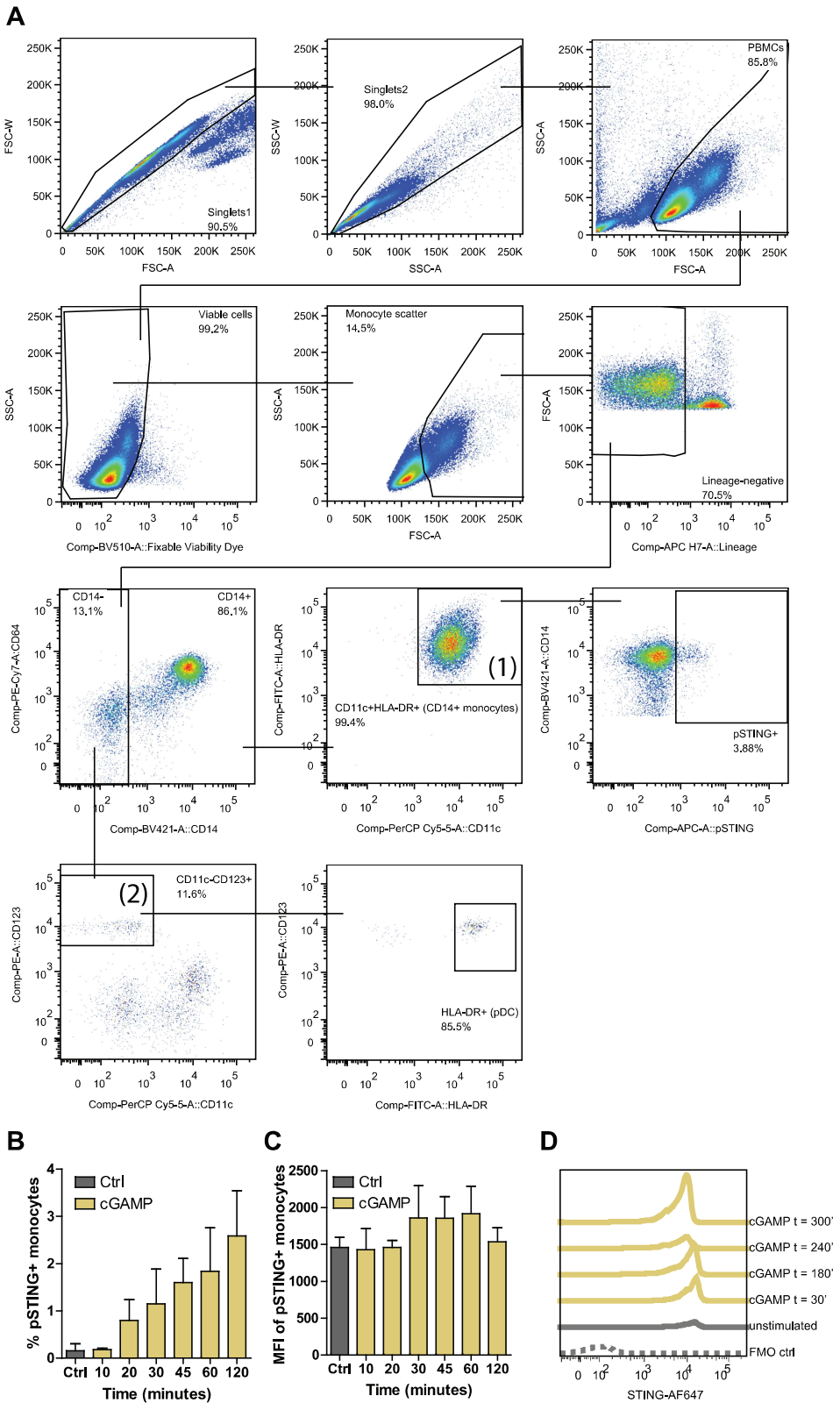


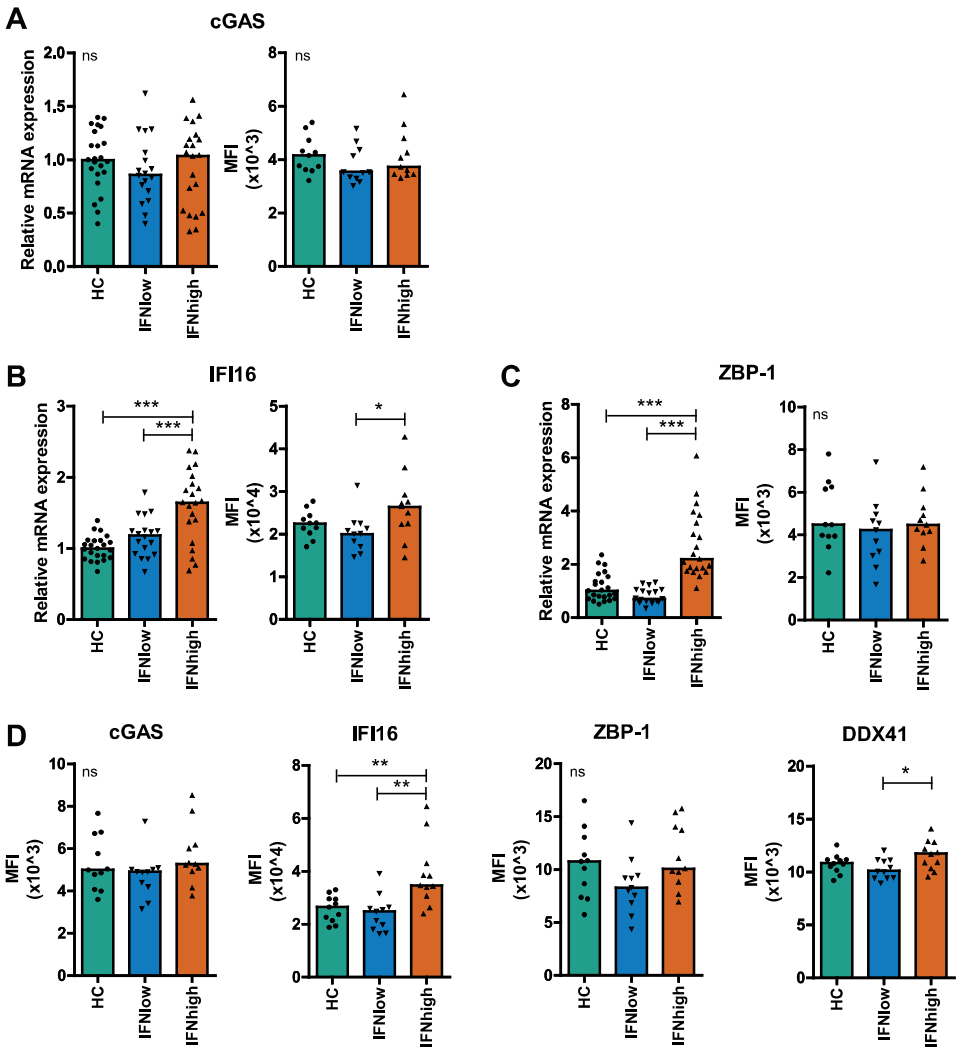
Supplementary Figure 2. Gating strategy for analysis of intracellular IFNα in monocytes and plasmacytoid dendritic cells.

Gating strategy for identification of live (1) Lineage$^-$ HLA-DR$^+$ CD11c$^+$ CD14$^+$ monocytes and (2) Lineage$^-$ CD14$^-$ CD11c$^-$ HLA-DR$^+$ CD123$^+$ pDCs in 6 hour PBMC cultures.

Supplementary Figure 3 (next page). cGAMP-induced STING phosphorylation in monocytes maximum at 30-60 minutes.

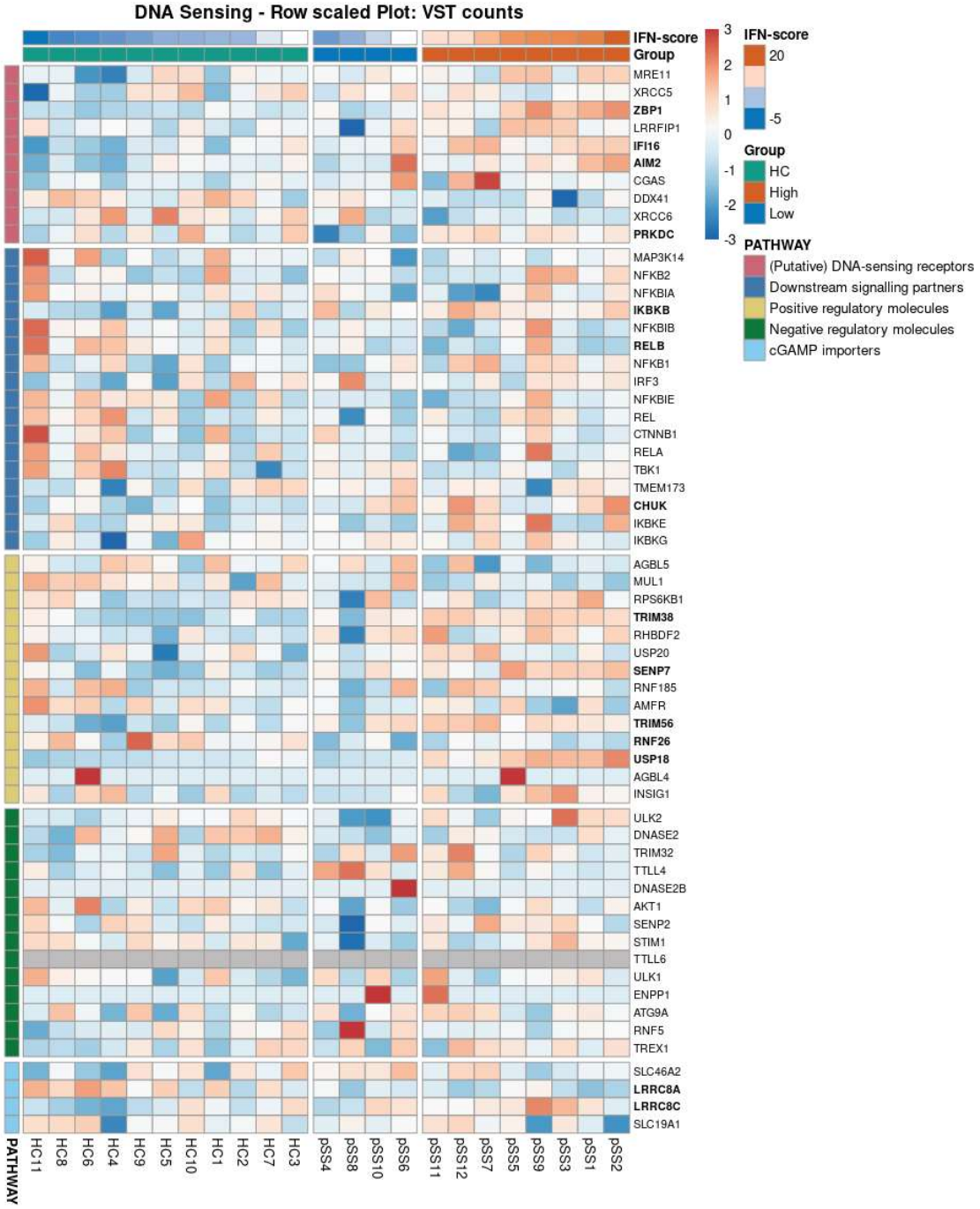
(A) Gating strategy for pSTING analysis in live (1) Lineage$^-$ CD14$^-$ CD64$^-$ CD11c$^-$ HLA-DR$^+$ CD123$^+$ pDCs and (2) Lineage$^-$ HLA-DR$^+$ CD11c$^+$ CD14$^+$ monocytes in PBMC cultures. **(B)** Frequency of pSTING+ monocytes of total monocytes, **(C)** median fluorescence intensity (MFI) of pSTING+ monocytes, and **(D)** histogram of total STING in monocytes at various time points after PBMC stimulation with 25 µg/mL 2'3'-cGAMP.


A
Singlets1
90.5%
Singlets2
98.0%
PBMCs
85.8%
Viable cells
99.2%
Monocyte scatter
14.5%
Lineage-negative
70.5%
CD14-
13.1%
CD14+
86.1%
(1)
CD11c+HLA-DR+ (CD14+ monocytes)
99.4%
pSTING+
3.88%
(2)
CD11c-CD123+
11.6%
HLA-DR+ (pDC)
85.5%
B
% pSTING+ monocytes
Ctrl
cGAMP
Time (minutes)
C
MFI of pSTING+ monocytes
D
cGAMP t = 300'
cGAMP t = 240'
cGAMP t = 180'
cGAMP t = 30'
unstimulated
FMO ctrl
STING-AF647



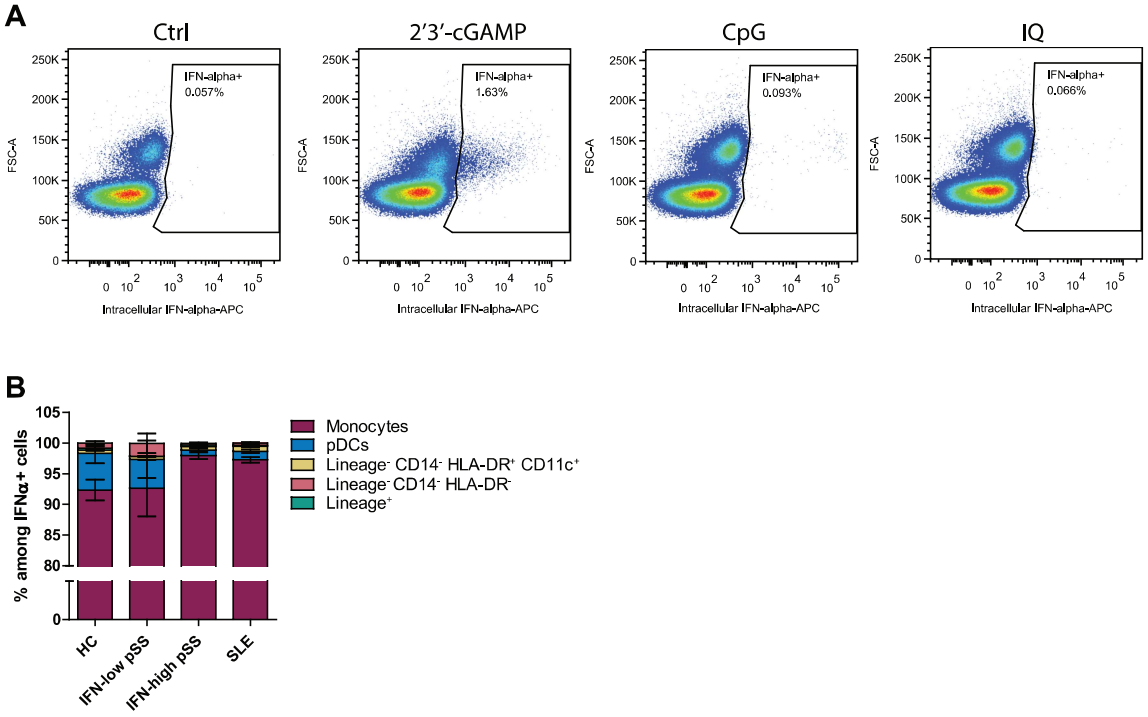
Supplementary Figure 4. Monocytes and pDCs from IFN-high pSS express higher IFI16.

Relative mRNA expression measured by RT-PCR (left) and median fluorescence intensity (right) of DNA-sensing receptors **(A)** cGAS, **(B)** IFI16, and **(C)** ZBP-1 in CD14+ monocytes from IFN-low and IFN-high pSS patients and HC. RT-PCR or flow cytometry was performed in different samples. **(D)** Median fluorescence intensity (MFI) of cGAS, IFI16, ZBP-1 and DDX41 in pDCs from IFN-low and IFN-high pSS patients and HC. Bars represent medians. Depending on the data distribution, One-way ANOVA + Tukey's HSD test or Kruskal-Wallis H + Dunn's Multiple Comparison test was used to compare means/medians between groups. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ns = not significant.



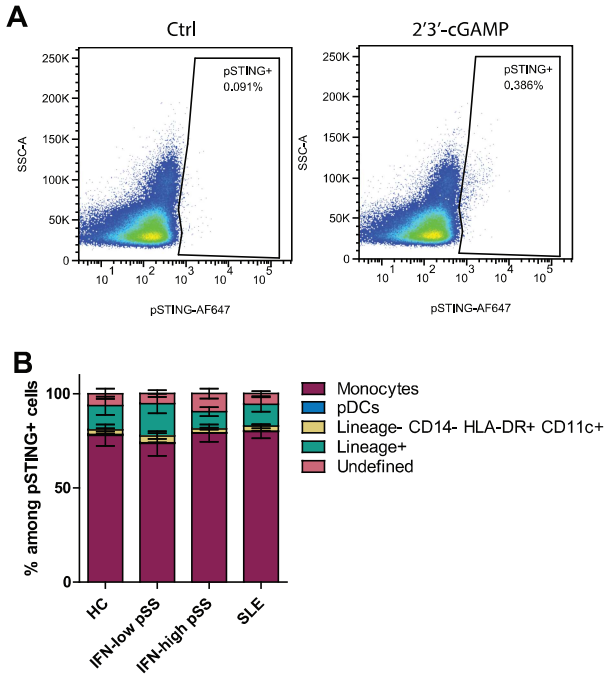
Supplementary Figure 5. Differential gene expression analysis of DNA-sensing receptors and STING regulators in pSS monocytes.

Heatmap of z-scores of DNA-sensing pathway related genes in RNAseq dataset (GSE173670) of *ex vivo* monocytes from pSS and HC. Patients were stratified based on their IFN-I score. Bold genes indicate differential expression in comparison analysis of either pSS versus HC or IFN-high vs IFN-low (details provided in Supplementary Table S2 and S3). TTLL6 was not expressed in any of the samples: indicated in grey.



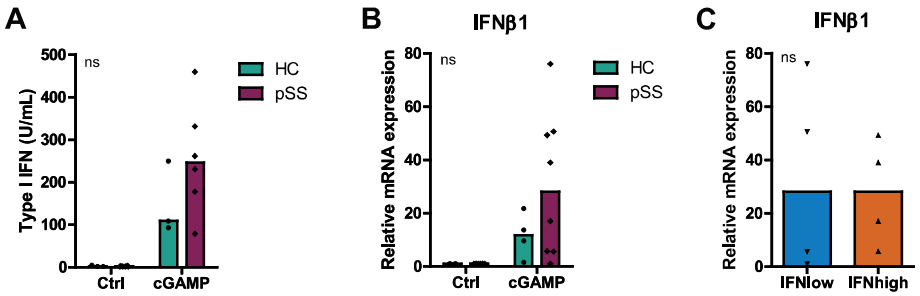
Supplementary Figure 6. Monocytes and pDCs produce IFNα upon 2'3'-cGAMP stimulation.

(A) Representative plot of intracellular IFNα staining in PBMCs (shown plot from FN-high pSS) after 6 hours of stimulation with 25 µg/mL 2'3'-cGAMP, 10 µg/mL CpG, or 5 µg/mL imiquimod (IQ) and 1 µg/mL GolgiPlug during the last 3 hours. **(B)** Distribution of cell types among IFNα-positive PBMCs from pSS, SLE or HC after 6 hour-stimulation with 25 µg/mL 2'3'-cGAMP. Bars represent mean ± SEM of n=8 observations.



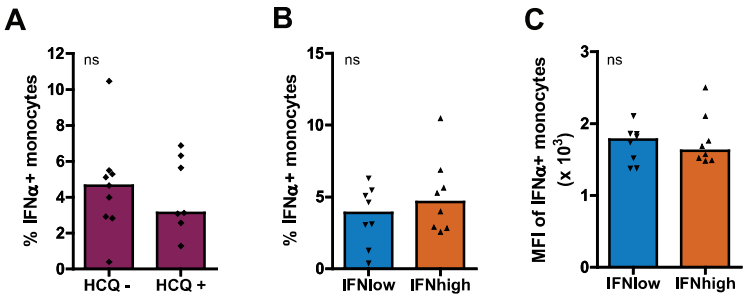
Supplementary Figure 7. Stimulation with 2'3'-cGAMP induces phosphorylation of STING in monocytes.

(A) Representative plot of pSTING in PBMCs after 45 minutes of stimulation with 25 µg/mL 2'3'-cGAMP. **(B)** Distribution of cell types among pSTING-positive PBMCs from pSS, SLE or HC after 45 minutes of stimulation with 25 µg/mL 2'3'-cGAMP. Bars represent median percentages of n=7-8 (SLE, HC) or n=16 (pSS) observations.



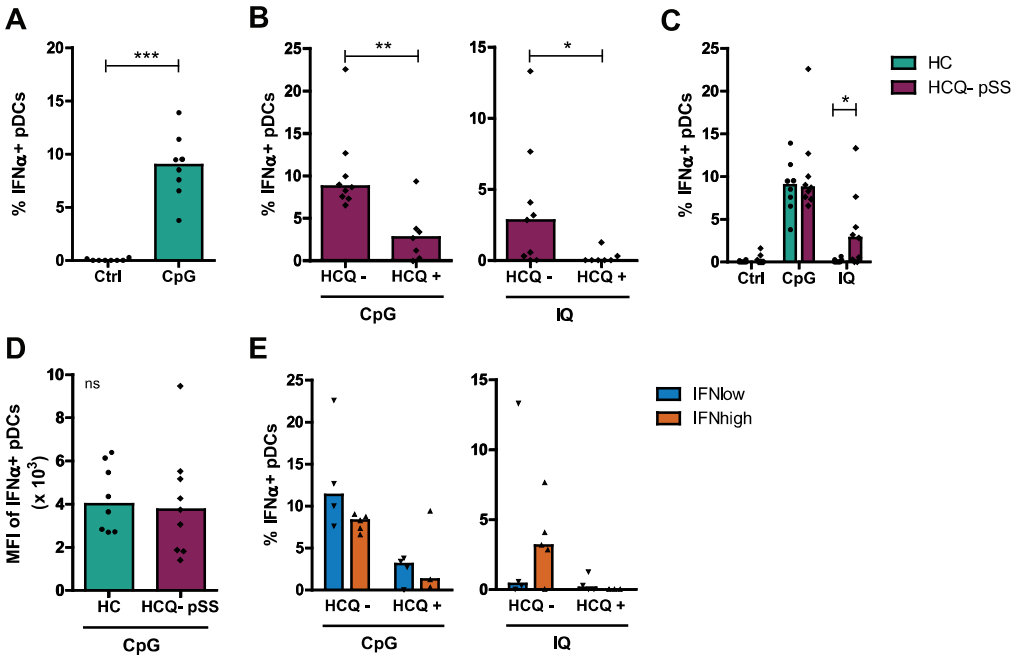
Supplementary Figure 8. Elevated IFN-I secretion by PBMCs from pSS patients upon 2'3'-cGAMP stimulation.

(A) Bioactive IFN-I in supernatants quantified by IFN-I reporter assay and **(B)** relative IFNβ1 transcript expression in PBMCs from HC and patients with pSS **(C)** stratified on IFN-I score after 24 hours of stimulation with 3.6 µg/mL of 2'3'-cGAMP. Bars represent medians. Independent-samples t-test was used to compare means between groups. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ns = not significant.



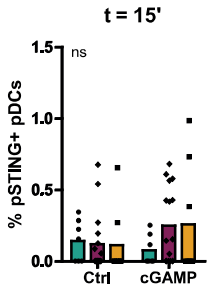
Supplementary Figure 9. Monocyte IFNα response is independent from use of HCQ or IFN-I score.

Proportion of IFNα-producing monocytes of total monocytes or median fluorescence intensity of IFNα+ monocytes in pSS PBMC cultures stimulated with 25 µg/mL 2'3'-cGAMP for 6 hours stratified based on **(A)** use of HCQ or **(B)** IFN-I score. Depending on the data distribution, independent-samples t-test or Mann-Whitney U test was used to compare medians/means between groups. ns = not significant.



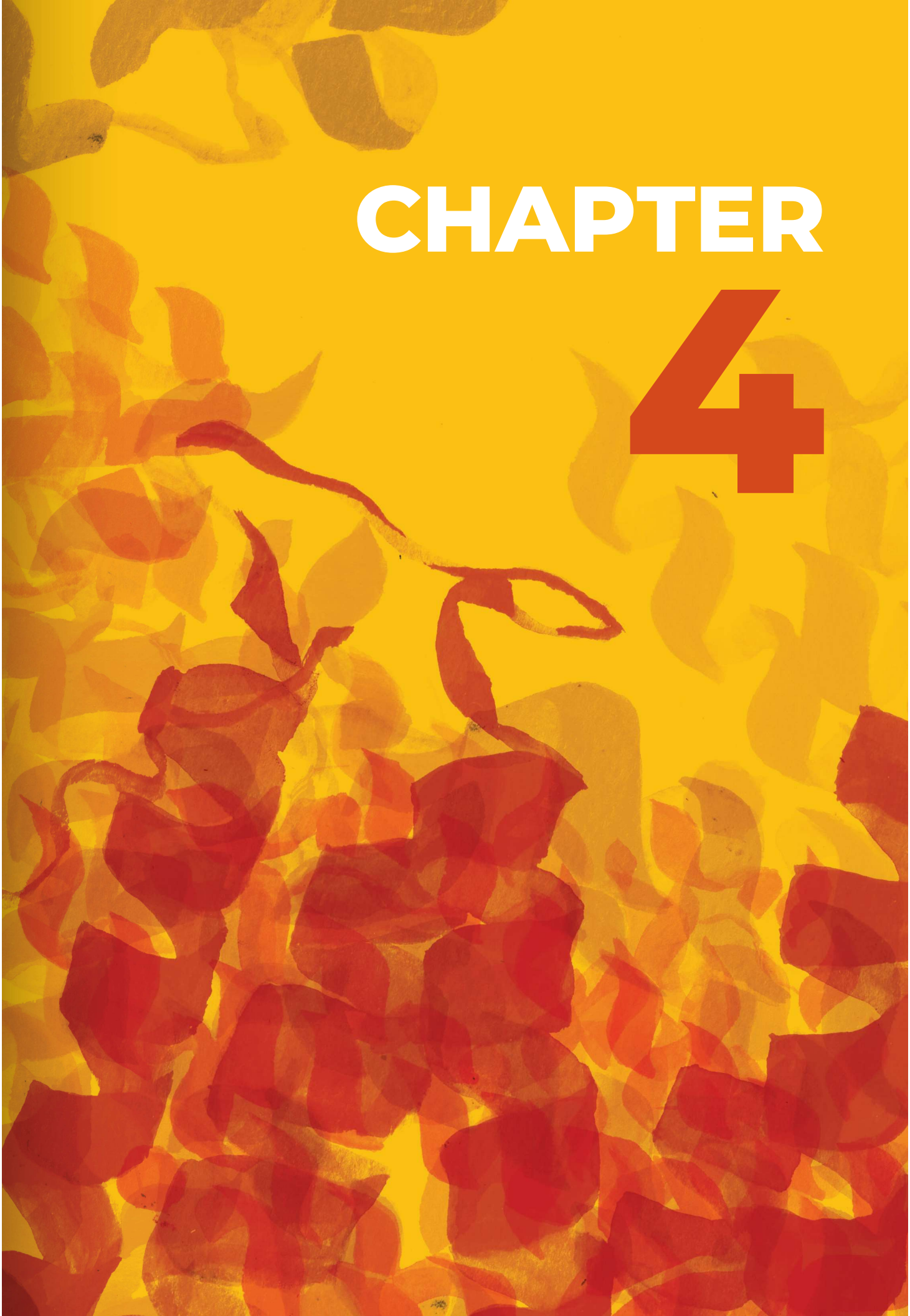
Supplementary Figure 10. Endosomal TLR-induced IFNα response by pDCs is strongly reduced in pSS patients using HCQ treatment.

(A) Frequency of IFNα+ pDCs of total pDCs in 10 µg/mL CpG-stimulated HC PBMC cultures. **(B)** Frequency of IFNα + pDCs of total pDCs in 10 µg/mL CpG- or 5 µg/mL IQ-stimulated pSS PBMCs stratified based on patient's use of HCQ. **(C)** Frequency of IFNα+ pDCs of total pDCs, or **(D)** median fluorescence intensity of IFNα+ pDCs in CpG- or IQ-stimulated PBMCs of pSS patients not using HCQ treatment and HC. **(E)** Frequency of IFNα+ pDCs of total pDCs in CpG- or IQ-stimulated pSS PBMC cultures stratified based on patient's IFN-I score and grouped by HCQ treatment. Bars represent medians. Depending on the data distribution, independent-samples t-test or Mann-Whitney U test was used to compare medians/means between groups. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ns = not significant.



Supplementary Figure 11. Plasmacytoid dendritic cells from pSS and SLE phosphorylate STING upon cGAMP stimulation.

Frequency of pSTING+ pDCs of total pDCs in 25 µg/mL 2'3'-cGAMP-stimulated PBMC cultures from patients with pSS, SLE or HC at t=15 minutes. Symbols represent individual samples and bars indicate means. ns; not significant.


CHAPTER
4

CHAPTER 4

Trained immunity and pro-atherogenic phenotype induced by type I interferons: potential implications for primary Sjögren's syndrome

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Frontiers in Immunology; under review

ABSTRACT

Background: Trained immunity – or innate immune memory – can be described as the long-term reprogramming of innate immune cells towards a hyperresponsive state which involves intracellular metabolic changes. Trained immunity has been linked to atherosclerosis. A subgroup of patients with primary Sjögren's syndrome (pSS) exhibits systemic type I interferon (IFN) pathway activation, indicating innate immune hyperactivation. Here, we studied the link between type I IFNs and trained immunity in an *in vitro* monocytic cell model and peripheral blood mononuclear cells (PBMCs) from pSS patients.

Methods: The training stimuli heat killed *Candida albicans*, muramyl dipeptide, IFNβ, and patient serum were added to THP-1 cells for 24 hours, after which the cells were washed, rested for 48 hours and subsequently re-stimulated with LPS, Pam3Cys, poly I:C, IFNβ or oxLDL for 4-24 hours. PBMCs from pSS patients and healthy controls were stimulated with LPS, Pam3Cys, poly I:C or IFNβ for 0.5-24 hours.

Results: Training with IFNβ induced elevated production of pro-atherogenic cytokines IL-6, TNFα and *CCL2*, differential cholesterol- and glycolysis-related gene expression, and increased glucose consumption and oxLDL uptake upon re-stimulation. Type I IFN production was increased in *Candida albicans*- and IFNβ-trained cells after LPS re-stimulation, but was reduced after poly I:C re-stimulation. Training with muramyl dipeptide and IFNβ, but not *Candida albicans*, affected the IFN-stimulated gene expression response to IFNβ re-stimulation. PBMCs from pSS patients consumed more glucose compared with healthy control PBMCs and tended to produce more TNFα and type I IFNs upon LPS stimulation, but less type I IFNs upon poly I:C stimulation.

Conclusions: Type I IFN is a trainer inducing a pro-atherogenic trained immunity phenotype in monocytes. Conversely, trained immunity also affects the production of type I IFNs and transcriptional response to type I IFN receptor re-stimulation. The phenotype of pSS PBMCs is consistent with trained immunity. This connection between type I IFN, trained immunity and cholesterol metabolism may have important implications for pSS and the pathogenesis of (subclinical) atherosclerosis in these patients.

INTRODUCTION

Trained immunity describes the ability of innate immune cells to mount increased responses to re-stimulation after initial exposure to inflammatory stimuli [1]. For instance, cell-wall polysaccharide β-glucan from *Candida albicans* is a well-described trainer that confers elevated cytokine responses in macrophages when these are re-stimulated with a secondary non-related stimulus such as LPS [2]. This hyperresponsive phenotype is long-lasting and is orchestrated by cellular metabolic and epigenetic reprogramming [2, 3]. The characteristics of innate immune memory have been demonstrated both *in vitro* and *in vivo* [1, 4, 5]. Trained immunity contributes to inflammatory processes and has been linked to human diseases, including atherosclerosis, and could potentially have immunopathobiological relevance in systemic autoimmune diseases [1, 6, 7].

The chronic rheumatic autoimmune disease primary Sjögren's syndrome (pSS) is typified by mononuclear cell infiltration in the salivary glands and symptoms of oral and ocular dryness [8]. Patients may additionally experience a diversity of extraglandular disease manifestations which can cause substantial morbidity [9]. Patients with pSS appear to exhibit accelerated (subclinical) atherosclerosis and other cardiovascular risk factors, which is also seen in other autoimmune rheumatic diseases, including systemic lupus erythematosus (SLE) [10-13].

Stronger cytokine responses by innate immune cells from pSS patients compared with healthy controls (HC) have been reported upon stimulation with diverse inflammatory stimuli [14-18]. These observations accentuate a hyperresponsive phenotype of innate immune cells in pSS. Innate immune hyperactivation in pSS is further exemplified by persistent systemic activation of the type I interferon (IFN) pathway in the majority of patients [19, 20]. This latter phenomenon is shared with related systemic autoimmune diseases, such as SLE [21]. All together, these features of innate immune hyperactivity are suggestive of trained immunity in pSS.

Type I IFNs are a family of cytokines with potent immunomodulatory properties. Recent studies have suggested that immunomodulatory effects of type I IFNs involve histone modifications of inflammatory genes that affect the transcriptional responses to TLR4 or secondary type I IFN stimulation [22-24]. Similar type of histone marks on cytokine genes drive the pro-inflammatory phenotype in β-glucan trained macrophages [3, 25]. Thus, type I IFN-driven immunomodulation might potentially share some of the regulatory mechanisms that are fundamental to trained immunity.

In this study, we examined the link between type I IFNs and trained immunity in pSS. For this we used an *in vitro* monocytic model for trained immunity and peripheral blood cells from pSS patients. We hypothesized type I IFNs in patients to function at three different levels within the framework of trained immunity: 1) as a trainer inducing a trained

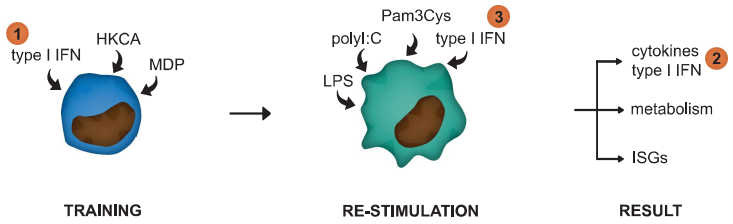

Figure 1. Graphical representation of hypothesized functions of type I IFNs within the framework of trained immunity.

(1) as a trainer inducing a trained immunity phenotype in innate immune cells, **(2)** as a result of trained immunity manifested by elevated type I IFN secretion upon re-stimulation, or **(3)** as a re-stimulus inducing the upregulation of IFN-stimulated genes (ISGs).

immunity phenotype in monocytes, 2) as a result of trained immunity manifested by elevated type I IFN secretion upon re-stimulation, or 3) as a re-stimulus inducing the upregulation of IFN-stimulated genes (ISGs; Figure 1).

MATERIALS AND METHODS

Trained immunity model

THP-1 cells were maintained in RPMI 1640 (Gibco, Thermo Fisher Scientific, Tilburg, The Netherlands) supplemented with 10% fetal calf serum (FCS) and antibiotics (Penicillin-Streptomycin; Gibco) in a humidified incubator at 37 °C / 5% CO_2. For the trained immunity model, 35.10e3 THP-1 cells per well were plated in 96-well flat bottom Nunclon Delta plates (Thermo Fisher Scientific) in RPMI 1640 + 10% (not heat-inactivated) FCS + Penicillin-Streptomycin (hereafter culture medium) and subsequently trained with heat-killed *Candida albicans* (HKCA; InvivoGen, San Diego, USA), Muramyl dipeptide (MDP; Sigma-Aldrich, Merck, Zwijndrecht, The Netherlands), IFNα-A(2a) or IFN-β1a (both from PBL Assay Science, Tebu-bio, Heerhugowaard, The Netherlands) or 50% human serum (final volume of 100 µl) for 24 hours in a humidified incubator at 37 °C / 5% CO_2. In some experiments 30 U/mL of the LPS neutralizing antibiotic Polymyxin B sulfate (Sigma-Aldrich) was added simultaneously with the training stimuli. After the first 7 hours of training, 25 nM Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was added for the remaining training time to the culture to initiate THP-1 adherence and differentiation. After 24 hours of training, the medium containing the training stimuli was removed and cells were washed once with pre-warmed PBS. Fresh culture medium containing 25 nM PMA was added and cells were rested for 30 hours. Thereafter, medium was removed, cells were washed once with pre-warmed PBS and cells were starved in RPMI 1640 + 0.5% FCS + Penicillin-Streptomycin (hereafter starvation medium) for 17 hours. Thereafter, cells were re-stimulated with LPS (50 ng/mL or 1 µg/mL; *E.coli* O55:B5; Sigma-Aldrich), Pam3Cys (10

µg/mL; EMC Microcollections, Tübingen, Germany), poly I:C HMW (5 µg/mL; InvivoGen) or IFN-β1a (100 U/mL) in starvation medium for 6 or 24 hours. Each condition was performed in triplicate. Supernatant was harvested for quantification of cytokine production, lactate and glucose concentrations and cells were lysed in RLT buffer for RNA isolation.

Patients and healthy controls

Patients with pSS (classified according to the 2016 ACR-EULAR Classification Criteria for primary Sjögren's Syndrome [26]) and (childhood) SLE (classified according to the 2019 ACR-EULAR Classification Criteria for SLE [27]) were recruited at the outpatient clinics of the Erasmus MC and Sophia Children's Hospital, Rotterdam University Medical Center, Rotterdam, the Netherlands. Disease activity at the time of inclusion was assessed using the EULAR Sjögren's syndrome disease activity index (ESSDAI) [28] or Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)-2K or SELENA-SLEDAI [29, 30]. Patient characteristics, use of medication and routine hematological and serological parameters were retrieved from patient records and are summarized in Supplementary Table 1. HC (age and sex-matched to pSS and adult SLE) were included at the Erasmus MC. The Medical Ethics Review Committee of the Erasmus MC (MEC-2011-116; MEC-2016-202; MEC-2019-0412) has approved of this study and written informed consent was provided by all participants in compliance with the declaration of Helsinki.

Blood sampling and processing

Peripheral blood was obtained from patients and HC in NH Sodium Heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Becton Dickinson, Vianen, The Netherlands) and BD Vacutainer™ SST™ II Advance Tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation and cryopreserved in liquid nitrogen until later use. Serum samples were stored at -80 °C until later use. Blood samples were processed in the laboratory within two hours of collection.

PBMC stimulations

PBMCs were first rested for 30 minutes in RPMI 1640 +10% heat-inactivated FCS + Penicillin-Streptomycin. Then, 4.10e5 PBMCs were plated in 96-well round bottom Nunclon Delta plates (Thermo Fisher Scientific) and stimulated with LPS (10 ng/mL), Pam3Cys (10 µg/mL) or poly I:C HMW (5 µg/mL) for 24 hours or IFN-β1a (100 U/mL) for 0.5, 2 or 6 hours (final volume 200 µl) in a humidified incubator at 37 °C / 5% CO_2. Harvested supernatants were kept on ice at all times and immediately stored at -20 °C until quantification of cytokine levels, or stored at -80 °C until lactate and glucose measurements. Cells were lysed in RLT buffer and stored at -20 °C until RNA isolation.

RT-PCR

RNA was isolated from PAXgene Blood RNA Tubes using the PAXgene Blood RNA Kit (PreAnalytiX GmbH) or from cultured cells using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and reverse transcribed to cDNA using High-Capacity Reverse Transcription Kit (Applied Biosystems, Bleiswijk, The Netherlands). RT-PCR was performed on a QuantstudioTM 5 Real-Time PCR System using predesigned primer/probe sets (Applied Biosystems). The housekeeping gene *ABL* was used to normalize CT values for each sample. Relative mRNA quantity was calculated using the $2^{-\Delta Ct}$ (relative copy number) or $2^{-\Delta\Delta Ct}$ (fold change) method as indicated in the figure legends.

Type I IFN score in peripheral blood cells

Whole blood expression of ISGs *MX1*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* was quantified from PAXgene Blood RNA tubes by RT-PCR, and a type I IFN score was calculated as previously described [31]. A cohort of 106 HC was used for the calculation of the type I IFN scores. The threshold for stratification of patients in IFN-low and IFN-high was determined by the $Mean_{HC} + 2*SD_{HC}$.

Cytokine quantification

The pro-inflammatory and pro-atherogenic cytokines TNFα and IL-6 were measured in culture supernatants with ELISA (Human TNF-alpha DuoSet; R&D systems and Human Interleukin 6 Cytoset kit; Invitrogen, Thermo Fisher, Scientific). Type I IFN bioactivity was measured in culture supernatants by a reporter assay system (HEK-Blue™ IFN-α/β cells; InvivoGen) according to the manufacturer's instructions. Recombinant human IFNβ1a was used for calibration.

Lactate and glucose measurements

Lactate and glucose concentrations in culture supernatants were quantified using the Lactate Assay Kit (Sigma-Aldrich) and Glucose Colorimetric Detection Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions.

Cell counting and viability

To assess the number of cells for each training condition, THP-1 cells were retrieved from 6 wells by 0.25% trypsin-0.02% EDTA (Gibco) 48 hours after the training, stained with trypan blue and counted in duplicate.

DiI-oxLDL flow cytometry

THP-1 cells were trained and differentiated according to the described training protocol. After removal of the differentiation medium, cells were washed in PBS and rested

overnight in serum-free RPMI 1640 + Penicillin-Streptomycin. Cells were incubated with 50 µg/mL DiI-oxLDL (Invitrogen) for 4 hours, washed with PBS three times, trypsinized with 0.25% trypsin-0.02% EDTA and stained with eBioscience Fixable Viability Dye eF506 (Thermo Fisher Scientific) in PBS for 15 minutes at 4 °C. Cells were analyzed on a LSR Fortessa SOP (BD Biosciences).

RNA Sequence analysis

Paired-end raw FASTQ files were downloaded from the GEO database using GEO Series accession number GSE173670 [17], and were analyzed with the nf-core/RNA-seq pipeline (v3.1) using Nextflow (21.05.0.edge) and its default settings [32, 33]. Quality of the sequencing was reported with FastQC (v11.9). Subsequently, bases with low Phred scores (≤ 30) were either trimmed or the complete reads were removed using Trim Galore! (v6.6). Trimmed FASTQ reads were mapped to the human reference genome version GRCh38 with the GRCh38 gencode 37 gene annotation file using RSEM (v1.3.1), which umbrellas STAR (v2.7.6a) as read aligner. Next, SAMtools (v 1.10) processed the alignment files and extracted mapping statistics of the post-alignment [34-36]. Quality of each sample alignment was visually inspected using reports derived from RSeQC (v3.0.1), Qualimap (v2.2.2-dev) and Preseq (v3.1.1), including read inner distance plots, splice junction annotations, the genomic origin of the mapped reads, and the estimated complexity of the sequencing library [37-39]. RSEM estimated transcript counts were imported into R (v4.1.0), transformed to gene counts using tximport (v1.20) and analyzed with DESeq2 (v1.32.0) [40, 41]. Only protein-coding genes were kept for subsequent analyses. Gene counts were transformed using the "varianceStabilizingTransformation" (VST) function of DESeq2. Differentially expressed genes were calculated using DESeq2. p-values were calculated using Wald statistical test and corrected with the Benjamini-Hochberg multiple hypothesis testing method for all protein-coding genes. For the comparison of pSS patients against HC, the HC were set as base reference. For the analysis between the IFN-high and IFN-low pSS patients the IFN-low subgroup was set as base reference. Fold Changes were shrunk with the DESeq2 function "lfcshrink" using method "apeglm" [42]°C. Heatmaps were made using the R package heatmap (v1.0.12), Z-scores were calculated per gene using the VST transformed counts.

Statistical analysis

Graphpad Prism 5.0 (Graphpad Software, La Jolla, CA, USA) was used for graph design and statistical analyses. Depending on the data distribution, independent-samples Student's t-test or Mann-Whitney U test, one-way ANOVA followed by Tukey's HSD test or Kruskal-Wallis H test followed by Dunn's Multiple Comparison test was used to compare two or more groups. One-sample Student's t-test or Wilcoxon signed rank test was used

to compare medians with a hypothetical 1. Friedman test followed by Dunn's Multiple Comparison test or repeated measures ANOVA followed by Dunnett's post hoc test were used for paired observations. Multiple logistic regression analysis was performed in R version 4.0.3 [43] and JMP Pro version 15 [44] to assess the relationship between type I IFN pathway activation and cardiovascular events including seven cardiovascular risk- or trained immunity-associated covariates: Age, BMI, current Statin use, current hydroxychloroquine use, current NSAID use, Smoking status (past or present) and Hypertension status. Effect likelihood ratio tests were used to determine a relationship between the covariates and type I IFN pathway activation.

RESULTS

Establishment of the in vitro trained immunity THP-1 model

To study the interaction between type I IFNs and trained immunity, we first established an *in vitro* model for trained immunity using the monocytic THP-1 cell line (Figure 2A). Training with *Candida albicans*-derived β-glucans or the bacterial cell wall component muramyl dipeptide (MDP) has been described to trigger a lasting pro-inflammatory phenotype in monocytes, which is exemplified by increased TLR2/4-induced IL-6 and TNFα production [25, 45]. Therefore, we used these well-established trainers to validate the induction of trained immunity in THP-1 cells. Heat-killed *Candida albicans* (HKCA) and MDP induced a significant dose-dependent increase in IL-6 and TNFα secretion upon re-stimulation with the TLR4 agonist LPS or the TLR2 agonist Pam3Cys (Figure 2B, C and Supplementary Figure 1A,B). Likewise, re-stimulation with TLR3 agonist poly I:C induced significantly elevated IL-6 and TNFα secretion by THP-1 cells trained with MDP (Figure 2D). These enhanced cytokine responses were not due to cell number differences between the different training conditions (Supplementary Figure 2). Altogether, these data support the induction of trained immunity by *Candida albicans* and MDP in THP-1 cells.

Type I IFN is a trainer in THP-1 cells

Using the THP-1 cell model, we subsequently explored the training-inducing capacity of type I IFNs. Training with IFNβ caused a significantly increased production of IL-6 and TNFα upon re-stimulation with LPS or Pam3Cys (Figure 3A,B and Supplementary Figure 3A,B). Similar to MDP, training with IFNβ also induced increased poly I:C-stimulated IL-6 and TNFα secretion (Figure 3C). The cytokine secretion of IFNβ-trained cells is likely underestimated as the number of cells per well was lower in IFNβ-trained compared with untrained conditions (Supplementary Figure 2). Importantly, LPS-induced gene expression of the pro-atherogenic chemokine *CCL2* was significantly enhanced in IFNβ-trained

THP-1 cells and to a lesser extent in HKCA- and MDP-trained cells (Figure 3D). The recombinant IFNβ protein used for the training experiments was produced in *E. coli*. Therefore, to excluded bystander effects of potential endotoxin contamination, an additional set of experiments was conducted in which the LPS-neutralizing polymyxin B was added during the training. This did not revert the observed phenotype (Supplementary Figure 4A-C). To evaluate whether other IFNα/β receptor (IFNAR) ligands also induce training, we tested the training-inducing capacity of IFNα2. Training with IFNα2 also resulted in elevated IL-6 and TNFα production upon re-stimulation with LPS or Pam3Cys (Supplementary Figure 5A,B). Together these data indicate type I IFN as a trainer that induces a pro-inflammatory and pro-atherogenic phenotype in monocytes.

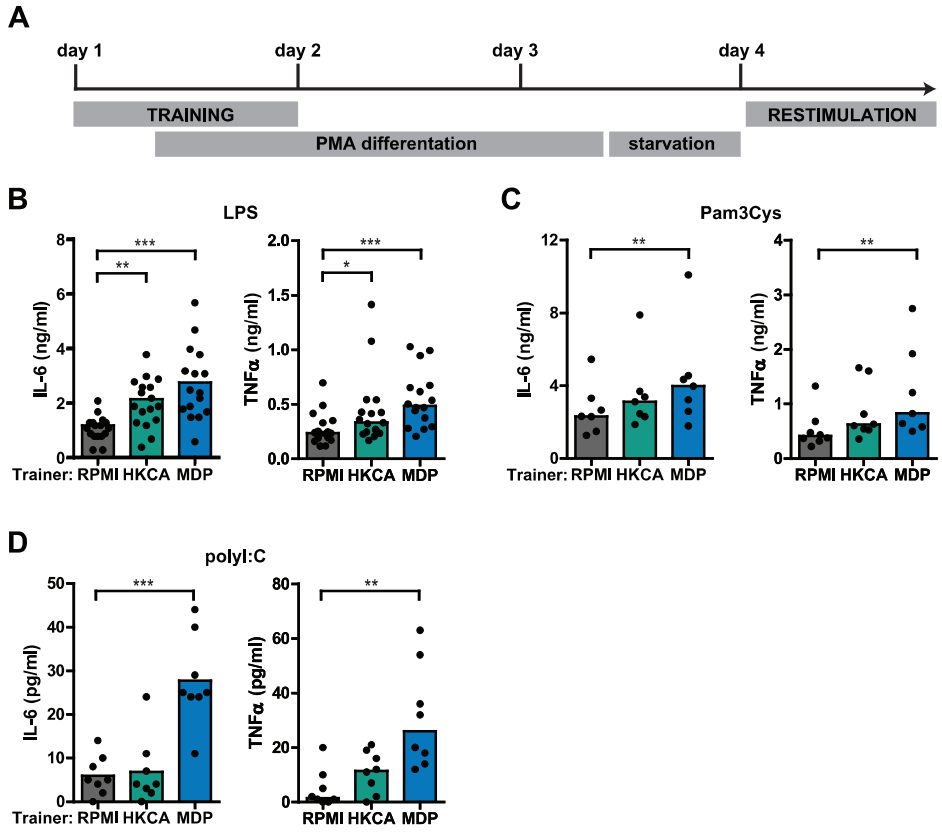

Figure 2. Training with *Candida albicans* and MDP prompts elevated cytokine responses in THP-1 cells.
(A) Schematic overview of *in vitro* THP-1 cell model for trained immunity. **(B-D)** Concentrations of IL-6 or TNFα quantified by ELISA in culture supernatants of THP-1 cells trained with heat-killed *Candida albicans* (HKCA; 10^6 cells/mL), muramyl dipeptide (MDP; 50 µg/mL) or RPMI and re-stimulated with **(B)** 50 ng/mL LPS, **(C)** 10 µg/mL Pam3Cys, or **(D)** 5 µg/mL poly I:C for 24 hours. Symbols represent the average of triplicates. Depending on the data distribution, bars represent means or medians and Friedman test or repeated measures ANOVA were performed to compare groups. * p<0.05, **p<0.01, ***p<0.001.

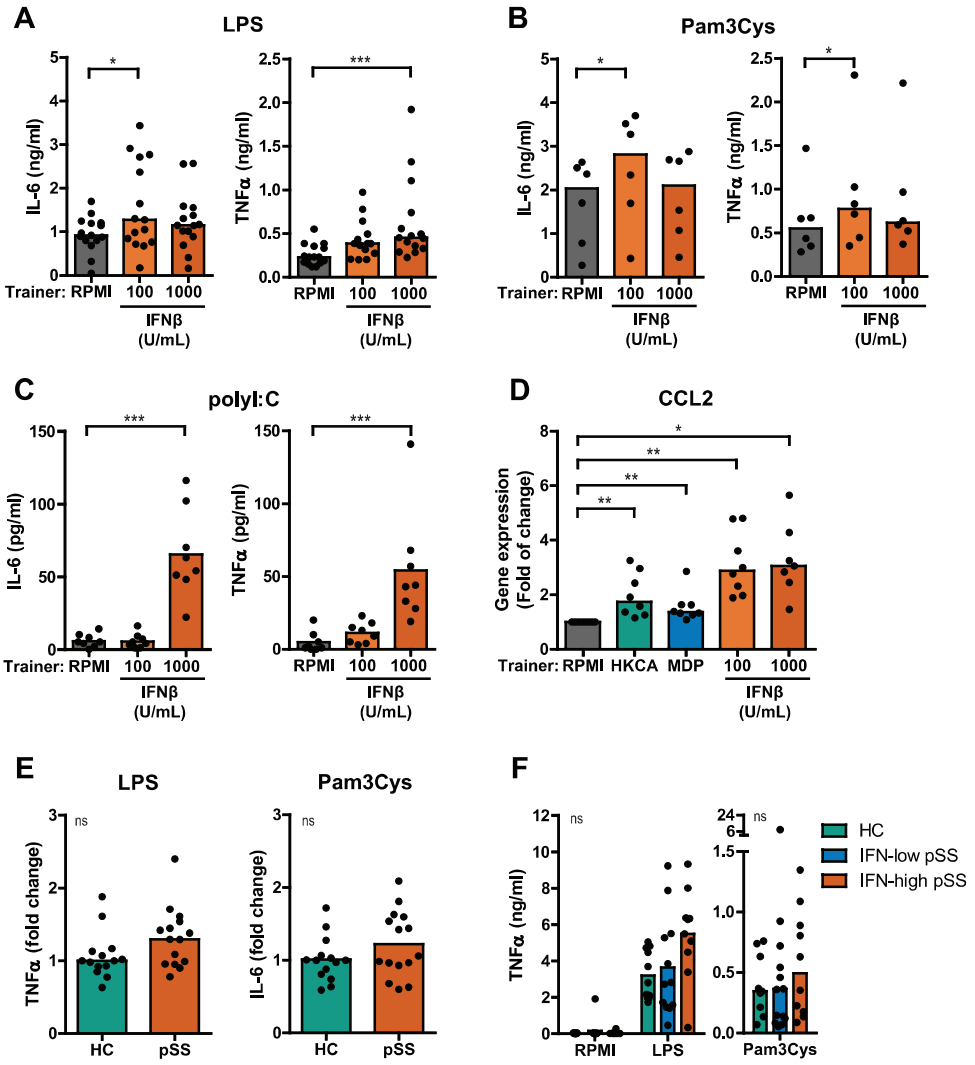

Figure 3. Type I IFNs induce training of THP-1 cells.

Concentrations of IL-6 or TNFα quantified by ELISA in culture supernatants of THP-1 cells trained with IFNβ or RPMI and re-stimulated with **(A)** 50 ng/mL LPS, **(B)** 10 µg/mL Pam3Cys, or **(C)** 5 µg/mL poly I:C for 24 hours. **(D)** Relative mRNA expression (2ΔΔCT) of CCL2 in HKCA-, MDP- or IFNβ-trained THP-1 cells re-stimulated with 50 ng/mL LPS for 24 hours. Fold change expression was calculated relative to LPS stimulated untrained (RPMI) THP-1 cells. **(E)** TNFα or IL-6 secretion by THP-1 cells trained with 50% serum from pSS patients or healthy controls (HC) upon re-stimulation with 50 ng/mL LPS or 10 µg/mL Pam3Cys for 24 hours. For each experiment, observations were normalized to the untrained condition and expressed relative to the median of HC (age and sex-matched to pSS) serum-trained conditions within the corresponding experiment. **(F)** TNFα concentrations in supernatants of PBMCs from HC and pSS patients stratified based on blood ISG expression stimulated with 10 ng/mL LPS or 10 µg/mL Pam3Cys for 24 hours. Symbols represent the average of duplicates (F) or triplicates (A-E). Depending on the data distribution, bars represent means or medians and Friedman test, repeated measures ANOVA, Mann-Whitney U test, student's t test or Kruskal-Wallis test were performed to compare groups. Wilxocon singed rank test was used to compare medians with a hypothetical 1. ns: not significant, * p<0.05, **p<0.01, ***p<0.001.

Elevated cytokine responses in pSS serum-trained THP-1 cells and patient's PBMCs

A substantial part of pSS patients is characterized by increased IFNα serum levels [20]. To investigate a potential contribution of serum type I IFN to trained immunity in patients, THP-1 cells were trained with serum from IFN-high pSS patients. Training of THP-1 cells with serum from IFN-high pSS patients induced a trend towards higher cytokine production in response to re-stimulation with LPS and Pam3Cys (Figure 3E and Supplementary Figure 6A). Similarly, THP-1 cells trained with serum from patients with childhood-onset SLE (cSLE) – another type I IFN-associated autoimmune disease – produced slightly higher levels of IL-6 after Pam3Cys re-stimulation (Supplementary Figure 6A). Next, we hypothesized that type I IFN would act as an *in vivo* trainer in patients affecting the responsiveness of PBMCs to *ex vivo* stimulation. PBMCs from patients and HC secreted highly variable levels of TNFα and IL-6 upon LPS and Pam3Cys stimulation (Figure 3F and Supplementary Figure 6B). PBMCs from pSS and adult SLE patients tended to secrete slightly higher levels of TNFα upon LPS stimulation than HC PBMCs, which was most pronounced in IFN-high patients (Figure 3F and Supplementary Figure 6B). Together, the trends observed using patient material support a role for type I IFN as inducer of a trained immunity phenotype in IFN-high pSS.

Altered type I IFN production is a result of trained immunity in THP-1 cells

Next, we tested whether trained immunity affects the type I IFN response to re-stimulation in a similar manner as observed for IL-6 and TNFα production. Re-stimulation with LPS or Pam3Cys in THP-1 cells trained with different trainers did not result in measurable type I IFN secretion in most of the experiments (Supplementary Figure 7A and data not shown), although HKCA-trained cells seemed to secrete slightly more type I IFNs (Supplementary Figure 7A). However, LPS re-stimulation did upregulate *IFNB* mRNA expression, which was significantly higher when THP-1 cells were trained with HKCA (Figure 4A), while IFNβ-trained cells showed at similar trend. No effects of LPS re-stimulation were observed on *IFNA2* gene expression (data not shown). The increased *IFNB* production in HKCA-trained cells and subsequent IFNAR-signaling upon LPS re-stimulation is further supported by enhanced induction of the ISG *IFI44* (Supplementary Figure 7B). Re-stimulation with poly I:C – which signals through TLR3 and IRF3 – strongly stimulated type I IFN secretion by THP-1 cells (Figure 4B). Surprisingly, and in contrast to LPS stimulation, poly I:C-induced type I IFN production was heavily reduced in cells trained with IFNβ and to a lesser extent in HKCA-trained cells compared with untrained cells (Figure 4B). Similarly, type I IFN secretion by pSS PBMCs tended to be higher than HC PBMCs in response to LPS stimulation, but tended to be lower in response to poly I:C stimulation (Figure 4C). The contrasting

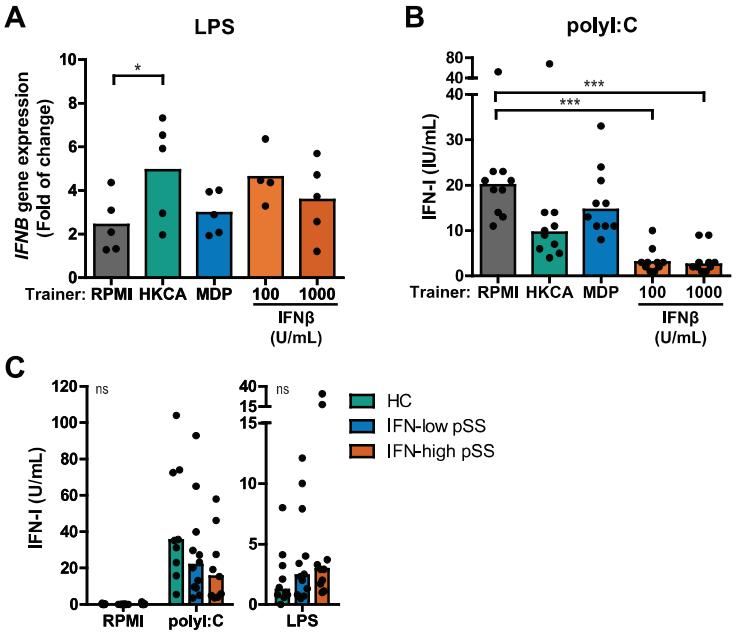

Figure 4. Training with HKCA en MDP induce differential type I IFN response upon re-stimulation.
(A) Relative mRNA expression ($2^{\Delta\Delta CT}$) of *IFNB* in THP-1 cells trained with heat-killed *Candida albicans* (HKCA; 10^6 cells/mL), muramyl dipeptide (MDP; 50 µg/mL) or IFNβ and re-stimulated with 1 µg/mL LPS + 10% fetal calf serum for 24 hours. Fold change expression was calculated relative to the corresponding unstimulated control. **(B-C)** Type I IFN bioactivity quantified by the HEK IFN-α/β reporter cell assay in culture supernatants of **(B)** HKCA-, MDP- and IFNβ-trained THP-1 cells re-stimulated with 5 µg/mL poly I:C for 24 hours or **(C)** PBMCs from pSS patients stratified based on blood ISG expression and healthy controls (HC) stimulated with 5 µg/mL poly I:C or 10 ng/mL LPS for 24 hours. Symbols indicate the average of duplicates (C) or triplicates (A,B) and bars represent means or medians. Friedman test or repeated measures ANOVA was performed to compare groups. ns: not significant, * p<0.05, ***p<0.001.

direction of type I IFN responses to poly I:C and LPS was most pronounced in IFN-high patients. PBMCs from SLE patients showed similar tendency (Supplementary Figure 7C). In conclusion, trained immunity affects type I IFN production in response to re-stimulation.

Trained immunity affects the cellular response to re-stimulation with IFNβ

Here, we investigated the effects of training with HKCA, MDP or IFNβ on the cellular response to re-stimulation with type I IFNs. A large proportion of patients with pSS display elevated expression of ISGs in peripheral blood cells [19, 31]. Training with IFNβ has been reported to induce transcriptional memory of specific ISGs in fibroblasts [23]. In these conditions, the ISGs *IFI44* and *MX1* showed transcriptional memory to IFNβ training, while *IRF1* did not. We hypothesized that similar principles may apply in our experimental conditions and assessed the transcriptional response of trained THP-1 cells to IFNβ re-stimulation for 4

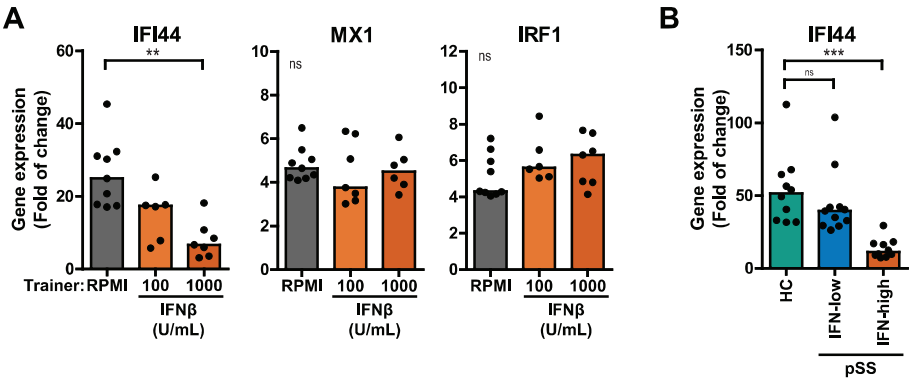

Figure 5. Differential ISG response to type I IFN in IFNβ-trained THP-1 and patient's PBMCs.

(A) Relative mRNA expression ($2^{\Delta\Delta CT}$) of *IFI44*, *MX1* and *IRF1* in IFNβ-trained THP-1 cells re-stimulated with 100 IU/mL IFNβ for 4 hours. **(B)** Relative mRNA expression ($2^{\Delta\Delta CT}$) of *IFI44* in PBMCs from pSS stratified based on blood ISG expression and HC stimulated with 100 IU/mL IFNβ for 2 hours. Fold change expression was calculated relative to unstimulated cells. Depending on the data distribution, bars represent means or medians and One-way ANOVA or Kruskal-Wallis test were used to compare groups. ns: not significant, **p<0.01, ***p<0.001.

hours. The peak transcriptional response of THP-1 cells to IFNβ re-stimulation was reached after 2 hours for *IRF1* and after 4-6 hours for *IFI44* and *MX1* (Supplementary Figure 8A). We first focused on the effects of training with HKCA or MDP on IFNβ-induced ISG expression. Increased transcriptional responses of *MX1* and *IRF1* to IFNβ re-stimulation were observed in MDP-trained THP-1 cells compared with untrained cells (Supplementary Figure 8B). In THP-1 cells trained with IFNβ, the fold change induction of *IFI44* upon IFNβ re-stimulation was reduced (Figure 5A). Yet, the IFNβ-induced relative *IFI44* mRNA count was slightly increased in IFNβ-trained cells (Supplementary Figure 8C). In addition, THP-1 cells trained with IFNβ exhibited higher unstimulated expression of *IFI44* and *MX1*, but not *IRF1*, relative to untrained cells (Supplementary Figure 8C). In patients, trained immunity might affect the response to type I IFN which could act as a re-stimulus *in vivo* inducing ISG expression in PBMCs. *Ex vivo* stimulation of PBMCs with IFNβ resulted in a peak induction of *IFI44* expression after 2 hours (Supplementary Figure 8D). The *IFI44* expression in unstimulated and IFNβ-stimulated PBMCs from IFN-high pSS and SLE mimicked the *IFI44* response in IFNβ-trained THP-1 cells (Figure 5B and Supplementary Figure 8E). Together, these data indicate that training with MDP and IFNβ, but not HKCA, affects the ISG response to type I IFN re-stimulation.

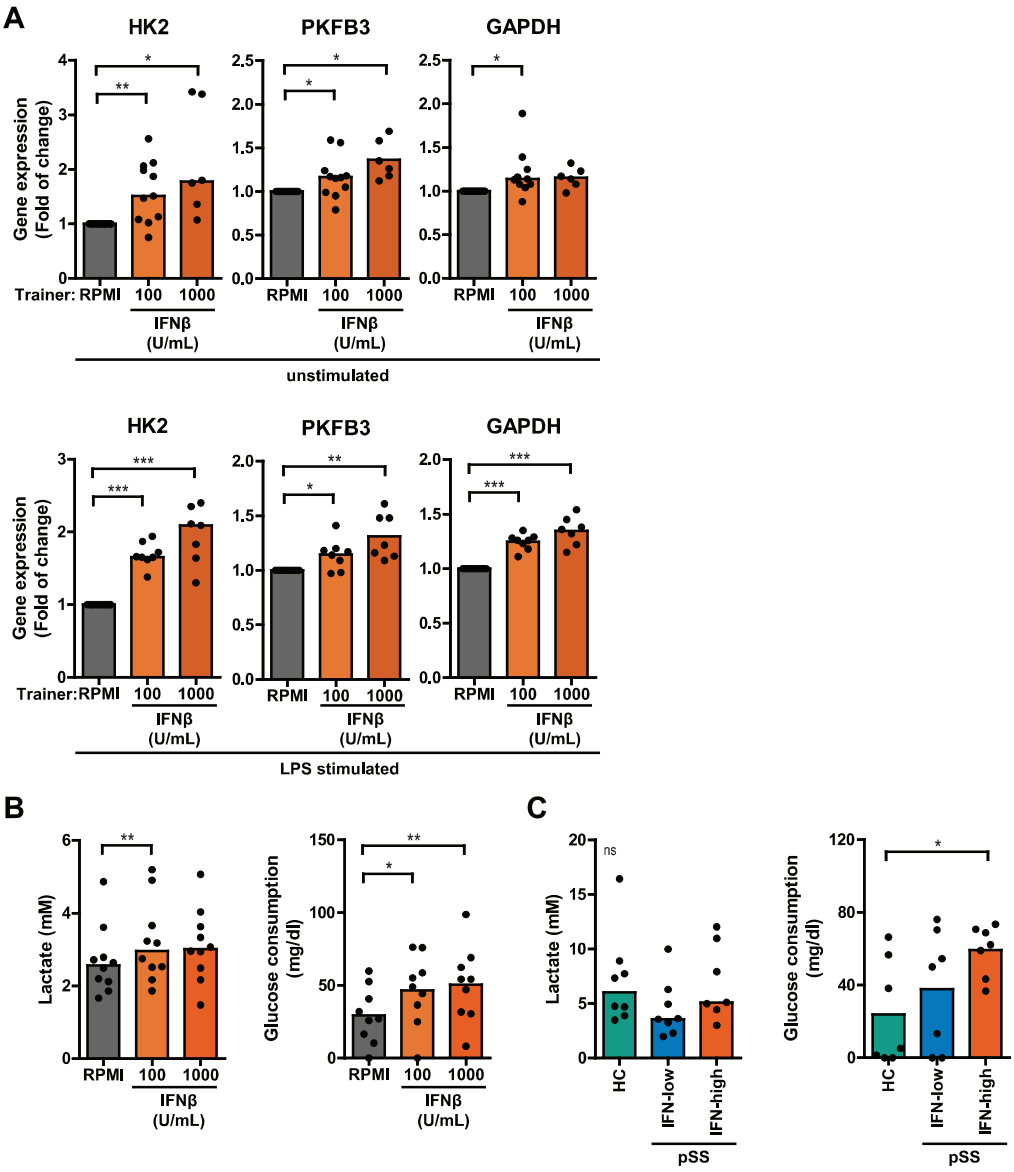

Figure 6. Altered glucose metabolism in type I IFN-trained THP-1 and patient's PBMCs.

(A) Relative mRNA expression ($2^{\Delta\Delta CT}$) of *HK2*, *PKFB3* and *GAPDH* in THP-1 cells trained with IFNβ either before re-stimulation (upper panel) or after 24 hour stimulation with 50 ng/mL LPS (lower panel). Fold change expression was calculated relative to the corresponding untrained (RPMI) cells. **(B-C)** Lactate concentrations (left) in culture supernatants and glucose consumption (right) by 24 hour LPS-stimulated **(B)** THP-1 cells trained with IFNβ or **(C)** PBMCs from pSS and HC. One sample t-test or Wilxocon singed rank test were used to compare means/medians with a hypothetical 1 and repeated measures ANOVA or Friedman test, or Kruskal-Wallis test was used to compare groups. ns: not significant, * p<0.05, **p<0.01, ***p<0.001.

Altered glucose metabolism in type I IFN-trained THP-1 and patient's PBMCs

Trained immunity is mediated by changes in cellular metabolism, including increased glycolysis [3]. Monocytic expression of key genes involved in cellular metabolic pathways associated with trained immunity were analyzed using a publicly available RNA sequencing dataset of pSS and HC monocytes [17]. Monocytes from pSS patients expressed significantly lower transcript levels of the mTOR signaling pathway components *PIK3R1* and *EIF4EBP1*, the glycolytic enzymes *PKM* and *PGM1* and the TCA-cycle enzymes *MDH2* and *CS* (Supplementary Figure 9 and Supplementary Tables 2 and 3). In THP-1 cells trained with IFNβ, the mRNA expression of *HK2*, *PKFB3* and *GAPDH* – key enzymes of glycolysis – was elevated relative to untrained cells, which was most evident in LPS-stimulated cells (Figure 6A). Although less pronounced, these glycolytic enzymes were also slightly upregulated in HKCA- and MDP-trained THP-1 cells (Supplementary Figure 10A). In accordance with the gene expression, THP-1 cells trained with IFNβ consumed significantly more glucose and secreted more lactate than untrained THP-1 cells (Figure 6B and Supplementary Figure 10B). Glucose consumption was also significantly higher in PBMC cultures from IFN-high pSS patients compared with HC PBMCs (Figure 6C).

Increased oxLDL uptake in type I IFN-trained THP-1

Trained immunity has also been linked to alterations in cellular cholesterol metabolism and atherosclerosis [25, 46]. Monocytes from pSS patients expressed significantly higher *NR1H3* – one of the key enzymes in the cholesterol biosynthesis pathway – compared with HC monocytes (Supplementary Figure 9 and Supplementary Tables 2 and 3). The scavenger receptor *MSR1* that mediates the uptake of modified low-density lipoprotein (LDL)-cholesterol was upregulated, while the other main oxLDL importer *CD36* was downregulated in pSS monocytes (Supplementary Figure 9 and Supplementary Tables 2 and 3). Differential gene expression of two principal cholesterol biosynthesis enzymes *MVK* and *NR1H3*, cholesterol efflux transporters *ABCA1* and *ABCG1,* and scavenger receptors *MSR1* and *CD36* was also observed in THP-1 cells trained with IFNβ, HKCA and MDP (Figure 7A and Supplementary Figure 11A). In addition to changes in the cholesterol metabolism pathway, increased uptake of cholesterol particles – such as oxidized LDL (oxLDL) – have also been described in trained monocytes, resulting in foam cell formation [25]. We tested the uptake of oxLDL in a functional assay of trained THP-1 cells. The median fluorescence intensity of DiI-oxLDL exposed THP-1 cells trained with IFNβ, as well as those trained with HKCA and MDP, was higher compared with untrained cells, indicating incremented internalization of oxLDL by trained THP-1 cells (Figure 7B and Supplementary Figure 11B). Finally, we analyzed the association between cardiovascular events and type I IFN pathway activation in an established pSS cohort [31, 47]. A history of cardiovascular events was reported for 7

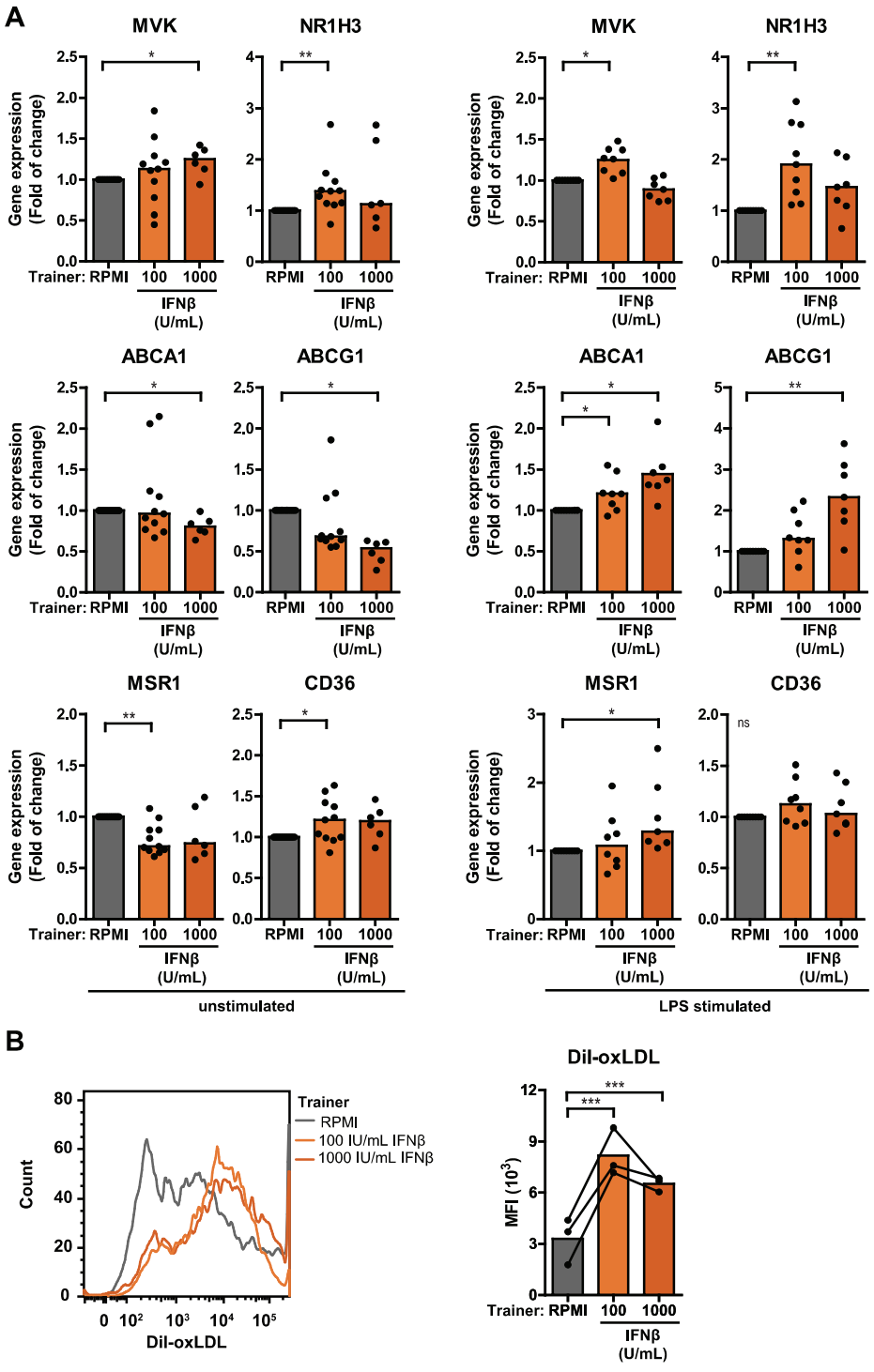
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Figure 7 (previous page). Differential cholesterol metabolism in type I IFN-trained THP-1 cells.
(A) Relative mRNA expression ($2^{\Delta\Delta CT}$) of *MVK*, *NR1H3*, *ABCA1*, *ABCG1*, *MSR1* and *CD36* in THP-1 cells trained with IFNβ either before re-stimulation (left panel) or after 24 hour stimulation with 50 ng/mL LPS (right panel). Fold change expression was calculated relative to the corresponding untrained (RPMI) cells. **(B)** Representative histograms (left panel) and median fluorescence intensity (MFI; right panel) of IFNβ-trained THP-1 cells incubated with 50 μg/mL DiI-oxLDL for 4 hours. Depending on the data distribution, bars represent means or medians. One sample t-test or Wilxocon singed rank test were used to compare means/medians with a hypothetical 1 and repeated measures ANOVA was used to compare groups. ns: not significant, * p<0.05, **p<0.01, ***p<0.001.

out of 86 patients (8.1%), all of which (7/7) were IFN-high patients. Logistic regression analysis showed a significant positive relationship between type I IFN pathway activation and cardiovascular events (p = 0.042) after adjustment for age, BMI, current or past smoking status, hypertension and current use of statins, HCQ and NSAIDs (Supplementary Table 4).

DISCUSSION

Hyperresponsiveness of innate immune cells is a hallmark characteristic of trained immunity [48]. A large subgroup of pSS patients exhibits type I IFN pathway activation, indicative of hyperactive innate immunity [19]. Here, we studied the link between type I IFNs and trained immunity. We show that type I IFNs induce a trained immunity phenotype in monocytes (Figure 1; hypothesis 1), including elevated production of the pro-inflammatory and pro-atherogenic cytokines CCL2, IL-6 and TNFα and enhanced cholesterol uptake. In addition, we show that training affects both the production of type I IFNs (Figure 1; hypothesis 2) and the ISG response to IFNβ re-stimulation (Figure 1; hypothesis 3).

Type I IFNs established a trained immunity phenotype in monocytes (hypothesis 1). In addition to pathogens and pathogen-associated molecular patterns [2, 49-51], various cytokines are able to prompt trained immunity [5, 52-55]. This study adds type I IFNs to the list of cytokine trainers that affect the response to re-stimulation which persists even when the initial challenge has been removed. Priming with type I IFN has previously been described to modulate the transcription of pro-inflammatory cytokines by myeloid cells upon LPS stimulation [22, 56, 57]. In these priming protocols, the primary and secondary stimulation are added simultaneously or sequentially close in time. This is different than the training models that allow the cells to return to homeostasis before re-stimulation [58]. Trained immunity also involves various metabolic pathways and alterations in cellular metabolism [5, 46, 49, 50, 54, 59]. Likewise, training with type I IFNs increased both glucose consumption and cholesterol import in THP-1 cells.

Type I IFN production upon re-stimulation was modulated by training with both HKCA and IFNβ (hypothesis 2). These results show for the first time that trained immunity can

affect type I IFN production. Further studies are needed to explain the contrasting effects of training on the type I IFN response to bacterial (LPS) and viral (poly I:C) re-stimulation.

Training with the microbial component MDP altered the ISG response to IFNβ re-stimulation (hypothesis 3). Related to this, training with HKCA has recently been demonstrated to cause increased expression of several ISGs and cytokines IL-6 and TNFα by monocytes upon type I IFN re-stimulation [51]. In addition to MDP, training with type I IFNs also affected the transcription of ISGs, indicating that both IFN and non-IFN trainers can confer transcriptional memory of the type I IFN pathway. Others have also shown effects of training with type I and type II IFN on the transcriptional response to IFNβ in immune and non-immune cells [23]. We observed a modest upregulation of basal ISG expression in type I IFN-trained cells two days after the removal of the trainer. Since type I IFN signaling is self-amplifying, auto- and/or paracrine IFNAR signaling could potentially be sustained after the elimination of the type I IFN training stimulus. However, limiting auto-/paracrine type I IFN signaling by inhibition of the IFNAR during the two-day resting period in a pilot experiment could only partially revert the elevated ISG expression (data not shown). *In vivo* observations in pSS and SLE patients of relatively stable type I ISG expression over time but more variable type I IFN protein levels [19, 31, 60, 61] (and unpublished observations) also support memory-like features for ISG transcript expression.

Ex vivo PBMC stimulations provided support for the hypothesized connections between type I IFN and trained immunity in pSS. PBMCs from pSS patients showed TLR-induced cytokine responses, type I IFN production, IFNβ-stimulated ISG expression patterns and metabolic alterations consistent with a trained immunity phenotype, indicating potential *in vivo* training. Very recently, the elevated TLR-stimulated TNFα production has also been described in monocytes from pSS patients [17]. The trained phenotype that we observed in pSS was most pronounced in IFN-high patients and could also be observed in patients with IFN-high SLE. In these IFN-high patients, type I IFNs could potentially act as a trainer *in vivo* (Figure 1, hypothesis 1). This hypothesis is supported by the elevated cytokine responses in THP-1 cells trained with serum from IFN-high pSS patients. Also, the ISG expression patterns and TLR-induced type I IFN production in PBMCs from IFN-high pSS patients indicate a role for type I IFN as a trainer. On the other hand, training affected the production of type I IFNs upon re-stimulation. Therefore, type I IFN production in pSS patients could potentially be modulated by trained immunity (Figure 1, hypothesis 2). Indeed, the type I IFN response to TLR stimulation was different between pSS and HC PBMCs. The produced type I IFN may subsequently induce further training, affecting cytokine production, cellular metabolism and ISG expression, creating a vicious loop in patients. Taken together, the upregulated ISG expression in pSS patients that is induced by the chronically elevated circulating type I IFNs could potentially be modulated by both type I IFN and non-IFN trainers. This could have consequences for the treatment of IFN-associated systemic autoimmune diseases.

Inhibition of ongoing type I IFN signaling might not be sufficient to overcome type I IFN-induced training, which might require metabolic or epigenetic intervention. Interestingly, the antimalarial hydroxychloroquine that is frequently used for treatment of pSS and SLE has recently been described to inhibit HKCA-induced training [51], providing a rationale for combination treatment of anti-IFNAR and hydroxychloroquine.

Patients with pSS are more prone to accelerated subclinical atherosclerosis, dyslipidemia and ischemic heart disease than the general population [11-13]. In SLE, the risk for cardiovascular disease has been associated with type I IFN pathway activation [62]. Although underpowered, cardiovascular events were only observed in patients with type I IFN pathway activation in the pSS cohort presented in this study, suggesting a similar association in pSS. A compelling body of evidence suggest a role for type I IFN in the pathogenesis of atherosclerosis [63]. Type I IFNs have been described to advance various pro-atherogenic processes, such as dysfunction of endothelial (progenitor) cells, monocyte infiltration, NETosis, uptake of modified LDL particles, foam cell formation and plaque rupture [63, 64].

Trained immunity contributes to atherosclerosis [7, 65]. Both microbial and non-microbial trainers have been described to promote a pro-atherogenic phenotype in monocytes [25, 65, 66]. In this study, we confirmed the induction of a similar phenotype in THP-1 cells trained with HKCA and MDP. Even more pronounced, training with type I IFNs caused increased production of the pro-atherogenic cytokines CCL2, IL-6 and TNFα, upregulation of cholesterol metabolism-related genes and increased oxLDL influx. Cholesterol is a critical regulator of cellular membrane fluidity controlling basic cellular functions, including cellular immune responses [67-69]. Type I IFN has been shown to shift the balance from cholesterol biosynthesis to cholesterol import in macrophages, which lowers the threshold for additional type I IFN production [70, 71]. Our data indicate that the effects of type I IFN on cholesterol metabolism can be maintained over time, even when the primary stimulus has been removed. The connection between type I IFN, trained immunity and cholesterol metabolism provides further insight into the pathogenesis of (subclinical) atherosclerosis in patients with pSS and other type I IFN-associated systemic autoimmune diseases.

This study describes the induction of a pro-atherogenic trained immunity phenotype and modification of cellular metabolism by type I IFNs in a monocytic cell line. However, the molecular basis of this phenotype including the causative role of metabolic and epigenetic reprogramming still requires further investigation. The high metabolic rate of THP-1 cells might impact trained immunity processes. Future studies should therefore validate these findings in freshly isolated primary human cells and further characterize the metabolic and epigenetic rewiring of type I IFN-trained cells and pSS innate immune cells. In addition, the association between *in vivo* type I IFN pathway activation and cardiovascular disease

in pSS should be further investigated in a larger cohort properly adjusting for potential confounders to strengthen the clinical implications of these findings.

In conclusion, immune cells from patients with (IFN-high) pSS have a trained immunity phenotype. *In vitro*, type I IFN is both a trainer inducing a pro-atherogenic trained immunity phenotype in monocytes and a result of trained immunity. The bidirectional link between type I IFN and trained immunity provides a rationale for alternative treatment strategies and contributes to the understanding of the pathogenesis of atherosclerosis in patients with pSS and other IFN-associated systemic autoimmune diseases.

ACKNOWLEDGMENTS

The authors would like to thank Vera P. Mourits for providing technical assistance with the *in vitro* cell model. The UK primary Sjogren's syndrome registry was established with funding support from the Medical Research Council (grant number: G0800629) and the British Sjogren's Syndrome Association.

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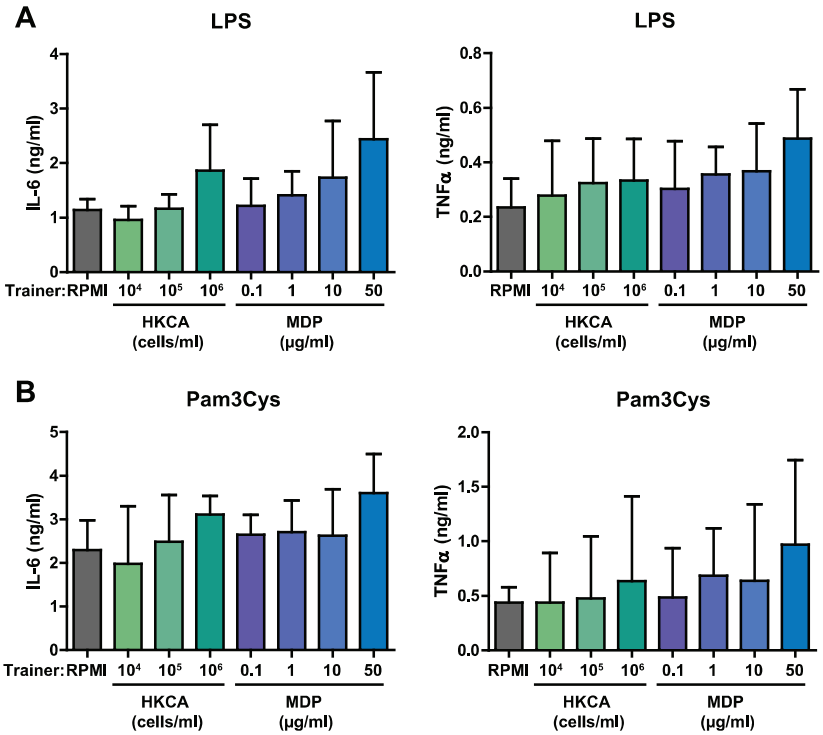
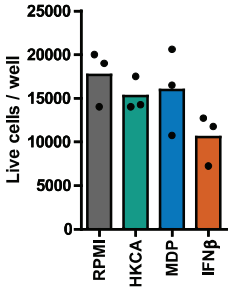
SUPPLEMENTARY DATA



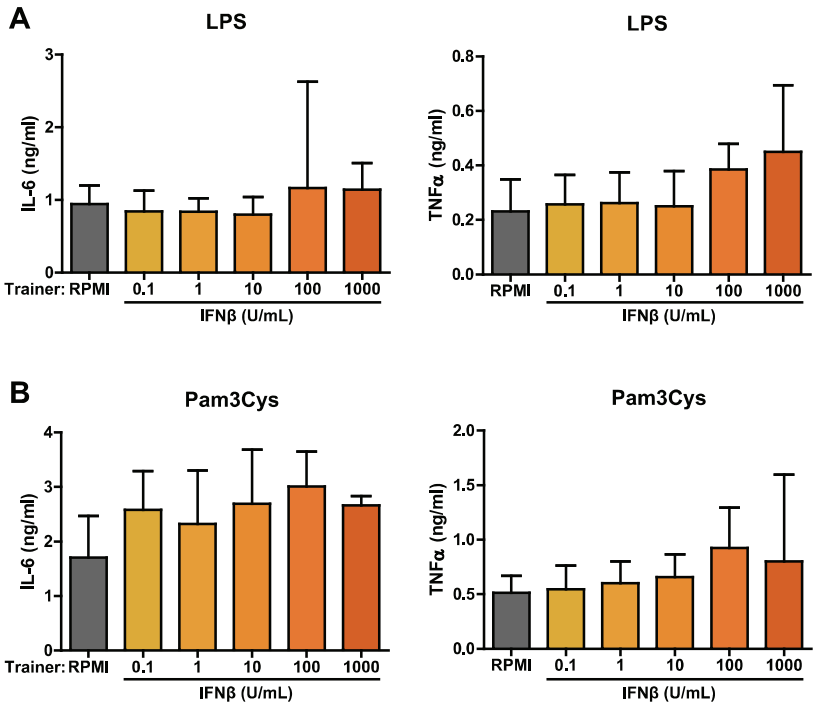
Supplementary Figure 1. Training with *Candida albicans* and MDP prompts elevated cytokine responses in THP-1 cells.

Concentrations of IL-6 or TNFα in culture supernatants of THP-1 cells trained with increasing concentrations of heat-killed *Candida albicans* (HKCA) or muramyl dipeptide (MDP) and re-stimulated with **(A)** 50 ng/mL LPS or **(B)** 10 µg/mL Pam3Cys for 24 hours as quantified by ELISA. Bars represent medians + Q3 (n=8-17).



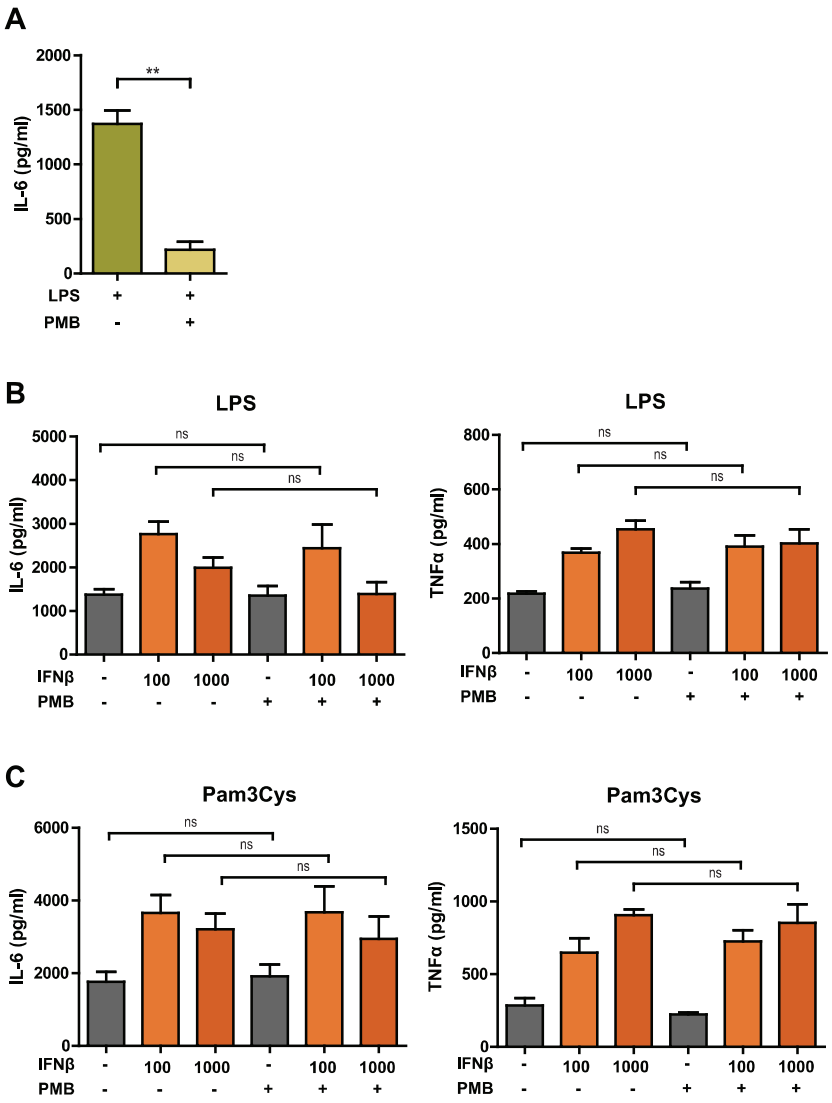
Supplementary Figure 2. Training of THP-1 cells did not induce higher number of cells.

Number of live cells recovered per well at the time point of re-stimulation after training of THP-1 cells with heat-killed *Candida albicans* (HKCA; 10^6 cells/mL), muramyl dipeptide (MDP; 10 µg/mL) or IFNβ (100 U/mL). Shown are averages of duplicate measurements of 6 wells combined. Bars represent means.



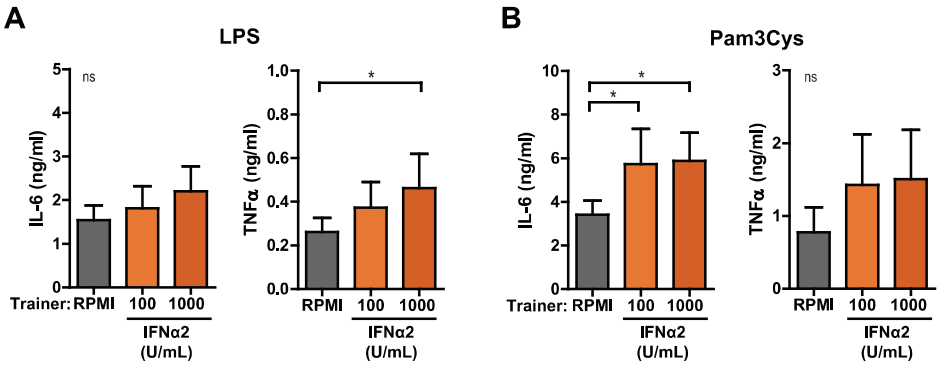
Supplementary Figure 3. Type I IFNs induce training of THP-1 cells.

Concentrations of IL-6 or TNFα quantified by ELISA in culture supernatants of THP-1 cells trained with increasing concentrations of IFNβ and restimulated with **(A)** 50 ng/mL LPS or **(B)** 10 µg/mL Pam3Cys for 24 hours. Bars represent medians + Q3 (n=8).

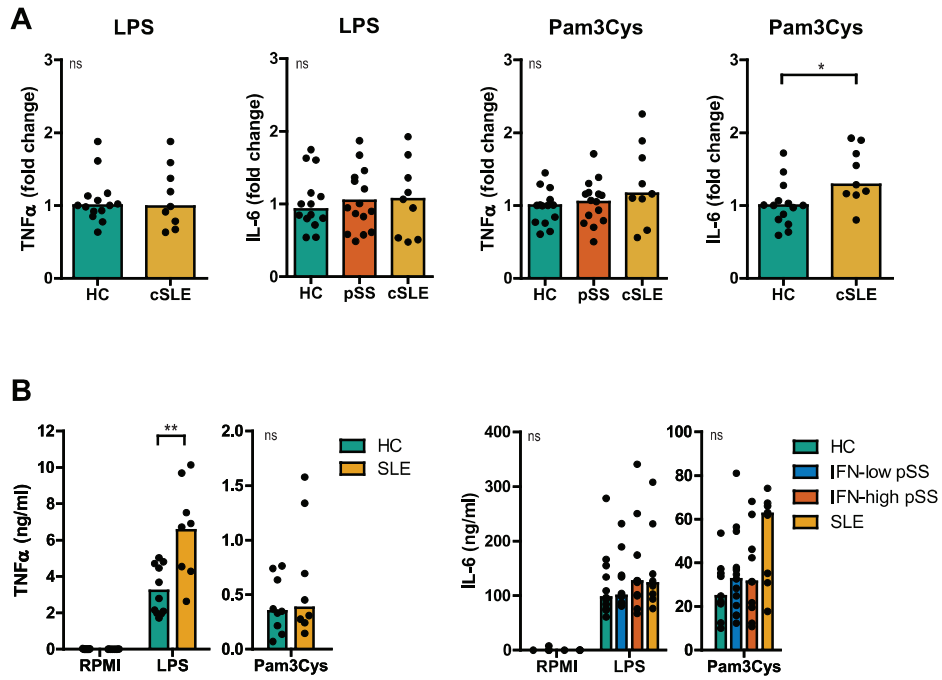


Supplementary Figure 4. Recombinant IFNβ-induced training cannot be explained by endotoxin contamination.

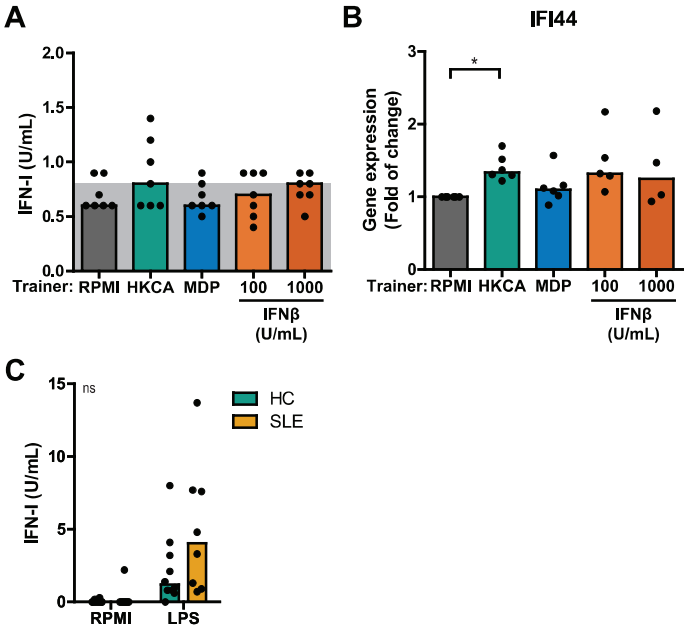
(A) Positive control for the effectiveness of polymyxin B (PMB) to inhibit LPS stimulation. Concentrations of IL-6 quantified by ELISA in culture supernatants of PMA-differentiated THP-1 cells stimulated with 50 ng/mL LPS in presence or absence of 30 IU/mL PMB for 24 hours. **(B/C)** Concentrations of IL-6 or TNFα quantified by ELISA in culture supernatants of THP-1 cells trained with IFNβ [U/mL] in the presence or absence of 30 IU/mL PMB and re-stimulated with **(A)** 50 ng/mL LPS or **(B)** 10 µg/mL Pam3Cys for 24 hours. Bars represent means + SEM (n=4). Paired t-test or Repeated measures ANOVA was used to compare the groups. ns: not significant, ** p<0.01.



Supplementary Figure 5. Training with IFNα2 prompts elevated cytokine responses in THP-1 cells.
Concentrations of IL-6 or TNFα quantified by ELISA in culture supernatants of THP-1 cells trained with IFNα2 and re-stimulated with **(A)** 50 ng/mL LPS or **(B)** 10 µg/mL Pam3Cys for 24 hours. Bars represent means + SEM (n=4). Repeated measures ANOVA was used to compare the groups. ns: not significant, * p<0.05.



Supplementary Figure 6. Elevated cytokine responses in pSS and cSLE serum-trained THP-1 cells and patient's PBMCs.
(A) TNFα or IL-6 secretion by THP-1 cells trained with 50% serum from pSS or cSLE patients or healthy controls (HC; age and sex matched to pSS patients) upon re-stimulation with 50 ng/mL LPS or 10 µg/mL Pam3Cys for 24 hours. For each experiment, observations were normalized to the untrained condition and expressed relative to the median of HC serum-trained conditions within the corresponding experiment. Symbols represent the average of triplicates. **(B)** TNFα or IL-6 concentrations in supernatants of PBMCs from HC, pSS stratified based on blood ISG expression, or SLE stimulated with 10 ng/mL LPS or 10 µg/mL Pam3Cys for 24 hours. Depending on the data distribution, bars represent medians or means and Mann-Whitney U test, student's t test or Kruskal-Wallis test was used to compare the groups. ns: not significant, * p<0.05, **p<0.01.

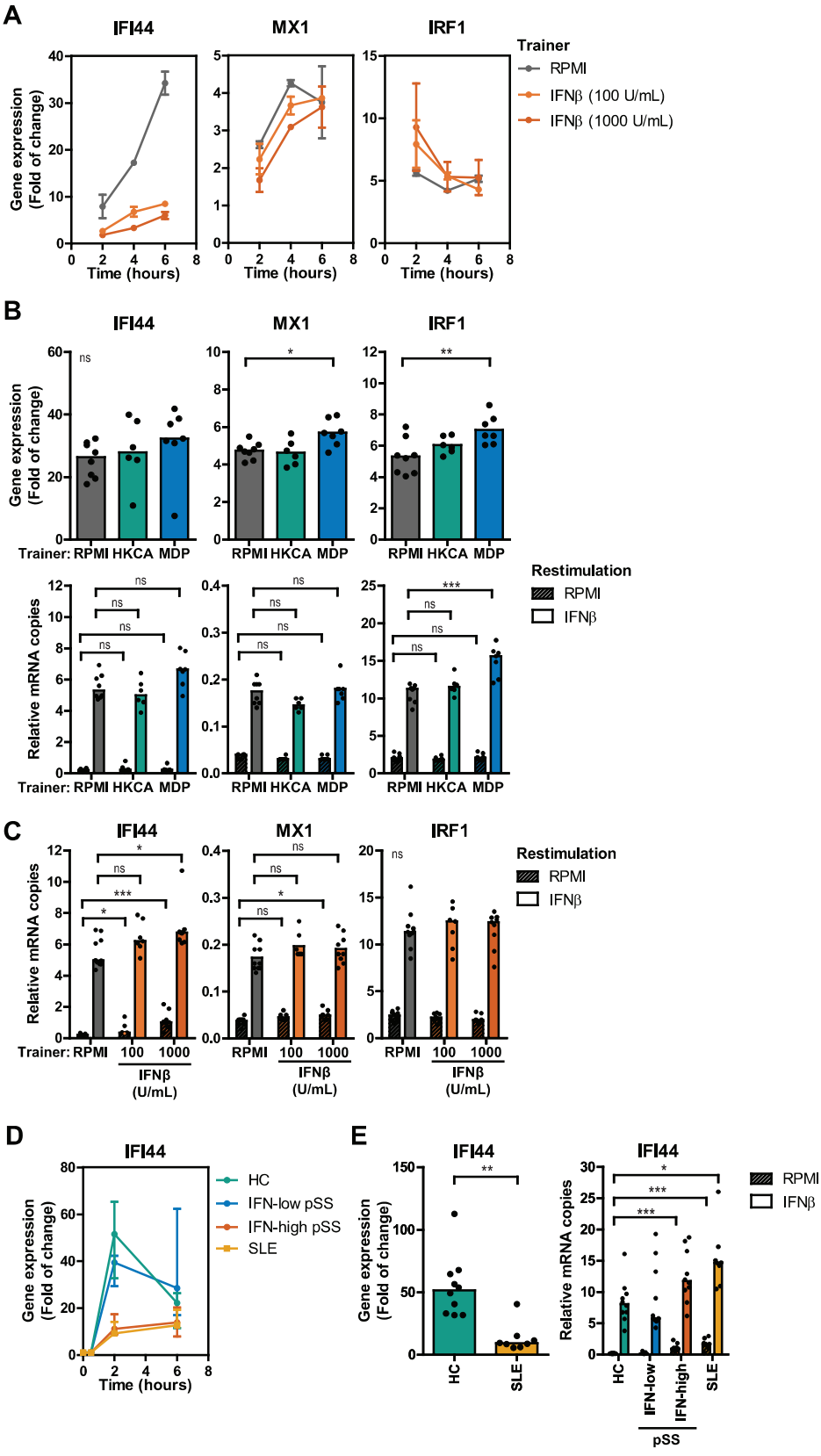


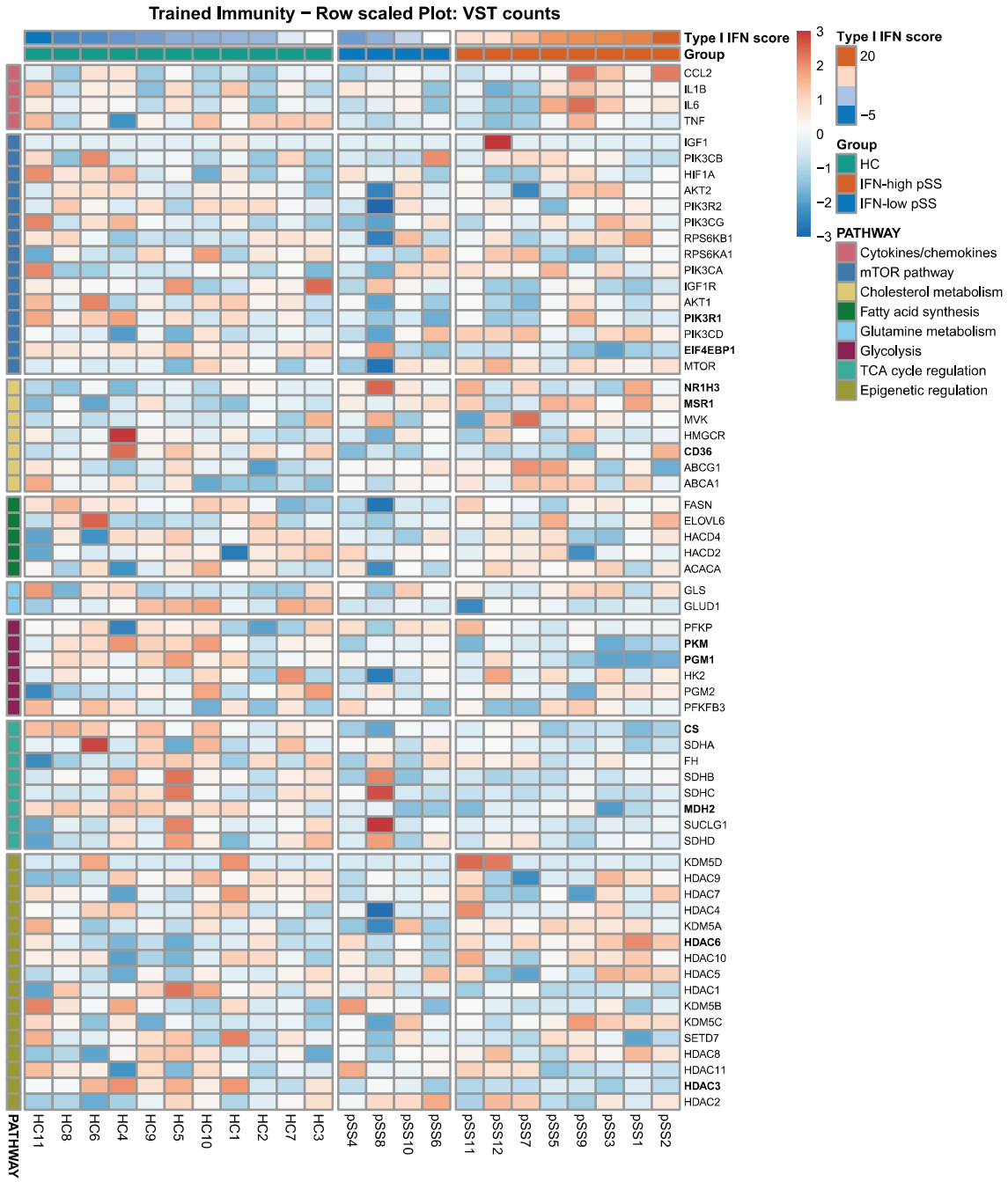
Supplementary Figure 7. Training with HKCA en MDP induce differential type I IFN response upon re-stimulation.

(A) Type I IFN bioactivity quantified by the HEK IFN-α/β reporter cell assay in culture supernatants of THP-1 cells trained with heat-killed *Candida albicans* (HKCA; 10^6 cells/mL), muramyl dipeptide (MDP; 50 µg/mL) or IFNβ and restimulated with 1 µg/mL LPS in the presence of 10% fetal calf serum for 24 hours. Lower limit of detection is approximately 0.8 U/mL (shaded area). **(B)** Relative mRNA expression ($2^{\Delta\Delta CT}$) of *IFI44* in HKCA-, MDP- or IFNβ-trained THP-1 cells re-stimulated with 1 µg/mL LPS + 10% fetal calf serum for 24 hours. Fold change expression was calculated relative to the LPS-stimulated untrained (RPMI) THP-1 cells. **(C)** Type I IFN bioactivity in supernatants of PBMCs from SLE patients and healthy controls (HC) stimulated with 10 ng/mL LPS for 24 hours. Symbols indicate averages of duplicates **(C)** or triplicates **(A,B)** and bars represent medians. Wilxocon singed rank test was used to compare medians with a hypothetical 1 and Mann-Whitney U test was used to compare groups. ns: not significant, * $p<0.05$.

Next page: Supplementary Figure 8. Differential ISG response to type I IFN in trained THP-1 and patient's PBMCs.

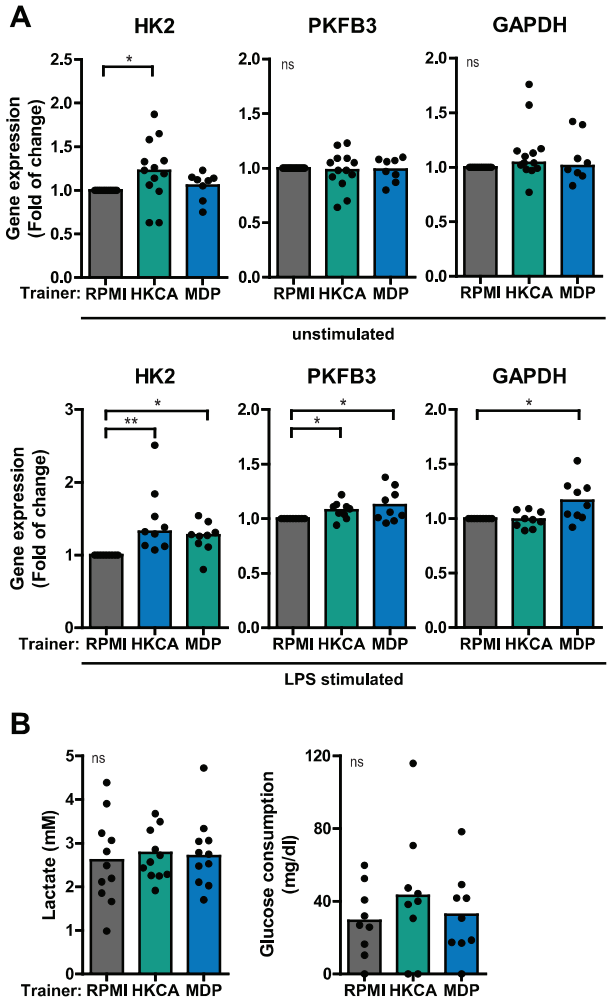
Transcript expression of *IFI44*, *MX1* and *IRF1* **(A)** over time in IFNβ-trained and untrained THP-1 cells or **(B-C)** at 4 hours in THP-1 cells trained with **(B)** heat-killed *Candida albicans* (HKCA; 10^6 cells/mL) or muramyl dipeptide (MDP; 50 µg/mL) or **(C)** IFNβ after re-stimulation with 100 IU/mL IFNβ indicated as fold change expression relative to unstimulated cells ($2^{\Delta\Delta CT}$) or relative mRNA copies normalized to household gene *ABL* ($2^{\Delta CT}$). **(D)** Relative mRNA expression ($2^{\Delta\Delta CT}$) of *IFI44* over time in PBMCs from patients with pSS stratified based on blood ISG expression, SLE and healthy controls (HC) stimulated with 100 IU/mL IFNβ. **(E)** Transcript expression of *IFI44* in SLE, pSS and HC PBMCs stimulated with 100 IU/mL IFNβ for 2 hours, indicated as fold change expression relative to unstimulated cells ($2^{\Delta\Delta CT}$) or relative mRNA count normalized to household gene *ABL* ($2^{\Delta CT}$). Depending on the data distribution, bars represent means or medians and One-way ANOVA, Kruskal-Wallis test OR Mann-Whitney U test were used to compare groups. ns: not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.


A
IFI44
MX1
IRF1
Trainer
RPMI
IFNβ (100 U/mL)
IFNβ (1000 U/mL)
Gene expression (Fold of change)
Time (hours)
B
IFI44
MX1
IRF1
Gene expression (Fold of change)
Trainer: RPMI HKCA MDP
Restimulation
RPMI
IFNβ
Relative mRNA copies
C
IFI44
MX1
IRF1
Restimulation
RPMI
IFNβ
Relative mRNA copies
Trainer: RPMI
100
1000
IFNβ (U/mL)
D
IFI44
HC
IFN-low pSS
IFN-high pSS
SLE
Gene expression (Fold of change)
Time (hours)
E
IFI44
Gene expression (Fold of change)
HC
SLE
IFI44
Relative mRNA copies
HC
IFN-low
IFN-high
SLE
pSS
RPMI
IFNβ



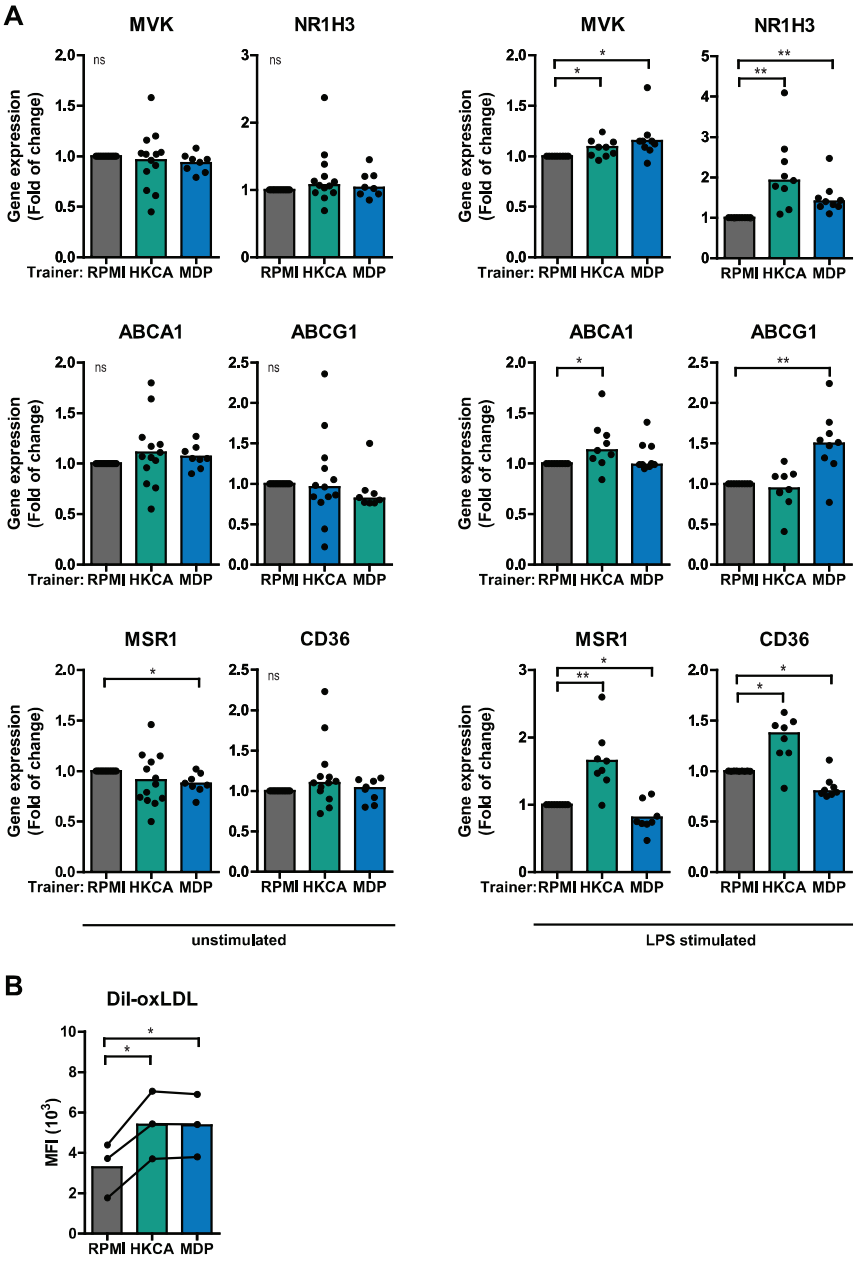
Supplementary Figure 9. Differential gene expression of genes associated with trained immunity in pSS monocytes.

Heatmap of z-scores calculated per gene using VST transformed counts of metabolism-related genes in RNAseq dataset (GSE173670) of *ex vivo* monocytes from pSS and HC. Patients were stratified based on their type I IFN score. Bold genes indicate significant differential expression (adjusted p-value < 0.1) in comparison analysis of either pSS versus HC or IFN-high vs IFN-low (details provided in Supplementary Table 2 and 3).



Supplementary Figure 10. Differential expression of genes related to glucose metabolism in HKCA- and MDP-trained THP-1 cells.

(A) Relative mRNA expression ($2^{\Delta\Delta CT}$) of *HK2*, *PKFB3* and *GAPDH* in THP-1 cells trained with heat-killed *Candida albicans* (HKCA; 10^6 cells/mL) or muramyl dipeptide (MDP; 50 µg/mL) either before re-stimulation (upper panel) or after 24 hour re-stimulation with 50 ng/mL LPS (lower panel). Fold change expression was calculated relative to the corresponding untrained (RPMI) cells. **(B)** Lactate concentrations (left) in culture supernatants and glucose consumption (right) by 24 hour LPS-stimulated THP-1 cells trained with HKCA or MDP. Depending on the data distribution, bars represent means or medians. One sample t-test or Wilxocon singed rank test were used to compare means/medians with a hypothetical 1 and repeated measures ANOVA was used to compare groups. ns: not significant, * p<0.05, **p<0.01.



Supplementary Figure 11. Differential cholesterol metabolism in HKCA- and MDP-trained THP-1 cells. **(A)** Relative mRNA expression ($2^{\Delta\Delta CT}$) of *MVK*, *NR1H3*, *ABCA1*, *ABCG1*, *MSR1* and *CD36* in THP-1 cells trained with heat-killed *Candida albicans* (HKCA; 10^6 cells/mL) or muramyl dipeptide (MDP; 50 µg/mL) either before re-stimulation (left panel) or after 24 hour re-stimulation with 50 ng/mL LPS (right panel). Fold change expression was calculated relative to the corresponding untrained (RPMI) cells. **(B)** Median fluorescence intensity (MFI) of HKCA- or MDP-trained THP-1 cells incubated with 50 µg/mL Dil-oxLDL for 4 hours. Depending on the data distribution, bars represent means or medians. One sample t-test or Wilxocon singed rank test were used to compare means/medians with a hypothetical 1 and repeated measures ANOVA was used to compare groups. ns: not significant, * $p<0.05$, ** $p<0.01$.

Supplementary Table 1. Demographic and clinical characteristics.

	HC	pSS		SLE (2)	
		IFN-low	IFN-high	adult	childhood
	n = 17	n = 11	n = 15	n = 9	n = 9
Demographics					
Female[a]	15/17 (94.1)	11/11 (100)	14/15 (93)	9/9 (100)	7/9 (77.8%)
Age [years][b]	53 (45-56)	64 (52.5-68)	58 (50-67.5)	42 (24-51)	14 (11-14)
Patient characteristics					
Disease duration [years][b]	-	6 (4-13)	15 (8.5-23)	17 (4-31)	0 (0-0)**
Disease activity[b] (1)	-	0 (0-6.5)	3 (2-8)	4 (2-4)	8 (5-14)
Laboratory parameters					
ANA[a]	-	6/11 (54.5)	15/15 (100)	9/9 (100)	9/9 (100)
Anti-SSA[a]	-	5/11 (45.5)	15/15 (100)	7/9 (77.8)	4/9 (44.4)
Anti-Ro52[a]	-	5/5 (100)	15/15 (100)	7/8 (87.5)*	4/4 (100)
Anti-Ro60[a]	-	4/5 (80)	14/15 (93.3)	7/8 (87.5)*	3/4 (75)
Anti-SSB[a]	-	2/11 (18.2)	13/15 (86.7)	5/9 (55.6)	2/9 (22.2)
Anti-dsDNA [IU/mL][b]	-	-	-	18 (1.4-62)	14 (1.3-182)
Anti-Sm[a]	-	-	-	2/9 (22.2)	4/9 (44.4)
Anti-RNP[a]	-	-	-	4/9 (44.4)	7/9 (77.8)
C3 [g/L][b]	-	1.15 (1.06-1.25)*	1.07 (1.03-1.19)*	0.97 (0.86-1.22)	1.07 (0.85-1.15)
C4 [g/L][b]	-	0.23 (0.18-0.26)*	0.18 (0.14-0.20)*	0.19 (0.13-0.21)	0.14 (0.11-0.16)
Current medication[a]					
HCQ	-	3/11 (27.3)	3/15 (20)	8/9 (88.8)	0/9 (0)
HCQ monotherapy	-	3/3 (100)	3/3 (100)	4/8 (50)	-
HCQ + corticosteroids / DMARDs	-	0/3 (0)	0/3 (0)	4/8 (50)	-
Corticosteroids/DMARDs	-	0/11 (0)	1/15 (6.7)	5/9 (55.5)	0/9 (0)
Corticosteroids + DMARDs	-	0/0 (0)	1/1 (100)	1/5 (20)	-
Corticosteroids only	-	0/0 (0)	0/1 (0)	2/5 (40)	-
DMARDs only	-	0/0 (0)	0/1 (0)	2/5 (40)	-
NSAIDs	-	2/11 (18.2)	1/15 (6.7)	3/9 (33.3)	2/9 (22.2)

Data are presented as number of patients (%)[a] or median (Q1-Q3)[b]. (1) Disease activity: EULAR Sjögren's syndrome disease activity index (ESSDAI) for pSS and Systemic Lupus Erythematosus disease activity index (SLEDAI) for SLE. (2) All (childhood) SLE patients were IFN-high. *Missing values for some of the patients. **All childhood-SLE patients were included at diagnosis and treatment naïve, except for NSAIDs. None of the pSS or (childhood) SLE patients were treated with statins or metformin. Abbreviations: ANA, anti-nuclear antibodies; DMARDs, disease-modifying anti-rheumatic drugs; HC, healthy controls; HCQ, hydroxychloroquine; NSAIDs, non-steroidal anti-inflammatory drugs; pSS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus.

Supplementary Table 2. Differential Gene Expression pSS vs HC.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000165029.17	chr9	1,05E+08	1,05E+08	-	protein_coding	ABCA1	827,2224	8,78E-06	0,001443	0,088672	0,270124
ENSG00000160179.19	chr21	42199689	42297244	+	protein_coding	ABCG1	201,0209	7,21E-06	0,001443	0,039829	0,17224
ENSG00000278540.5	chr17	37084992	37406836	-	protein_coding	ACACA	303,0225	2,36E-07	0,001443	0,72494	0,858021
ENSG00000142208.17	chr14	1,05E+08	1,05E+08	-	protein_coding	AKT1	4074,608	-8,5E-05	0,001444	0,061234	0,218808
ENSG00000105221.18	chr19	40230317	40285536	-	protein_coding	AKT2	2588,785	6,71E-05	0,001443	0,680424	0,831798
ENSG00000108691.9	chr17	34255218	34257203	+	protein_coding	CCL2	153,806	3,42E-06	0,001443	0,015876	0,102988
ENSG00000135218.19	chr7	80369575	80679277	+	protein_coding	CD36	13634,89	-5,5E-05	0,001443	0,012519	0,089443
ENSG00000062485.19	chr12	56271699	56300391	-	protein_coding	CS	3733,867	-0,13666	0,060462	0,000649	0,015179
ENSG00000187840.5	chr8	38030534	38060365	+	protein_coding	EIF4EBP1	937,9822	-0,30573	0,132313	0,000568	0,013912
ENSG00000170522.10	chr4	1,1E+08	1,1E+08	-	protein_coding	ELOVL6	13,7378	4,95E-08	0,001443	0,991369	
ENSG00000169710.9	chr17	82078338	82098294	-	protein_coding	FASN	632,5709	-7,5E-06	0,001443	0,168572	0,390723
ENSG00000091483.7	chr1	2,41E+08	2,42E+08	-	protein_coding	FH	653,7212	-1,7E-05	0,001443	0,646794	0,809227
ENSG00000115419.13	chr2	1,91E+08	1,91E+08	+	protein_coding	GLS	894,351	4,44E-06	0,001443	0,413467	0,642548
ENSG00000148672.9	chr10	87050202	87094843	-	protein_coding	GLUD1	5070,729	-3,8E-05	0,001443	0,015974	0,103281
ENSG00000206527.10	chr3	1,23E+08	1,24E+08	-	protein_coding	HACD2	1177,55	2,56E-06	0,001443	0,789932	0,893024
ENSG00000188921.14	chr9	20999509	21031640	-	protein_coding	HACD4	4014,552	-7,6E-06	0,001443	0,44216	0,665633
ENSG00000116478.12	chr1	32292083	32333635	+	protein_coding	HDAC1	2366,029	-3E-05	0,001443	0,044664	0,184341
ENSG00000100429.18	chr22	50245183	50251405	-	protein_coding	HDAC10	1258,13	2,76E-05	0,001443	0,365921	0,60026
ENSG00000163517.15	chr3	13479724	13506424	+	protein_coding	HDAC11	109,2857	-2,2E-06	0,001443	0,866556	0,935509
ENSG00000196591.12	chr6	1,14E+08	1,14E+08	-	protein_coding	HDAC2	1021,509	1,55E-05	0,001443	0,104099	0,296805
ENSG00000171720.10	chr5	1,42E+08	1,42E+08	-	protein_coding	HDAC3	1401,003	-0,23756	0,077806	7,5E-05	0,003877
ENSG00000068024.17	chr2	2,39E+08	2,39E+08	-	protein_coding	HDAC4	1012,384	-3,7E-06	0,001443	0,427291	0,652725
ENSG00000108840.15	chr17	44076746	44123702	-	protein_coding	HDAC5	1701,144	1,38E-05	0,001443	0,153233	0,36976
ENSG00000094631.21	chrX	48801377	48824982	+	protein_coding	HDAC6	1022,455	0,317338	0,172628	0,001436	0,024184
ENSG00000061273.18	chr12	47782722	47833132	-	protein_coding	HDAC7	1686,498	-7,4E-06	0,001443	0,395787	0,626171
ENSG00000147099.21	chrX	72329516	72573101	-	protein_coding	HDAC8	267,2832	1,61E-05	0,001443	0,281722	0,522785
ENSG00000048052.23	chr7	18086949	19002416	+	protein_coding	HDAC9	604,9947	-5,3E-06	0,001443	0,309094	0,548474
ENSG00000100644.17	chr14	61695513	61748259	+	protein_coding	HIF1A	8074,215	-3,3E-06	0,001443	0,269224	0,510691
ENSG00000159399.10	chr2	74834127	74893359	+	protein_coding	HK2	3648,353	-2,9E-06	0,001443	0,719458	0,854285

Supplementary Table 2 (continued). Differential Gene Expression pSS vs HC.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000113161.17	chr5	75336329	75364001	+	protein_coding	HMGCR	1545,374	-6,2E-06	0,001443	0,394003	0,624068
ENSG00000017427.17	chr12	1,02E+08	1,02E+08	-	protein_coding	IGF1	0,347234	2,21E-07	0,001443	0,887791	
ENSG00000140443.15	chr15	98648539	98964530	+	protein_coding	IGF1R	375,528	-9,4E-06	0,001443	0,022157	0,125209
ENSG00000125538.12	chr2	1,13E+08	1,13E+08	-	protein_coding	IL1B	5258,49	-5,3E-07	0,001443	0,752346	0,872085
ENSG00000136244.12	chr7	22725884	22732002	+	protein_coding	IL6	23,26775	1,34E-06	0,001443	0,256092	0,49561
ENSG00000073614.13	chr12	280057	389320	-	protein_coding	KDM5A	1955,384	-8,9E-05	0,001444	0,535489	0,736273
ENSG00000117139.18	chr1	2,03E+08	2,03E+08	-	protein_coding	KDM5B	582,7864	-8,6E-06	0,001443	0,385152	0,616681
ENSG00000126012.12	chrX	53191321	53225422	-	protein_coding	KDM5C	2432,619	1,62E-05	0,001443	0,062544	0,220948
ENSG00000012817.16	chrY	19703865	19744939	-	protein_coding	KDM5D	0,241385	1,54E-07	0,001443	0,798082	
ENSG00000146701.12	chr7	76048051	76067508	+	protein_coding	MDH2	2522,976	-6E-05	0,001443	3,87E-05	0,002408
ENSG00000038945.15	chr8	16107878	16567490	-	protein_coding	MSR1	623,1403	1,04521	0,418009	0,000346	0,01043
ENSG00000198793.13	chr1	11106535	11262551	-	protein_coding	MTOR	1221,023	8,04E-06	0,001443	0,210214	0,444142
ENSG00000110921.14	chr12	1,1E+08	1,1E+08	+	protein_coding	MVK	101,5056	7,13E-05	0,001444	0,211088	0,445382
ENSG00000025434.19	chr11	47248300	47269033	+	protein_coding	NR1H3	189,666	1,27E-05	0,001443	0,01286	0,091081
ENSG00000170525.21	chr10	6144934	6254644	+	protein_coding	PFKFB3	8127,08	-1,1E-06	0,001443	0,626671	0,796776
ENSG00000067057.18	chr10	3066333	3137718	+	protein_coding	PFKP	437,5825	3,58E-06	0,001443	0,529533	0,732172
ENSG00000079739.17	chr1	63593411	63660245	+	protein_coding	PGM1	828,6456	-4,5E-05	0,001443	0,007746	0,067386
ENSG00000169299.14	chr4	37826660	37862937	+	protein_coding	PGM2	1152,005	-3,9E-06	0,001443	0,944345	0,974563
ENSG00000121879.6	chr3	1,79E+08	1,79E+08	+	protein_coding	PIK3CA	845,4326	7,69E-06	0,001443	0,155889	0,372551
ENSG00000051382.9	chr3	1,39E+08	1,39E+08	-	protein_coding	PIK3CB	1284,814	-4E-05	0,001443	0,640697	0,805109
ENSG00000171608.16	chr1	9651731	9729114	+	protein_coding	PIK3CD	4898,363	-3,8E-06	0,001443	0,021621	0,124074
ENSG00000105851.11	chr7	1,07E+08	1,07E+08	+	protein_coding	PIK3CG	1621,753	-3,6E-06	0,001443	0,625234	0,795712
ENSG00000145675.15	chr5	68215756	68301821	+	protein_coding	PIK3R1	2145,902	5,27E-05	0,001443	0,011734	0,086538
ENSG00000105647.19	chr19	18153163	18170532	+	protein_coding	PIK3R2	290,2064	-4,3E-05	0,001443	0,210526	0,444439
ENSG00000067225.19	chr15	72199029	72231822	-	protein_coding	PKM	28970,43	-0,19922	0,07482	0,000235	0,008142
ENSG00000117676.14	chr1	26529761	26575030	+	protein_coding	RPS6KA1	4528,852	-5,9E-06	0,001443	0,818774	0,908676
ENSG00000108443.14	chr17	59893046	59950574	+	protein_coding	RPS6KB1	699,0111	-3,2E-06	0,001443	0,783276	0,889577
ENSG00000073578.17	chr5	218241	257082	+	protein_coding	SDHA	1729,95	-1,4E-05	0,001442	0,250564	0,490326
ENSG00000117118.10	chr1	17018722	17054032	-	protein_coding	SDHB	1933,785	-2E-05	0,001443	0,048581	0,193748

Supplementary Table 2 (continued). Differential Gene Expression pSS vs HC.

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000143252.16	chr1	1,61E+08	1,61E+08	+	protein_coding	SDHC	1699,93	-5,1E-06	0,001443	0,275724	0,51667
ENSG00000204370.13	chr11	1,12E+08	1,12E+08	+	protein_coding	SDHD	2747,383	-1,5E-06	0,001443	0,845341	0,923996
ENSG00000145391.14	chr4	1,39E+08	1,4E+08	-	protein_coding	SETD7	1608,061	-2E-05	0,001443	0,071301	0,237185
ENSG00000163541.12	chr2	84423528	84460045	-	protein_coding	SUCLG1	1121,824	1,49E-07	0,001443	0,952543	0,977987
ENSG00000232810.4	chr6	31575565	31578336	+	protein_coding	TNF	288,8001	-7,9E-06	0,001443	0,03037	0,148414

Supplementary Table 3. Differential Gene Expression IFN-high vs IFN-low PSS

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000165029.17	chr9	1,05E+08	1,05E+08	-	protein_coding	ABCA1	956,6842	5,84E-06	0,001443	0,134928	0,55007
ENSG00000160179.19	chr21	42199689	42297244	+	protein_coding	ABCG1	246,9822	1,18E-06	0,001443	0,632372	0,897901
ENSG00000278540.5	chr17	37084992	37406836	-	protein_coding	ACACA	299,217	5,37E-06	0,001443	0,088548	0,48085
ENSG00000142208.17	chr14	1,05E+08	1,05E+08	-	protein_coding	AKT1	3966,724	0,000138	0,001446	0,475261	0,830905
ENSG00000105221.18	chr19	40230317	40285536	-	protein_coding	AKT2	2608,2	2,49E-06	0,001443	0,671698	0,912961
ENSG00000108691.9	chr17	34255218	34257203	+	protein_coding	CCL2	228,9842	3,49E-06	0,001443	0,007955	0,227138
ENSG00000135218.19	chr7	80369575	80679277	+	protein_coding	CD36	12576,71	-2E-06	0,001443	0,290303	0,721229
ENSG00000062485.19	chr12	56271699	56300391	-	protein_coding	CS	3565,183	2E-05	0,001443	0,921411	0,979859
ENSG00000187840.5	chr8	38030534	38060365	+	protein_coding	EIF4EBP1	831,3081	-1,2E-05	0,001443	0,107237	0,511603
ENSG00000170522.10	chr4	1,1E+08	1,1E+08	-	protein_coding	ELOVL6	13,06553	2,62E-06	0,001443	0,027094	0,331468
ENSG00000169710.9	chr17	82078338	82098294	-	protein_coding	FASN	606,7884	1,11E-05	0,001443	0,004678	0,182668
ENSG00000091483.7	chr1	2,41E+08	2,42E+08	-	protein_coding	FH	681,2073	5,45E-07	0,001443	0,751157	0,934963
ENSG00000115419.13	chr2	1,91E+08	1,91E+08	+	protein_coding	GLS	1066,15	-2,4E-06	0,001443	0,802701	0,948924
ENSG00000148672.9	chr10	87050202	87094843	-	protein_coding	GLUD1	4901,224	-3,8E-06	0,001443	0,882801	0,971219
ENSG00000206527.10	chr3	1,23E+08	1,24E+08	-	protein_coding	HACD2	1212,87	-4,3E-06	0,001443	0,558181	0,867894
ENSG00000188921.14	chr9	20999509	21031640	-	protein_coding	HACD4	3985,042	8,35E-06	0,001443	0,661458	0,909232
ENSG00000116478.12	chr1	32292083	32333635	+	protein_coding	HDAC1	2281,307	1,07E-05	0,001443	0,354782	0,76217
ENSG00000100429.18	chr22	50245183	50251405	-	protein_coding	HDAC10	1334,58	7,84E-06	0,001443	0,248116	0,685343
ENSG00000163517.15	chr3	13479724	13506424	+	protein_coding	HDAC11	108,6144	-2E-06	0,001443	0,320185	0,743667
ENSG00000196591.12	chr6	1,14E+08	1,14E+08	-	protein_coding	HDAC2	1094,198	-1,4E-05	0,001443	0,230589	0,669568
ENSG00000171720.10	chr5	1,42E+08	1,42E+08	-	protein_coding	HDAC3	1304,791	-6,5E-06	0,001443	0,597819	0,884493
ENSG00000068024.17	chr2	2,39E+08	2,39E+08	-	protein_coding	HDAC4	1001,587	5,93E-06	0,001443	0,051859	0,401728
ENSG00000108840.15	chr17	44076746	44123702	-	protein_coding	HDAC5	1849,306	-1,8E-05	0,001443	0,610384	0,890086
ENSG00000094631.21	chrX	48801377	48824982	+	protein_coding	HDAC6	1176,869	2,77E-06	0,001443	0,035484	0,356562
ENSG00000061273.18	chr12	47782722	47833132	-	protein_coding	HDAC7	1675,489	-1,8E-06	0,001443	0,910034	0,977302
ENSG00000147099.21	chrX	72329516	72573101	-	protein_coding	HDAC8	286,654	6,05E-06	0,001443	0,37606	0,772755
ENSG00000048052.23	chr7	18086949	19002416	+	protein_coding	HDAC9	559,9909	5,14E-06	0,001443	0,397528	0,783797
ENSG00000100644.17	chr14	61695513	61748259	+	protein_coding	HIF1A	7324,636	-4,6E-07	0,001443	0,702176	0,921555
ENSG00000159399.10	chr2	74834127	74893359	+	protein_coding	HK2	3693,382	1,23E-05	0,001443	0,030628	0,340457

Supplementary Table 3 (continued). Differential Gene Expression IFN-high vs IFN-low PSS

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000113161.17	chr5	75336329	75364001	+	protein_coding	HMGCR	1557,228	0,000144	0,001446	0,652572	0,906849
ENSG00000017427.17	chr12	1,02E+08	1,02E+08	-	protein_coding	IGF1	0,564419	1,78E-07	0,001443	0,829938	
ENSG00000140443.15	chr15	98648539	98964530	+	protein_coding	IGF1R	323,7296	-6E-06	0,001443	0,072617	0,451582
ENSG00000125538.12	chr2	1,13E+08	1,13E+08	-	protein_coding	IL1B	5052,055	1,44E-06	0,001443	0,730102	0,929266
ENSG00000136244.12	chr7	22725884	22732002	+	protein_coding	IL6	30,08453	7,1E-07	0,001443	0,159911	0,588553
ENSG00000073614.13	chr12	280057	389320	-	protein_coding	KDM5A	2086,172	1,49E-05	0,001443	0,044717	0,384941
ENSG00000117139.18	chr1	2,03E+08	2,03E+08	-	protein_coding	KDM5B	563,4534	-1,5E-05	0,001443	0,292502	0,722412
ENSG00000126012.12	chrX	53191321	53225422	-	protein_coding	KDM5C	2741,996	-4,6E-06	0,001443	0,323549	0,747808
ENSG00000012817.16	chrY	19703865	19744939	-	protein_coding	KDM5D	0,409929	2,6E-07	0,001443	0,877708	
ENSG00000146701.12	chr7	76048051	76067508	+	protein_coding	MDH2	2373,968	1,22E-05	0,001443	0,68586	0,918814
ENSG00000038945.15	chr8	16107878	16567490	-	protein_coding	MSR1	899,0994	-8,3E-06	0,001443	0,329668	0,751493
ENSG00000198793.13	chr1	11106535	11262551	-	protein_coding	MTOR	1324,912	8,93E-06	0,001443	0,060896	0,421898
ENSG00000110921.14	chr12	1,1E+08	1,1E+08	+	protein_coding	MVK	113,8473	8E-07	0,001443	0,805908	0,950093
ENSG00000025434.19	chr11	47248300	47269033	+	protein_coding	NR1H3	231,5444	-3,5E-06	0,001443	0,283499	0,716614
ENSG00000170525.21	chr10	6144934	6254644	+	protein_coding	PFKFB3	7638,744	-2,3E-05	0,001443	0,971038	0,992425
ENSG00000067057.18	chr10	3066333	3137718	+	protein_coding	PFKP	471,5435	2,61E-05	0,001443	0,694012	0,92047
ENSG00000079739.17	chr1	63593411	63660245	+	protein_coding	PGM1	762,6009	-1,2E-05	0,001443	0,08477	0,474033
ENSG00000169299.14	chr4	37826660	37862937	+	protein_coding	PGM2	1164,816	-3,2E-06	0,001443	0,820474	0,95453
ENSG00000121879.6	chr3	1,79E+08	1,79E+08	+	protein_coding	PIK3CA	903,7166	3,25E-06	0,001443	0,413492	0,793562
ENSG00000051382.9	chr3	1,39E+08	1,39E+08	-	protein_coding	PIK3CB	1343,898	4,99E-07	0,001443	0,910854	0,977418
ENSG00000171608.16	chr1	9651731	9729114	+	protein_coding	PIK3CD	5498,368	8,53E-06	0,001443	0,15962	0,588381
ENSG00000105851.11	chr7	1,07E+08	1,07E+08	+	protein_coding	PIK3CG	1661,638	2,09E-06	0,001443	0,234686	0,672455
ENSG00000145675.15	chr5	68215756	68301821	+	protein_coding	PIK3R1	1894,606	1,97E-05	0,001443	0,088181	0,480605
ENSG00000105647.19	chr19	18153163	18170532	+	protein_coding	PIK3R2	267,1642	2,81E-06	0,001443	0,421617	0,799213
ENSG00000067225.19	chr15	72199029	72231822	-	protein_coding	PKM	27095,07	-9,9E-05	0,001444	0,490426	0,837167
ENSG00000117676.14	chr1	26529761	26575030	+	protein_coding	RPS6KA1	4580,177	2,04E-06	0,001443	0,881792	0,971163
ENSG00000108443.14	chr17	59893046	59950574	+	protein_coding	RPS6KB1	732,3753	3,3E-06	0,001443	0,296862	0,72613
ENSG00000073578.17	chr5	218241	257082	+	protein_coding	SDHA	1727,962	-1,3E-05	0,001442	0,373079	0,77193
ENSG00000117118.10	chr1	17018722	17054032	-	protein_coding	SDHB	1825,299	-1,8E-05	0,001443	0,515653	0,849055

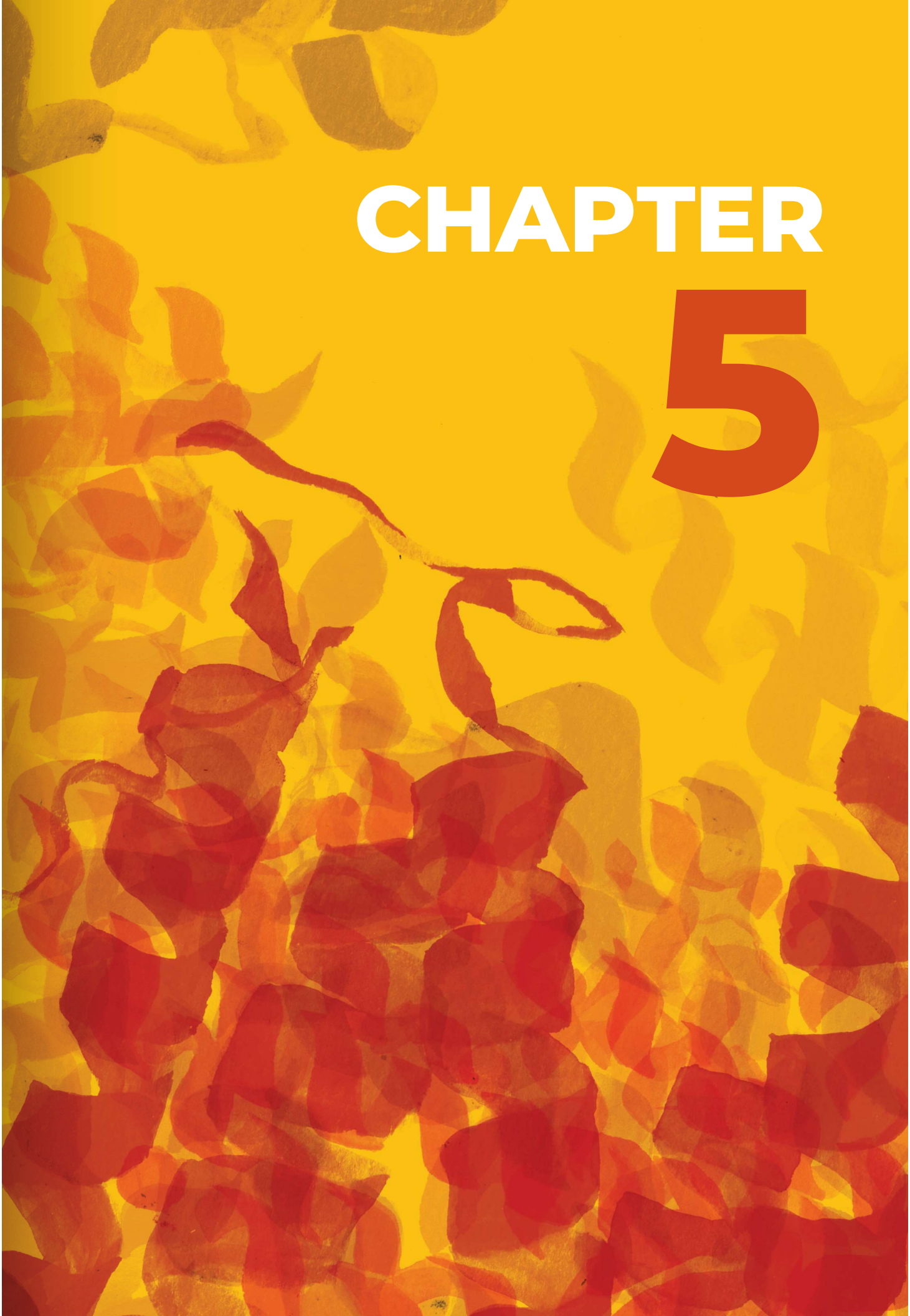
Supplementary Table 3 (continued). Differential Gene Expression IFN-high vs IFN-low PSS

Gene_ID	chromosome	start	end	strand	gene_type	gene_name	baseMean	log2FoldChange	lfcSE	pvalue	padj
ENSG00000143252.16	chr1	1,61E+08	1,61E+08	+	protein_coding	SDHC	1666,464	-2,8E-06	0,001443	0,067367	0,43917
ENSG00000204370.13	chr11	1,12E+08	1,12E+08	+	protein_coding	SDHD	2786,111	-1,6E-05	0,001443	0,37811	0,77335
ENSG00000145391.14	chr4	1,39E+08	1,4E+08	-	protein_coding	SETD7	1657,052	-1,8E-06	0,001443	0,768125	0,938568
ENSG00000163541.12	chr2	84423528	84460045	-	protein_coding	SUCLG1	1144,965	-6,5E-06	0,001443	0,073423	0,453306
ENSG00000232810.4	chr6	31575565	31578336	+	protein_coding	TNF	237,8759	1,94E-06	0,001443	0,500141	0,843868

Supplementary Table 4. Multivariable logistic regression analysis.

Type I IFN pathway activation (IFN-low / IFN-high) ~	P value
Cardiovascular events	**0.042**
Hypertension	0.111
Smoking (past or current)	0.320
Body mass index	0.256
Age	0.843
NSAID (current)	0.824
Hydroxychloroquine (current)	0.213
Statins (current)	0.331

Shown are p-values of effect likelihood ratio tests used to determine a relationship between type I IFN pathway activation (IFN-low vs IFN-high) and the covariates.


CHAPTER
5

SUMMARY AND GENERAL CONCLUSIONS

Assays measuring type I IFN pathway activation

I. The Myxovirus resistance protein 1 (MxA) is upregulated in response to type I and type III IFNs. Protein expression of MxA measured in whole blood by an immunoassay is highly correlated (r_s=0.735 to r_s=0.854, p≤0.003) to the blood type I ISG transcript expression (type I IFN score) in patients with SLE and SSc. The MxA-immunoassay shows excellent performance (AUC 0.938-0.991) to discriminate low and high type I IFN score and is therefore a suitable alternative for blood type I ISG expression analysis in SLE and SSc.

II. Blood type I IFN score and MxA expression in pSS, as well as in SLE and SSc, are correlated with circulating IFNα2 protein detected by single-molecule array (Simoa) technology. Serum IFNα2 levels in pSS are lower compared with SLE. Serum IFNα2 and blood MxA have comparable ability to identify pSS patients with a high type I IFN score. Circulating IFNα2 in pSS associates with disease-relevant serological parameters, treatment and systemic disease manifestations.

Cytosolic nucleic acid-sensing pathways

III. The nucleic acid receptor signaling component TBK1 shows higher levels of phosphorylation – indicative of signaling activity – in pDCs from IFN-high pSS, SLE and SSc patients. Inhibition of TBK1 reduces TLR7-stimulated and spontaneous ISG expression in PBMCs from IFN-high pSS, SLE and SSc.

IV. Monocytes from pSS patients show hyperresponsive IFNα production and STING phosphorylation upon stimulation of the cytosolic DNA-sensing pathway. Several regulators of DNA-sensing pathway activation are differentially expressed in pSS monocytes compared with healthy controls (HC). In contrast to HC pDCs from pSS patients are sensitive to STING pathway stimulation. Similar observations were made in SLE, indicating that this hyperresponsiveness is not pSS specific.

Trained immunity

V. Type I IFNs induce trained immunity in an *in vitro* monocytic model exemplified by increased pro-atherogenic cytokine responses and altered cellular glucose and cholesterol metabolism. Training with heat-killed *Candida albicans*, MDP and type I IFNs alters type I IFN responses to secondary immune stimulation. In addition, training affects the ISG transcript expression patterns after re-stimulation with type I IFN. *Ex vivo* PBMCs from pSS patients, most clearly the IFN-high pSS, show cellular response patterns consistent with type I IFN-induced trained immunity.

This chapter discusses the pathophysiological and clinical implications of these findings, related recent developments and future perspectives.

TYPE I IFN ASSAYS IN RESEARCH

Aberrant activation of the type I IFN pathway has been a consistent finding in a subgroup of patients with pSS and other systemic autoimmune rheumatic diseases. A considerable body of research suggests a pathogenic role for type I IFNs in these diseases [1]. The most recent development that further supports this notion is the clinical benefit and FDA approval of anifrolumab – an IFNAR blocking antibody – for treatment of active SLE [2, 3].

Principles of type I IFN assays

Type I IFN pathway activation encompasses a broad concept from the transcription of type I IFNs to the downstream effects of IFNAR signaling. Assays for quantification of type I IFN activation can be divided into two main categories. The first group of assays concentrate around the type I IFN proteins. These methods either directly detect the type I IFN proteins in (ultrasensitive) immunoassays or quantify the biological effects of type I IFNs using reporter gene systems, cytopathic effect assays or plaque reduction assays. The second group of assays measure the *in vivo* downstream consequences of type I IFN signaling ranging from analysis of DNA methylation to ISG protein expression.

Up to now, these assays have mainly been used in a research setting to study the role of type I IFNs in disease pathogenesis and clinical disease activity. In this context, the quantified biomarker should accurately and specifically reflect type I IFN pathway activity. The IFN assays used in this thesis cover both ends of the spectrum of type I IFN pathway activation and are highly specific for the type I IFN pathway [4-6]. The assays used in research should preferably have a low limit of detection, although this prerequisite may be less relevant for some research questions. Recently, the ultrasensitive Simoa technology has been adopted for direct measurement of IFNα protein, primarily in SLE [7-10]. Using this method in two independently collected cohorts, **chapter 2.2** describes elevated circulating IFNα2 in pSS patients with high blood ISG expression for the first time.

Diversity and level of agreement between type I IFN assays

Although abundant literature exists reporting individual type I IFN assays, side-by-side comparison of multiple type I IFN assays in paired samples is scarce [9, 11, 12]. **Chapter 2** shows moderate to high correlations between assays in a side-by-side comparison of the type I IFN score with the MxA immunoassay, IFNα2 Simoa, and type I IFN reporter assay in pSS. Of these assays, the blood type I IFN score appeared the most sensitive to measure

type I IFN pathway activation in patients, closely followed by the MxA immunoassay and IFNα2 Simoa. The type I IFN reporter assay was far less sensitive to detect low levels of type I IFNs. The most relevant level of assay sensitivity will dependent on the research question.

In addition, side-by-side comparison of multiple type I IFN assays between distinct disease entities are also limited so far [8, 11, 13-16]. **Chapter 2** revealed positive correlations between the four type I IFN assays used throughout this thesis in pSS, SLE and SSc. Yet, the parallel measurements also indicated slight differences in type I IFN pathway activation between these systemic autoimmune diseases. In accordance with literature [8, 11], SLE was found to have the strongest type I IFN pathway activation, both in terms of percentage IFN-high patients as well as the average level of MxA, IFNα2 and type I IFN score among the IFN-high patients. Interestingly, when considering samples from pSS and SLE patients with comparable type I IFN scores and MxA levels, the serum IFNα2 concentrations appeared lower in pSS (Figure 1). In pediatric SLE patients with moderately high type I IFN scores or MxA levels, the average serum IFNα2 concentration also seemed to be higher than those observed in pSS in **chapter 2.2** (unpublished results, Wahadat, *et al.*).

Various hypotheses could explain this subtle diversity in type I IFN pathway activation between pSS and SLE of which five are discussed below.

I. Cellular composition in whole blood might differ between pSS and SLE. Indeed, single cell analysis has suggested expansion of certain cell types in SLE, for example the low-density granulocytes, an expansion not seen in pSS [17, 18]. Single cell analysis in SLE patients has shown variable ISG expression between different cell types in peripheral blood [19]. Therefore, expansion or contraction of specific cell types may impact the overall blood type I IFN score.

II. The cell-intrinsic responsiveness of blood cells to type I IFN stimulation might be different between pSS and SLE. However, the results presented in **chapter 4** and Figure 2 do not support this hypothesis, since PBMCs from IFN-high pSS and SLE

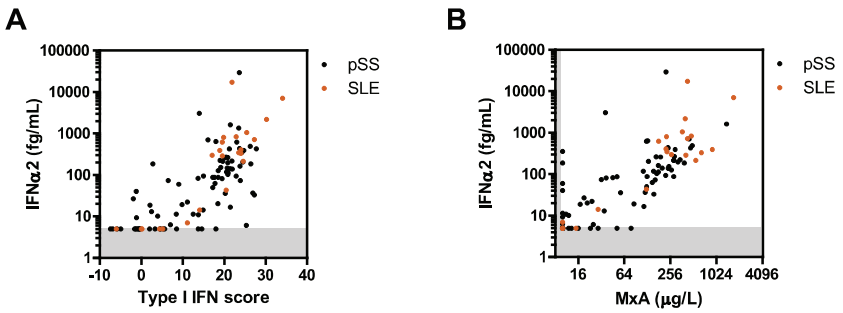


Figure 1. Higher serum IFNα2 concentrations in SLE than pSS with comparable blood ISG expression.
Correlation between serum IFNα2 concentrations and blood **(A)** type I IFN score or **(B)** MxA levels in pSS and SLE. Data extracted from Chapter 2.2, Rotterdam cohort.

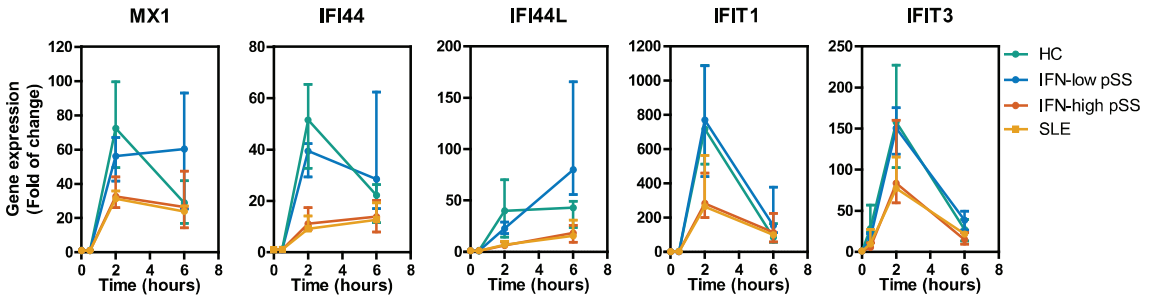


Figure 2. Comparable transcriptional ISG response in PBMCs from pSS and SLE.

Relative mRNA expression ($2^{-\Delta\Delta CT}$) of *MX1*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* in PBMCs from pSS (IFN-low, n=11; IFN-high, n=10), IFN-high SLE (n=8) and HC (n=10) stimulated with 100 U/mL of recombinant IFN-β1a for 0.5, 2 or 6 hours. Fold change expression was calculated normalized to household gene *ABL* and relative to unstimulated cells. Data represent medians ± interquartile range.

stimulated with IFNβ displayed highly similar response patterns of the type I IFN score genes *IFI44*, *IFI44L*, *IFIT1*, *IFIT3* and *MX1*.

III. The relative abundance of type I IFN subtypes other than the quantified IFNα2 could differ between pSS and SLE, also depending on the in vivo stimulus and involved cell types [20]. For instance, keratinocytes in SLE skin have been shown to produce IFNκ upon UV light exposure [21], which has not been implicated in pSS thus far. The individual type I IFN subtypes differ from each other with regard to their induction kinetics, amino acid sequence, post-translational modifications and IFNAR affinity [22-28]. Recently, Bondet, et al. compared ultrasensitive quantification of IFNα2 and pan-IFNα in patients with (chronic) viral infection and systemic autoimmune diseases [20]. In this analysis, the ratio between pan-IFNα and IFNα2 concentrations was highly variable between individual SLE and pSS patients. This indicates that although IFNα2 correlates with pan-IFNα on a cohort level in pSS and SLE patients, IFNα2 may not accurately reflect pan-IFNα concentrations for each individual patient. Interestingly, pan-IFNα/IFNα2 ratios higher than 1 were more frequently observed in pSS than SLE. This suggests that involvement of IFNα subtypes other than IFNα2 is more specifically associated with pSS than SLE.

IV. Other type I IFN signaling modulating factors might vary between pSS and SLE. The correlations between serum IFNα2 and the ISG response in the luciferase reporter assay or normal PBMCs described in **chapter 2.2,** and illustrated in Figure 3, are imperfect. In fact, pSS sera with the highest IFNα2 concentrations did not elicit a luciferase signal in the reporter assay whereas SLE sera with equal IFNα2 levels induced strong ISG expression. These data suggest that factors other than IFNα2 – either other (type I) IFN subtypes or non-IFN components – in serum modulate the downstream ISG response. Literature indicates that anti-IFNα autoantibodies

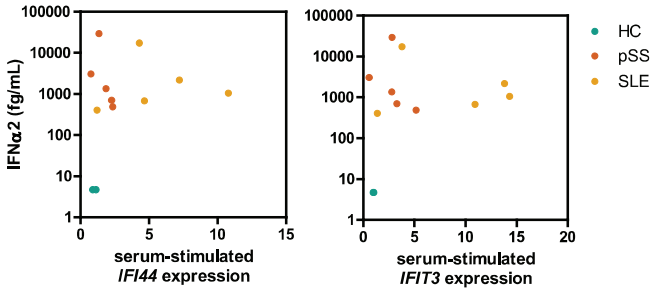


Figure 3. Poor correlation between serum IFNα2 concentrations and *in vitro* serum-stimulated ISG expression.

Relative mRNA expression ($2^{-\Delta CT}$) of *IFI44* and *IFIT3* in cryopreserved PBMCs from a healthy donor (4.10e5 cells/well) stimulated with 50% serum from pSS, SLE and HC for 5 hours plotted against the corresponding Simoa-quantified IFNα2 concentrations in these sera

in serum from pSS and SLE might explain these effects [20, 29, 30]. However, anti-IFNα autoantibodies were not detected in the samples used in **chapter 2.2**. To further investigate differential modulation of type I IFN signaling by serum from pSS and SLE patients, sera were spiked with recombinant IFNα2 and analyzed in the type I IFN reporter assay. Interestingly, the quantified levels of IFNα2 (corrected for levels in unspiked serum) were higher in SLE than pSS serum (Figure 4). These observations suggest that currently uncharacterized components in SLE serum enhance type I IFN signaling, while pSS serum suppresses type I IFN signaling.

V. The anatomical location of *in vivo* ISG induction in circulating immune cells could be different between pSS and SLE. The cellular source of IFNα2 and anatomical location of production in these patients have not been fully elucidated, but are assumed to be located in the tissues [19]. This would imply the type I IFNs in serum have spilled over from the tissues. The extend of spill-over and produced type I IFN subtypes might depend on the specific tissues that are involved. **Chapter 2.2** shows the type I IFNs in serum from some SLE patients, and to a lesser extend pSS, are capable of inducing ISG expression in normal PBMCs *in vitro*. However, a poor correlation exists between the *in vitro* ISG induction and *in vivo* type I IFN score

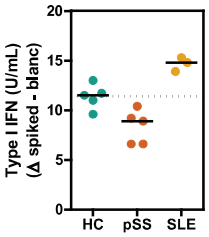


Figure 4. Serum from pSS and SLE contain type I IFN signaling modulating factors.

Quantified levels of type I IFN in serum from HC, pSS and SLE spiked with 20 U/mL recombinant IFNα2 using HEK-3C11 ISRE-reporter cell assay. Concentrations are corrected for the background signal of blanc serum for each subject. Dashed line indicates the theoretical quantified type I IFN level determined by the average of background signal of the HC sera. Symbols represent average of triplicate measurements.

(Figure 5). Therefore, the whole blood type I IFN score presumably results from exposure of immune cells to type I IFN in both the circulation and tissues. Both blood type I IFN score and type I IFN concentrations in serum presumably reflect processes occurring within the affected tissues, but their proportionality could be dependent on variables specific for the involved tissues (e.g. tissue size, number of type I IFN-producing cells in tissue).

Assessment of type I IFN pathway activity using complementary assays in multiple diseases in parallel will advance our knowledge on type I IFN biology in systemic autoimmune diseases. Future studies should also consider post-translational modifications – such as glycosylation and enzymatic cleavage – of type I IFNs or the IFNAR, which may modulate type I IFN biological activity without affecting the type I IFN concentrations [31-33].

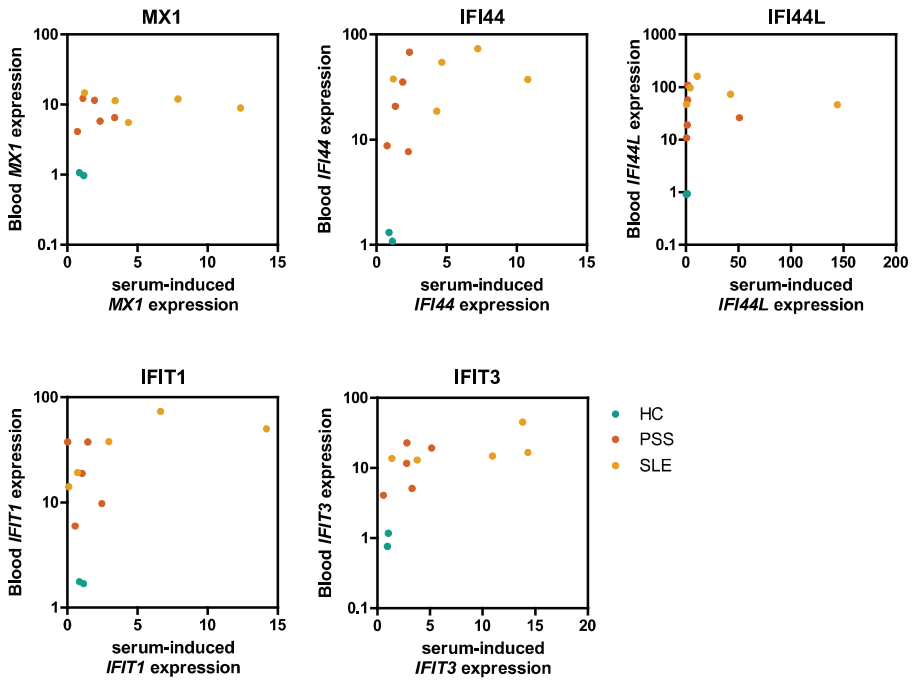


Figure 5. Correlation between *in vivo* blood ISG expression and *in vitro* serum-stimulated ISG expression. Relative mRNA expression ($2^{-\Delta CT}$) of *MX1*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* in PAXgene blood plotted against the ISG expression in cryopreserved PBMCs from a healthy donor (4.10e5 cells/well) stimulated with 50% serum from pSS, SLE and HC for 5 hours.

TYPE I IFN ASSAYS IN CLINICAL PRACTICE

Type I IFN pathway activation could also be relevant as a biomarker for the clinical management of systemic autoimmune diseases. In general, type I IFN assays could have clinical utility for diagnosis, prognosis or monitoring.

Diagnosis

The ability of an assay to discriminate between disease cases and controls is imperative for its value in diagnostics. The discriminatory power of an assay is commonly evaluated by ROC analysis and summarized by the area under the curve (AUC). In early stages of biomarker exploration, this analysis is often performed using healthy individuals as the control population. Overexpression of type I ISGs has consistently been found in patients with systemic autoimmune diseases relative to HCs. However, diagnostic measures of type I IFN assays are almost never reported. **Chapter 2.2** reports moderate to high classification accuracy of type I IFN score, MxA levels and IFNα2 protein concentrations to discriminate pSS and HC. Related to the percentage of patients characterized by type I IFN pathway activation, the ability of type I IFNs assay to discriminate cases from HC will fluctuate between various systemic autoimmune diseases. However, this approach using HC will poorly estimate the diagnostic performance in a clinical setting in which symptom-free individuals are never encountered [34, 35]. Because type I IFN pathway activation is a common feature in multiple systemic autoimmune diseases with overlapping clinical features, the value of type I IFN assays for the diagnosis of specific systemic autoimmune diseases in symptomatic patients will likely be limited. Literature suggests potential diagnostic value for specific ISG expression patterns to discriminate SLE from other systemic autoimmune diseases [36-38]. Type I IFN signature expression in salivary glands is associated with biopsy positivity and correlated with peripheral blood IFN signature [39]. Therefore, the potential value of blood type I IFN assays as a screening tool prior to biopsy should be evaluated in patients with sicca symptoms. Current studies have a high risk of disease progression bias as they mainly include patients with established diagnosis and treatment regimens [35]. Future studies into the diagnostic potential of type I IFNs assay should include the relevant target populations and assess the added value of these assays to the currently used diagnostic tools. Outside the field of rheumatology, type I IFNs may be a useful screening tool in patients suspected of viral infections or genetic monogenic interferonopathies prior to performing more elaborate diagnostics.

Prognosis and risk stratification

Measurement of type I IFN pathway activation at baseline in addition to the currently used markers might be useful for prediction of disease progression, exacerbations or

treatment response. In a prospective study of ANA+ patients suspected of systemic autoimmune diseases but without sufficient criteria for classification, blood ISG expression was higher in patients that progressed to full classification within 12 months compared with non-progressors [40]. In clinical practice, patients with undifferentiated connective tissue diseases are commonly seen and type I IFN assays might provide valuable information whether these patients should be monitored for disease progression.

High level of type I IFN pathway activation in patients with SLE is predictive of a disease flare in the following months, independent of and superior to anti-dsDNA [10, 41]. Associations between type I IFN pathway activation and future disease exacerbations have not been studied in pSS. The natural history of pSS is relatively stable in most patients and the incidence of disease 'flares' is much lower than in SLE [42-44]. Therefore, these studies are more challenging in pSS, requiring large number of patients and long follow-up time. The retrospective data presented in **chapter 2.2** indicated a more severe disease history in pSS patients with type I IFN pathway activation. Although challenging, prospective studies should be performed in patients with early pSS as a large proportion of new systemic disease manifestations presents around the time of diagnosis [44, 45].

Perhaps the most promising application for type I IFN assays is the prognosis of response to type I IFN-targeting treatments. This has been illustrated in the latest clinical trials of anifrolumab that showed the highest response rate in SLE patients with a high baseline ISG expression [46, 47]. Hitherto, links between type I IFN pathway activation and response to treatment have not been found in pSS. Baseline whole blood type I ISG expression in pSS was not associated with clinical response to treatment with HCQ or the anti-BAFF antibody belimumab [48, 49]. The short duration of the HCQ trial might have contributed to the lack of significant clinical response to HCQ. A clinical trial of anifrolumab in pSS will be conducted in the near future and it will be interesting to see whether response rates associate with baseline type I IFN pathway activation.

Monitoring of disease activity and treatment response

In contrast to biomarkers for diagnosis or risk stratification, those used for monitoring should be sensitive to change over time. Type I ISG expression seems to be relatively stable over time in patients with systemic autoimmune diseases [5, 50]. Longitudinal studies in SLE have indicated that type I ISG transcript expression does not reflect changes in disease activity, while the expression of ISGs that are induced by both type I and type II IFNs fluctuates in parallel with disease activity [5, 51]. Opposed to stably expressed type I ISG transcripts, changes in type I IFN-induced chemokine levels are correlated to disease activity in a proportion of SLE patients [52]. Longitudinal data in pediatric SLE patients also indicated parallel fluctuations of disease activity and IFNα2 protein in serum (Wahadat, *et al.*, unpublished data). Together these observations indicate that type I IFN(-induced) protein

levels may be more sensitive to change over time compared with ISG transcript expression and might therefore be better suited for monitoring purposes. However, it should be investigated whether type I IFN-induced protein as a biomarker for disease activity in SLE patients adds any value to the currently used anti-dsDNA titers to monitor disease activity. An association between type I ISG expression and disease activity at a cross-sectional level has been less consistent in pSS [12, 50, 53-56]. Longitudinal studies analyzing this association have not been performed in pSS patients.

Type I IFN assays could potentially be used to monitor response to treatment. Downregulation of type I ISGs was achieved in pSS patients treated with HCQ, but this was not associated with clinical response [48, 57]. In contrast, ISG expression remained stable in pSS and SLE patients treated with anti-B cell therapy [41, 49, 58]. As expected, blockade of IFNAR with anifrolumab reduced type I IFN-induced chemokine levels in SLE [59]. Thus, treatment can affect type I ISG expression depending on the pathway that is being targeted. Therefore, type I IFN assays could be used to monitor the biological response to these treatments.

Implementation of type I IFN assays in clinical practice

Although available research data is suggestive of potential clinical value, type I IFN assays have not been translated into routine clinical practice thus far. The most important requirement for clinical implementation is the added value to patient care [60]. The benefit of type I IFN assays in rheumatology for clinical decision making and patient outcomes as well as test performance measures that are transferable to real life settings have been poorly reported, explaining the lack of transition into clinical practice. To ensure transferability of the results and facilitate clinical implementation, future studies into clinical utility of type I IFN assays should consider the elements of the QUADAS and QUIPS tools for the design of diagnostic accuracy and prognosis studies [35, 61-63]. A few individual institutes have nonetheless implemented type I IFN assays in a diagnostic setting [64]. Since 2018, the PAXgene type I IFN score that was developed in our research group is available at the diagnostic Laboratory Medical Immunology of the Erasmus MC. The test is requested multiple times each month. The performance of this assay in a real-life setting, the clinical questions, test outcomes and added information of the performed tests is currently being analyzed.

A wide range of type I IFN assays exist of which some are highly specific for the type I IFN pathway activation, while others can also be induced by non-type I IFN stimuli. The required level of type I IFN pathway specificity will be dictated by the desired clinical application of the assay. Type I IFN assays for screening of patients that are suspected of monogenic interferonopathies or for the monitoring of the biological response to type I IFN-targeting treatments should preferably accurately reflect type I IFN pathway activation.

Depending on the specific part of the pathway – e.g. the production of type I IFNs or their downstream effects – that is being targeted by these treatments, some type I IFN assays may be more suitable than others. A high specificity for the type I IFN pathway is most likely less relevant in assays intended for diagnosis, risk stratification or monitoring of disease activity in systemic autoimmune diseases. More important, the assay should provide clinically relevant information in the target population and add value to the current standard of care. Therefore, it cannot be excluded that different clinical settings require the use of different classes of type I IFN assays.

Another important aspect for clinical implementation of an assay is the feasibility in routine diagnostic laboratories [60]. Assays should preferable be automated to limit technical variation and errors. The most widely used type I IFN assays are based on gene expression analysis by quantitative PCR [65]. Although these are currently quite laborious and expensive to perform, protocols for sample preparation and qPCR can be adapted for automatic analysis. Literature suggests that ISG protein expression might provide more clinically relevant information than ISG transcript expression. Quantification of proteins is most commonly performed by immunoassays and flow cytometry. Flow cytometers may not be available in all laboratories. However, automated immunoassays are routinely performed in diagnostic laboratories. Therefore, implementation of immunoassays for type I IFN pathway activation appears feasible. In fact, a point-of-care immunoassay that detects elevated MxA in a fingerstick blood sample is already available for clinical diagnostics [66]. Ultrasensitive immunoassays, such as the Simoa applied in **Chapter 2.2**, operate mostly automated. The detection of ultralow levels of proteins for diagnostic purposes is receiving attention [67], but as of yet, the equipment is present in only a few large research facilities and is not designed for efficient analysis of individual samples.

PATHOPHYSIOLOGY OF TYPE I IFN PATHWAY ACTIVATION IN PSS

Tight control mechanisms ensure a proper resolution of the type I IFN response after initial triggering [68]. In more than fifty percent of pSS patients, the type I IFN pathway is persistently activated. The contribution of different cell types and molecular pathways to the initiation and sustainment of the type I IFN signature in pSS are discussed below.

Initiation of type I IFN pathway activation in pSS

The mechanisms and exact timing of initiation of type I IFN pathway activation in pSS remains obscure. However, it seems to be a relatively late phenomenon in the disease development of pSS and other systemic autoimmune diseases, becoming evident only

just before the onset of clinical disease and/or disease classification [40, 69, 70]. In contrast, autoantibodies can be detected in serum up to 20 years before diagnosis [71]. Therefore, although some of the pSS-associated autoantibodies are able to provoke IFNα production by pDCs, the presence of these autoantibodies does not necessarily coincide with type I IFN pathway activation in preclinical disease. Although not investigated yet, interferonogenic capacity of autoantibodies could potentially be acquired during the preclinical phase of pSS by epitope spreading. Additionally, (viral) infections and expression of endogenous retro-elements may contribute to the initiation of type I IFN pathway activation [72]. The results presented in **Chapter 4** suggest prolonged effects of pathogens on the responsiveness of the type I IFN pathway through the induction of trained immunity. Through this mechanism, prior infections could potentially contribute to exacerbation of immune responses, including type I IFN production, stimulating the progression of autoimmunity in individuals at risk.

Cellular source of type I IFNs in pSS

The pDC – the professional IFNα-producing cell – is the principal suspected cellular source of type I IFN that drives the ISG expression in pSS [73]. The administration of anti-BDCA2 antibodies led to a partial and heterogeneous reduction of blood ISG expression in SLE [74], strongly suggesting a contribution of pDCs to the type I IFN signature. The heterogeneous effects of anti-BDCA2 treatment could be a reflection of inter-individual variation in pharmacodynamics, but could also indicate the contribution of other cell types to the type I IFN signature in SLE. Indeed, other cell types – including epithelial cells – have also been suggested as potential type I IFN-producing cells in SLE [75-79]. In the pristane-induced lupus mouse model, monocytes have been identified as the primary type I IFN-producing cells [80]. Illustrated in chapter 3.3, albeit producing less type I IFN molecules per cell compared with pDCs, monocytes can produce substantial amounts of type I IFN in response to TLR or cytosolic nucleic acid-sensing receptor stimulation [81]. Alternative cellular sources of type I IFNs besides pDCs, including monocytes, have been poorly studied in pSS. Chapter 3.3 indicates that the proportion of monocytes that produces IFNα in response to cytosolic DNA-sensing pathway stimulation is higher in pSS PBMCs than HC. Whether this is intrinsic to the monocytes or driven by interaction between monocytes and other cell types remains to be elucidated. Although challenging, future studies should aim to detect spontaneous signaling activity of type I IFN-inducing pathways in pSS monocytes and identify potential in vivo type I IFN-provoking stimuli.

Hitherto, the role of various cell types in type I IFN production in pSS and SLE has mainly been deduced from *in vitro* stimulation experiments with circulating immune cells. Detection of endogenous type I IFN transcription in patients appears extremely challenging. Even in virus-infected individuals, type I IFN mRNAs are hard to detect in circulating

immune cells [82]. The unstable nature of type I IFN mRNA [83] or restriction of type I IFN-producing cells to a specific anatomical location – as has been shown for chronic SIV infection in rhesus macaques [84] – could potentially explain the lack of detectable type I IFN transcription in pSS and SLE. Single-cell RNA sequencing is a useful tool to study the transcriptome of individual cells, which could aid the identification of type I IFN-producing cells in pSS and SLE [17-19, 85-87].

Molecular pathways contributing to type I IFN pathway activation in pSS

The leading hypothesis on the mechanism underlying sustained type I IFN signaling involves TLR7 stimulation of pDCs by RNA-containing immune complexes [88-90]. HCQ that is routinely used in the treatment of pSS and SLE effectively inhibits TLR7 en TLR9-induced IFNα production [91, 92]. This was confirmed in PBMC cultures from HCQ-treated pSS patients, presented in **chapter 3.3**. Although blood ISG expression and serum IFNα2 concentrations were lower in pSS patients treated with HCQ (**chapter 2.2**), residual type I IFN pathway activity could still be observed in a proportion of patients. Partial inhibition of TLR7-stimulated IFNα production by clinically relevant concentrations of HCQ [93] could not be excluded as plasma concentrations of HCQ in patients were not measured in **chapter 2.2**. However, residual type I IFN pathway activity in HCQ-treated individuals may also indicate a contribution of alternative type I IFN-inducing pathways in pSS.

Cytosolic nucleic acid-sensing pathways and ligands

Cytosolic nucleic acid-sensing receptors induce potent type I IFN responses. However, the potential involvement of these pathways in type I IFN activation in pSS is only beginning to be investigated. Chapter 3.1 provides a comprehensive overview of the research in pSS related to this topic and presents a hypothetical model for the activation of intracellular nucleic acid-sensing pathways in pSS. The results presented in this thesis further support an involvement of cytosolic nucleic acid-sensing pathways. Yet, the relative contribution of the RNA-sensing pathways and DNA-sensing pathways in pSS is difficult to specify at this point. TBK1 is the central signaling hub of the cytosolic nucleic acid-sensing receptors [94]. Chapter 3.2 shows increased levels of phosphorylated TBK1 in pDCs from IFN-high pSS patients, suggesting signaling of the RIG-I-like receptors (RLRs) and/or the cGAS-STING pathway. TLR7 and TLR9 are the dominant type I IFN-inducing receptors in quiescent pDCs, while RLR and cGAS-STING pathways are upregulated in activated pDCs [95, 96]. These cytosolic nucleic acid-sensing pathways are both functional in pDCs and contribute to their type I IFN production [95-98]. Chapter 3.3 provides evidence for hyperresponsiveness of the STING pathway in both pDCs and monocytes from pSS patients. This hyperresponsiveness was however not restricted to patients with type I IFN pathway

activation, indicating that both IFN-low and IFN-high pSS monocytes and pDCs have the potential to mount stronger type I IFN responses upon STING stimulation.

Although the evidence is currently lacking, the availability of cytosolic nucleic-acid sensing receptor ligands could potentially be different between IFN-low and IFN-high pSS patients. Potential exogenous and endogenous ligands of RLR and cGAS-STING pathways have been extensively discussed in **chapter 3.1**. To screen for the presence of RLR-stimulating RNAs, the RNA content of pSS and HC PBMCs was transfected into *IFNB* reporter cells (Figure 6). Transfection of PBMC-derived RNAs did not induce reporter gene expression, indicating absence of abundant RLR-activation RNAs in pSS PBMCs. Yet, low abundant RIG-I/MDA5 ligands might have been overshadowed by predominating inert RNA from bulk PBMCs.

Availability of cGAS-STING ligands in pSS – which mainly manifests in the middle-aged to older population – may relate to immune aging. Aging of immune cells is associated with genomic instability, loss of nuclear envelop integrity, reactivation of endogenous retro-elements and mitochondrial dysfunction. These processes provide ligands for cGAS-STING and promote a type I IFN response and age-related inflammation [100-105]. Type I IFN itself has been described to inhibit telomerase activity and stimulates ROS production, which further aggravates genomic instability and cellular senescence [105]. Aging of the immune system promotes senescence in other organs [106]. Interestingly, both accumulation of senescence-associated T cells and prematurely aged stem cells in salivary glands have been reported in (mouse) SS [107, 108]. In addition, miR-146a – which is upregulated

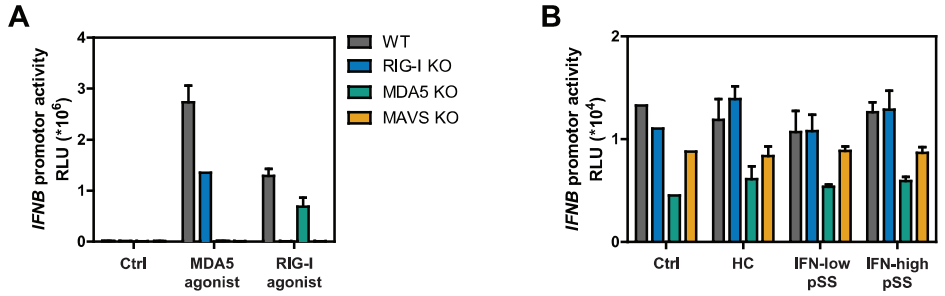


Figure 6. PBMC-RNAs from pSS do not stimulate RLRs.

Cellular RNAs were isolated from cryopreserved PBMCs (10x10^6 live cells) from 5 IFN-low pSS, 5 IFN-high pSS and 6 HC using the miRNeasy Mini Kit (Qiagen) with on-filter DNAse treatment. p125-HEK293 IFNβ reporter cells (WT, RIG-I KO, MDA5 KO or MAVS KO [99]; 5.10e4 cells/well) were pre-treated with 30 U/mL recombinant IFN-A/D for 24 hours and subsequently transfected with 100 ng of **(A)** selective RIG-I and MDA5 agonists (IVT RNA and HeLa-EMCV-RNA, respectively) or **(B)** PBMC-RNA complexed with 0.2 µL of Lipofectamine 2000. *IFNB* promotor activity was quantified (RLU, Relative Luminescence Units) 24 hours later using the OneGlo luciferase assay (Promega). Shown are means + SD of n = 5-6 PBMC-RNA samples per group, each transfected in triplicate. Transfection experiment was kindly performed by Natalia Sampaio and Jan Rehwinkel (University of Oxford).

in pSS PBMCs [109-111] – has recently been suggested to induce senescence in bone marrow stem cells [112]. Therefore, the role of aging immune cells, senescence and age-related type I IFN production in pSS is an interesting avenue for future research.

Trained immunity

Trained immunity affects the production of pro-inflammatory cytokines IL-6 and TNFα [113]. **Chapter 4** describes that trained immunity also modulates type I IFN responses by monocytes. In this light, trained immunity might also take part in the hyperresponsive IFNα production of pSS monocytes upon STING stimulation (**chapter 3.3**). Intriguingly, some trainers enhanced LPS-induced type I IFN production, while reducing poly I:C-induced type I IFN production, indicating pathway-specific effects of trained immunity on type I IFN responses. Gene expression analysis of *TLR3* and downstream signaling molecules of the NFκB and IRF3 pathways in trained monocytes could not explain the observed phenotype (Figure 7). The underlying mechanism of this observation requires further investigation, which should include analysis of (LPS-/poly I:C-induced) TLR3/4 protein expression and intracellular trafficking of TLRs in trained monocytes. In patients, trained immunity could potentially increase the type I IFN response to certain inflammatory stimuli, while reducing the type I IFN response to other stimuli.

In addition to type I IFN production, transcriptional response to type I IFN stimulation can be modulated by training (**chapter 4**). In patients, trained immunity might affect the level and stability of ISG mRNA expression, which might contribute to the steady ISG expression over time that has been observed in pSS patients. Type I IFN pathway activation in pSS is associated with DNA hypomethylation of ISGs [114, 115]. As opposed to the well-defined involvement of specific histone modifications, the role of DNA methylation in trained immunity is currently unknown [116]. Future studies should determine whether

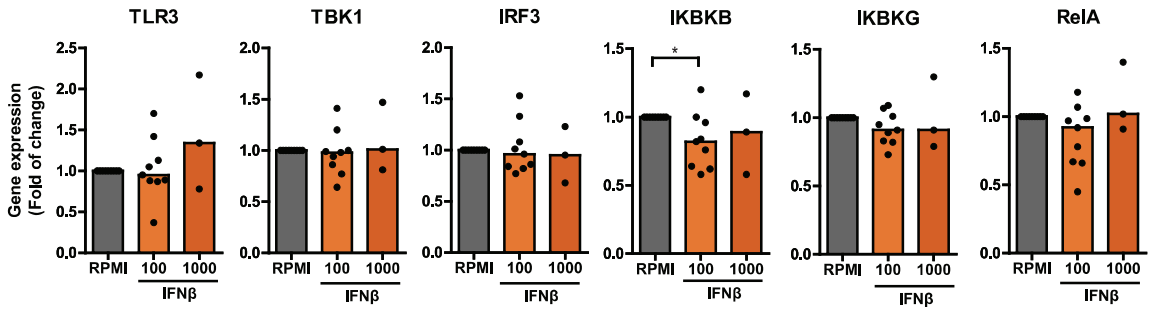


Figure 7. Comparable expression of TLR3 signaling-related genes in IFNβ-trained and untrained monocytes.

Relative mRNA expression ($2^{-\Delta\Delta CT}$) of *TLR3*, *TBK1*, *IRF3*, *IKBKB*, *IKBKG* and *RELA* in THP-1 cells trained with IFNβ. Fold change expression was calculated relative to the corresponding untrained (RPMI) cells.

epigenetic histone or DNA modifications of ISGs explain the effects of training and could be exploited as potential treatment targets.

Chapter 4 shows that training with type I IFN itself also modulates the stimulation-induced type I IFN response and ISG expression. Both type I IFN production upon poly I:C stimulation and the upregulation of ISGs in response to type I IFN stimulation was impaired in PBMCs from IFN-high pSS compared with HC. These data may suggest compensatory negative regulation of the type I IFN pathway in IFN-high pSS patients. This potentially limits further aggravation of type I IFN response and might possibly increase the susceptibility to certain viral infections [117-123]. Altogether, trained immunity could possibly both contribute to and be induced by type I IFN pathway activation in pSS.

Cellular metabolism

Immunometabolism is an emerging field of research that studies the connection between metabolism of immune cells and their function [124, 125]. In the context of type I IFN, dysbalanced cholesterol homeostasis has been shown to reduce the sensitivity of the STING pathway causing phosphorylation of TBK1 and a type I IFN signature in macrophages [126]. **Chapters 3.2** and **3.3** of this thesis demonstrate both higher levels of pTBK1 in pSS pDC and increased responsiveness of the STING pathway in pDCs and monocytes from pSS patients. Interestingly, inhibition of mTOR – a key regulator of cellular metabolism – by rapamycin suppressed the elevated IFNα production in STING-stimulated monocytes from SLE patients [78]. Cellular metabolic pathways are also important mediators of trained immunity [127, 128]. **Chapter 4** describes multiple indications of altered cellular metabolism in trained monocytes and pSS PBMCs. Taken together, altered cellular metabolism of immune cells may contribute to both the hyperresponsive phenotype of immune cells and type I IFN signature in pSS. Inversely, type I IFN also influences fatty acid synthesis, oxidative phosphorylation and cholesterol balance, highlighting the central position of type I IFN in immunometabolism [126, 129]. The reciprocal interaction between type I IFN and immunometabolism uncovers metabolic intervention (e.g. by rapamycin or metformin [130]) as a potential novel treatment strategy to limit type I IFN pathway activation in pSS.

In summary, nucleic acid-sensing pathways, trained immunity and cellular metabolism may each have a role in type I IFN pathway activation in pSS (Figure 8). Reciprocally, each of these pathways are regulated by type I IFN, representing positive and negative feedback mechanisms. Therefore, the underlying mechanisms of type I IFN pathway activation in patients cannot be viewed independently from the modulation of the same pathways by the perpetual presence of type I IFN.

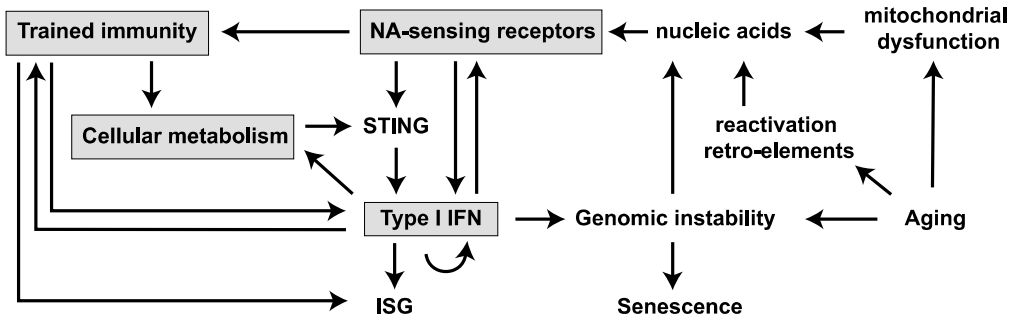


Figure 8. Hypothetical model of pathways contributing to type I IFN pathway activation in pSS.
Schematic overview of interactions between type I IFN and nucleic acid (NA)-sensing receptors, cellular metabolism and trained immunity.

TBK1 INHIBITION AS A TREATMENT STRATEGY TO LIMIT TYPE I IFN PATHWAY ACTIVATION

The elevated levels of phosphorylated TBK1 in pSS pDCs and the hyperresponsive STING pathway in pSS monocytes and pDCs (**chapter 3**) support a potential role for cytosolic nucleic acid-sensing pathways in type I IFN activation in pSS and provide a rationale for inhibition of TBK1.

Mechanism of action

Inhibitors of TBK1/IKKε modulate multiple pathways that are involved in type I IFN activation simultaneously. First, TBK1 is a critical signaling component of the RLR and cGAS-STING pathways [94]. Second, TBK1/IKKε inhibitors inhibit IFNAR signaling (Figure 9A), presumably through inhibition of IKKε which modulates IFNAR signaling [131, 132]. Third, **chapter 3.2** and Figure 9B show effective inhibition of TLR7-stimulated type I IFN and ISG expression by TBK1/IKKε inhibitors. These results are surprising given that TBK1 is not generally accepted as a downstream component of TLR7 signaling pathway. However, TBK1/IKKε kinase activity seems to be required for TLR7-stimulated TANK SUMOylation and the consequent release of active TRAF6 [133]. TRAF6 is involved in both TLR7-induced NFκB activation and TLR7/9-induced IRF7 activation [134-136]. Accordingly, TBK1/IKKε inhibitors may modulate TLR7-stimulated production of type I IFN and NFκB-induced cytokines. Taken together, inhibition of TBK1/IKKε modulates the activity of multiple type I IFN-inducing pathways as well as downstream IFNAR signaling (Figure 9C).

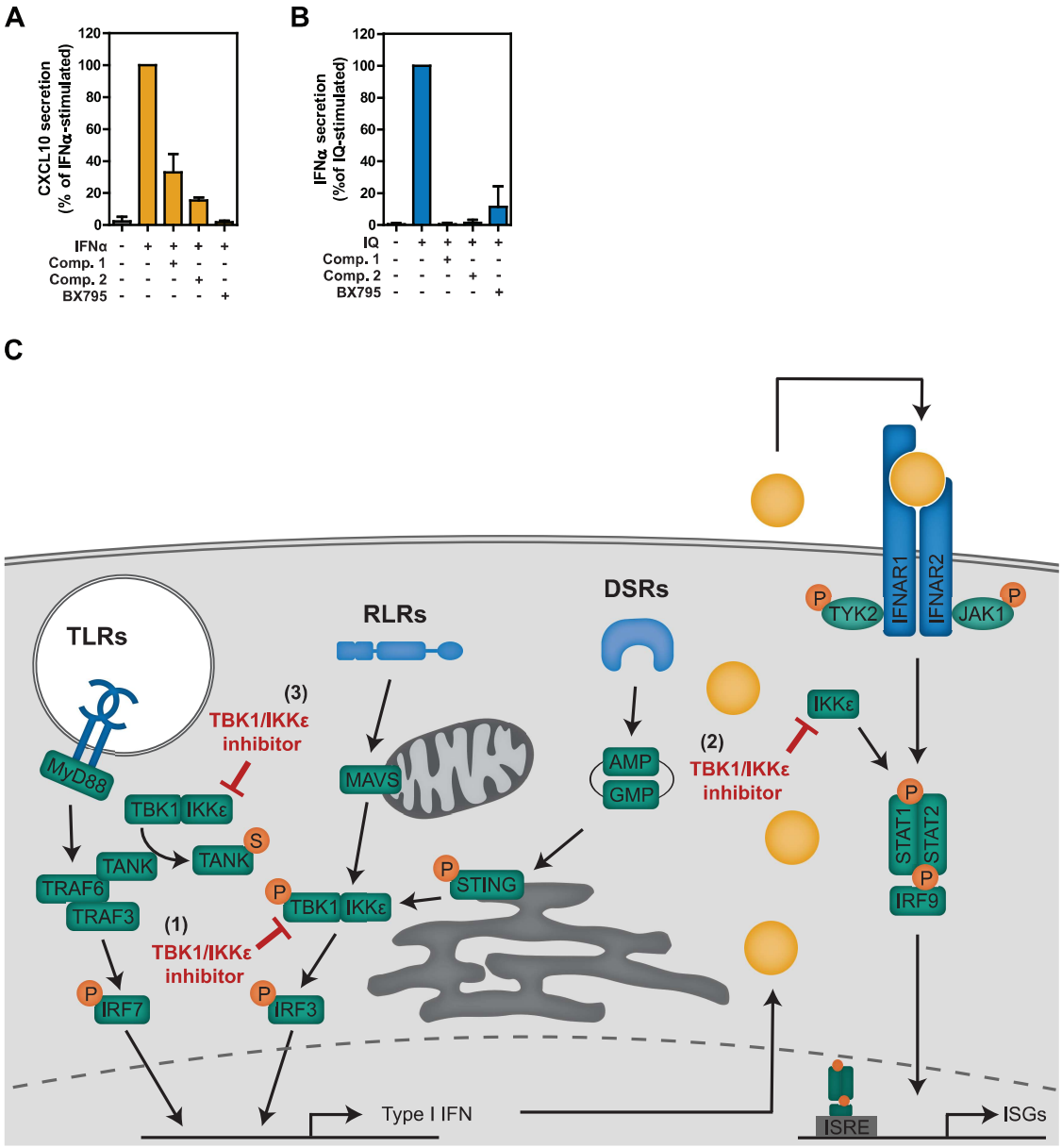


Figure 9. TBK1/IKKε inhibitors modulate the type I IFN pathway at multiple levels.

(A) CXCL10 secretion by HC-PBMCs stimulated with 500 U/mL of recombinant IFNα (PeproTech) for 24 hours and **(B)** TNFα and IFNα secretion by HC-PBMCs stimulated with 1 μg/mL of TLR7-agonist imiquimod (IQ) for 24 hours in presence or absence of TBK1/IKKε inhibitors compound 1*, compound 2* or BX795. * Identity of compounds 1 and 2 are confidential. Shown are means + SD of 5 independent experiments. **(C)** Schematic overview of type I IFN-related pathways modulated by TBK1/IKKε inhibitors. TBK1/IKKε inhibitors interfere with **(1)** the induction of type I IFNs by RLRs and cytosolic DNA-sensing receptors (DSRs), **(2)** IFNAR signaling, presumably through modulation of IKKε-mediated STAT1 phosphorylation, and **(3)** the induction of type I IFNs by TLR7, potentially through inhibition of TBK1/IKKε-mediated TANK-SUMOylation that is required for the continuation of TRAF6 signaling.

Pros and cons of therapeutic TBK1 inhibition

TBK1 has received much attention as a potential treatment target in multiple inflammatory diseases, cancer, metabolic diseases and neurological disorders [137-141]. During the last decade, numerous small-molecule TBK1 inhibitors have been patented [142, 143]. Because of the low molecular weight and uncomplicated structure, small molecule treatments can usually be taken orally and have simple dosing regimens [144]). For treatment of IFN-high autoimmune diseases in which multiple type I IFN-stimulating pathways can be involved, the broad action of TBK1/IKKε inhibitors on the type I IFN pathway could be an advantage. On the other hand, the involvement of TBK1/IKKε in numerous other biological processes related to immune responses and metabolism [145-147] could potentially also cause wide-ranging side effects of TBK1/IKKε inhibitors. Illustrating potential adverse effects of TBK1 inhibition,

inadequate TBK1 kinase activity has been associated with neuroinflammatory diseases, autoinflammation and severe viral infections in humans [141, 148-152]. In addition, TBK1 inhibition might lower the activation threshold of the NLRP3 inflammasome and promote the development of IgA-related nephropathy [146, 153]. Future studies will reveal whether the complexity of TBK1 function causes any insuperable adverse events of TBK1 inhibition in humans.

CONCLUDING REMARKS

This chapter discussed pathophysiological mechanisms, clinical and therapeutic implications and future directions arising from the results presented in this thesis. This thesis highlights several cellular pathways that are involved in the regulation of type I IFN pathway activation and pinpoints various potential treatment targets. Measurement of type I IFN pathway activation may aid the identification of patients eligible for these treatments, but clinical validation of type I IFN assays is urgently needed. To minimize confounding effects of treatment, disease-related damage or disease severity on research findings, it is important to study patients with short disease duration, minimal immunomodulatory treatment and variable disease severity. Further research into the pathophysiological mechanisms that trigger and regulate type I IFN pathway activation in systemic autoimmune diseases will lead to the identification of novel therapeutic strategies.

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ADDENDUM

Abbreviations

Summary

Samenvatting

Dankwoord

Curriculum Vitae

PhD Portfolio

Publications

ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette sub-family G member 1
ACR	American College of Rheumatology
AGS	Aicardi-Goutières syndrome
AIM2	absent in melanoma 2
ANA	anti-nuclear antibodies
APS-1	autoimmune polyglandular syndrome type I
AQP	aquaporin
ATP	adenosine triphosphate
AUC	area under the curve
BAFF	B cell activating factor
BDCA	blood dendritic cell antigen
BMI	body mass index
BTK	Bruton's tyrosine kinase
CARD	caspase-associated recruitment domain
CCL	CC chemokine ligand
CD	cluster of differentiation
cGAMP	cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cGAMP-synthase
CMV	human cytomegalovirus
CNS	central nervous system
CS	citrate synthase
DC	dendritic cell
DDX	DEAD-box
DMARD	disease-modifying anti-rheumatic drug
DNA	deoxyribonucleic acid
DSR	DNA-sensing receptor
EDTA	ethylenediamine tetraacetic acid
EGM	extraglandular manifestations
EIA	enzyme-immunoassay
EIF4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
ENA	extractable nuclear antigen
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ESSDAI	EULAR Sjögren's syndrome disease activity index
EULAR	European Alliance of Associations for Rheumatology

FCS	fetal calf serum
FDA	Food and Drug Administration
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTP	guanosine triphosphate
HBV	hepatitis B virus
HC	healthy control
HCQ	hydroxychloroquine
HCV	hepatitis C virus
HDV	hepatitis D virus
HIV	human immunodeficiency virus
HK2	hexokinase 2
HKCA	heat-killed Candida albicans
HLA	human leukocyte antigen
HMGB	high-mobility group box
IFI	interferon-inducible protein
IFIT	interferon-induced protein with tetratricopeptide repeats
IFN	interferon
IFNAR	interferon-alpha receptor
IFN-I	type I interferon
Ig	immunoglobulin
IKK	inhibitor of kappa B kinase
IL	interleukin
ILC	innate lymphoid cells
IQ	imiquimod
IQR	interquartile range
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISRE	interferon-stimulated response element
JAK	Janus tyrosine kinase
LGP2	laboratory of genetics and physiology 2
LINE	long interspersed nuclear element
LPS	lipopolysaccharide
M3R	cholinergic receptor muscarinic 3
MAVS	mitochondrial antiviral-signaling protein
MDA5	melanoma differentiation-associated protein 5
MDH2	malate dehydrogenase 2
MDP	muramyl dipeptide
MFI	mean/median fluorescence intensity

MHC	major histocompatibility complex
MSR1	macrophage scavenger receptor 1
mTOR	mammalian target of rapamycin
MVK	mevalonate kinase
MxA	Myxovirus resistance protein 1
NA	nucleic acid
NFκB	nuclear factor kappa B
NK	natural killer
NLRP	nucleotide-binding domain-like receptor protein
NPV	negative predictive value
NR1H3	nuclear receptor subfamily 1 group H member 3
NSAID	non-steroidal anti-inflammatory drug
OAS	oligoadenylate synthase
oxLDL	oxidized low-density lipoprotein
PS	penicillin streptomycin
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PD-L1	programmed death-ligand 1
PGM1	phosphoglucomutase 1
PIK3R1	phosphoinositide-3-kinase regulatory subunit 1
PKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PKM	pyruvate kinase M
PKR	protein kinase R
PMA	phorbol 12-myristate 13-acetate
PMB	polymyxin B
PNS	peripheral nervous system
PPV	positive predictive value
PRR	pattern recognition receptor
pSS	primary Sjögren's syndrome
RAGE	receptor for advanced glycation end products
RIG-I	retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNF	ring finger protein
RNP	ribonucleoprotein
ROC	receiver operating characteristic
ROS	reactive oxigen species
SAD	systemic autoimmune disease

SAVI	STING associated vasculopathy with onset in infancy
SENP	sentrin-specific protease
Simoa	single-molecule array
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
SOCS	suppressor of cytokine signaling
SS	Sjögren's syndrome
SSA	Sjögren's syndrome-associated autoantigen A
SSB	Sjögren's syndrome-associated autoantigen B
SSc	systemic sclerosis
STAT	signal transducer and activator of transcription
(p)STING	(phosphorylated) stimulator of interferon genes
TANK	TNF receptor-associated factor NF-κB activator
(p)TBK1	(phosphorylated) TANK-binding kinase 1
TCA	tricarboxylic acid
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TREM	triggering receptor expressed on myeloid cells
TRIM	tripartite motif
USP	universal stress protein
ZBP-1	Z-DNA-binding protein 1

SUMMARY

Primary Sjögren's syndrome (pSS) is a systemic autoimmune rheumatic disease characterized by focal mononuclear cell infiltrates in the exocrine glands and symptoms of dryness of the eyes and mouth. The disease affects around 1 in 1700 individuals worldwide, typically manifests between the ages 40 and 60 and is approximately ten times more prevalent in females than males. Apart from the glandular symptoms, a large proportion of patients additionally presents with extraglandular disease manifestations involving different organ systems. Fatigue, depressive symptoms and chronic pain are prevalent.

The current consensus hypothesis on the etiology of pSS is that a combination of environmental and internal factors in a genetically susceptible individual triggers chronic inflammation, both in target organs and systemically. **Chapter 1** describes the contribution of both the innate and adaptive arms of the immune system to the chronic inflammation in pSS. The most prominent sign of innate immune activation in pSS is the persistent type I interferon (IFN) pathway activation, both in the circulation and salivary glands. This feature is present in the majority of pSS patients and is also observed in subgroups of patients with other systemic autoimmune diseases, such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). Multiple lines of evidence suggest a pathogenic role for type I IFNs in the initiation and perpetuation of systemic autoimmune diseases, including pSS. The overall aim of this thesis is to generate better insight into the pathophysiological mechanisms that trigger and regulate type I IFN pathway activation in pSS as wells as the clinical and therapeutic implications of type I IFN pathway activation.

Therapeutic inhibition of type I IFN pathway has proven clinical benefit in SLE and will soon be evaluated in pSS. Measurement of type I IFN will likely assist in the selection of candidates for these targeted treatments, urging the development and validation of robust and easy-to-perform assays. Type I IFN protein concentrations are difficult to quantify in biological samples by routinely used laboratory techniques, such as ELISA, because of the diversity of subtypes and low circulating levels. Therefore, the most frequently used method to assess type I IFN pathway activation is the measurement of type I IFN-stimulated genes (ISG). Chapter 2 describes the performance of two immunoassays to detect systemic type I IFN pathway activation in patients with pSS, SLE and SSc. ***Chapter 2.1*** demonstrates a strong correlation between intracellular protein expression of type I IFN-inducible Myxovirus resistance protein 1 (MxA) measured in whole blood by an immunoassay and blood ISG transcript expression (type I IFN score) in patients with SLE and SSc. The MxA-immunoassay showed excellent performance to discriminate low and high type I IFN scores and is therefore a reliable alternative for blood type I ISG expression analysis. Ultrasensitive single-molecule array (Simoa) is a novel technology, that facilitates the measurement of low concentrations of IFNs. In ***chapter 2.2***, this Simoa technology was

used for the direct quantification of IFNα2 protein concentrations in serum from patients with pSS, SLE and SSc. Circulating IFNα2 protein levels were correlated with the blood type I IFN score and MxA protein expression in pSS, as well as in SLE and SSc. Serum IFNα2 levels in pSS were lower compared with SLE. Serum IFNα2 and blood MxA had comparable ability to identify pSS patients with a high type I IFN score. Circulating IFNα2 in pSS was associated with disease-relevant serological parameters, treatment and systemic disease manifestations.

Insight in the mechanisms that mediate the persistent type I IFN pathway activation in patients will aid the design of new targeted treatments. Research on type I IFN activation in pSS has primarily been focused on endosomal Toll-like receptors (TLRs) and immune complexes, while alternative type I IFN inducers have received little attention. **Chapter 3** demonstrates the involvement of cytosolic nucleic acid-sensing pathways in type I IFN pathway activation in pSS. Based on review of the literature, ***chapter 3.1*** presents an hypothetical model, which describes a role for both intra- and extracellular nucleic acids in the persistent systemic type I IFN pathway activation in pSS. Nucleic acids when highly abundant, incorrectly processed or mislocalized may activate cytosolic pattern recognition receptors (PRRs) or enter the extracellular compartment from which they can be internalized by immune cells and activate endosomal PRRs. Dysregulation of endolysosomal digestion and autophagic trafficking under inflammatory pressure may integrate signaling via the endosomal and cytosolic nucleic acid-sensing pathways in pSS. Further supporting a contribution of RIG-I-like receptors or DNA-sensing receptors, ***chapter 3.2*** shows increased levels of phosphorylation of the nucleic acid receptor signaling component TBK1 in plasmacytoid DCs (pDCs) from pSS, SLE and SSc patients with systemic type I IFN pathway activation. Furthermore, the TBK1/IKKε inhibitor BX795 reduced TLR7-stimulated and spontaneous ISG expression in PBMCs from IFN-high pSS, SLE and SSc. In ***chapter 3.3***, the responsiveness of pSS monocytes and pDCs to DNA-sensing pathway stimulation was investigated in relation to systemic type I IFN pathway activation and compared to SLE. Monocytes from pSS patients showed hyperresponsive IFNα production and STING phosphorylation upon stimulation of STING compared with monocytes from healthy controls. Potentially contributing to the increased responsiveness of the STING pathway, several positive and negative regulators of DNA-sensing pathway activation were differentially expressed in pSS monocytes. In contrast to healthy controls, pDCs from pSS patients were sensitive to STING pathway stimulation. The hyperresponsiveness was not restricted to patients with type I IFN pathway activation. Furthermore, this phenomenon was also observed in SLE.

Priming with type I IFNs can alter the functional state of cells. Trained immunity is a long-lasting form of cellular adaptation involving elevated cytokine production, altered cellular metabolism and epigenetic reprogramming that persists after removal of the initial

stimulus. **Chapter 4** shows a link between type I IFN and trained immunity. Type I IFNs induced a trained immunity phenotype in monocytes, including elevated production of the pro-inflammatory and pro-atherogenic cytokines CCL2, IL-6 and TNFα and enhanced cholesterol uptake. Conversely, trained immunity also affected the production of type I IFNs and transcriptional response to type I IFN receptor re-stimulation. The phenotype of pSS PBMCs was consistent with a trained immunity phenotype. This connection between type I IFN, trained immunity and cholesterol metabolism may have important implications for pSS and the pathogenesis of (subclinical) atherosclerosis in these patients.

Chapter 5 discusses the application of type I IFN assays in research and clinical settings. Measurement of type I IFN pathway activation could be relevant for the diagnosis, prognosis and monitoring of systemic autoimmune diseases. However, clinical validation of type I IFN assays is urgently needed. Integrating the findings from this thesis, this chapter elaborates on the pathophysiological mechanisms that trigger and regulate type I IFN pathway activation in pSS. In addition, TBK1 inhibition is discussed as a treatment strategy to limit type I IFN pathway activation. Taken together, this thesis highlights the involvement of nucleic acid-sensing pathways, trained immunity and cellular metabolism in the regulation of type I IFN pathway activation in pSS and pinpoints various potential treatment targets.

SAMENVATTING

Het primaire syndroom van Sjögren (pSS) is een systemische reumatische auto-immuunziekte die gekarakteriseerd wordt door focale infiltraten van mononucleaire cellen in de exocriene klieren en symptomen van droge ogen en/of een droge mond. Deze ziekte treft ongeveer 1 op de 1700 individuen, openbaart zich doorgaans tussen het 40e en 60e levensjaar en komt ongeveer tien keer vaker voor in vrouwen dan in mannen. Naast de glandulaire symptomen heeft een grote groep patiënten last van extraglandulaire ziektemanifestaties, waar verschillende orgaansystemen bij betrokken kunnen zijn. Vermoeidheid, depressieve klachten en chronische pijn komen vaak voor.

Momenteel wordt verondersteld dat pSS ontstaat door een combinatie van omgevingsfactoren en interne factoren in een individu met genetische predispositie en leidt tot chronische inflammatie. **Hoofdstuk 1** beschrijft de bijdrage van zowel het aangeboren als het verworven immuunsysteem aan de chronische inflammatie in pSS. Een duidelijke aanwijzing voor een geactiveerd aangeboren immuunsysteem in pSS is de continue activiteit van de type I interferon (IFN) signaalroute (hierna type I IFN activatie genoemd) in de circulatie en in de speekselklieren. Deze type I IFN activatie is aanwezig in het merendeel van de pSS patiënten en wordt ook gezien in een gedeelte van patiënten met andere systemische auto-immuunziekten, zoals systemische lupus erythematosus (SLE) en systemische sclerose (SSc). Meerdere onderzoeken wijzen op een pathogene rol voor type I IFN in de ontwikkeling en instandhouding van systemische auto-immuunziekten, waaronder pSS. Het hoofddoel van dit proefschrift is het verkrijgen van meer inzicht in de pathofysiologische mechanismen van de type I IFN signaalroute in pSS en de klinische en therapeutische implicaties van type I IFN activatie.

Het remmen van de type I IFN signaalroute is klinisch effectief gebleken in SLE en zal in de nabije toekomst worden getest in pSS. Bepaling van type I IFN kan mogelijk bijdragen aan de selectie van kandidaten voor deze gerichte behandelingen. Dit vraagt om de ontwikkeling en validatie van robuuste en eenvoudig uit te voeren bepalingen. Vanwege de diversiteit in subtypen en de lage concentraties type I IFN in de circulatie is het moeilijk om type I IFN eiwitconcentraties te bepalen in biologische monsters met behulp van veelgebruikte laboratoriumtechnieken, zoals ELISA. Daarom wordt veelal gebruik gemaakt van de type I IFN-gestimuleerde genexpressie (ISG) als maat voor type I IFN activatie. In **hoofdstuk 2** worden twee verschillende immunoassays om systemische type I IFN activatie te detecteren in patiënten met pSS, SLE en SSc beschreven. ***Hoofdstuk 2.1*** laat een sterke correlatie zien tussen de expressie van het intracellulaire type I IFN-induceerbare eiwit Myxovirus resistance protein 1 (MxA) – gemeten in volbloed met een immunoassay – en de expressie van ISGs (hierna type I IFN score genoemd) in het bloed van patiënten met SLE en SSc. De MxA-immunoassay vertoonde een uitstekend vermogen om lage en hoge

type I IFN scores te onderscheiden en is daarom een betrouwbaar alternatief voor de type I IFN score in bloed. De ultrasensitieve single-molecule array (Simoa) technologie heeft het mogelijk gemaakt om lage concentraties type I IFN te kunnen meten. In ***hoofdstuk 2.2*** werd deze nieuwe Simoa technologie gebruikt om de IFNα2 eiwitconcentraties in het serum van patiënten met pSS, SLE en SSc te kwantificeren. IFNα2 eiwitconcentraties in het serum waren gecorreleerd met de type I IFN score en de MxA concentratie in volbloed van zowel pSS als SLE en SSc patiënten. De IFNα2 concentraties in serum waren lager in pSS dan in SLE patiënten. De bepaling van IFNα2 in serum had een vergelijkbaar vermogen om pSS patiënten met een hoge type I IFN score te kunnen identificeren als de bepaling van MxA in volbloed. Circulerend IFNα2 was geassocieerd met voor de ziekte relevante serologische parameters, behandeling en systemische ziektemanifestaties in pSS.

Inzicht in de mechanismen die ten grondslag liggen aan de persisterende type I IFN activiteit bevordert de ontwikkeling van nieuwe gerichte behandelingen. Het onderzoek naar activiteit van de type I IFN-signaalroute in systemische auto-immuunziekten is met name gericht op de rol van endosomale Toll-like receptoren (TLRs) en immuuncomplexen, terwijl alternatieve mechanismen van type I IFN activatie tot dusver weinig aandacht hebben gekregen. **Hoofdstuk 3** toont aan dat cytoplasma-gelokaliseerde nucleïnezuur-receptoren betrokken zijn in de type I IFN activatie in pSS. Gebaseerd op de literatuur beschrijft ***hoofdstuk 3.1*** een hypothetisch model waarin zowel intra- als extracellulaire nucleïnezuren betrokken zijn bij de persisterende type I IFN activiteit in pSS. Nucleïnezuren kunnen 'pattern recognition receptors' (PRRs) activeren of naar het extracellulaire compartiment lekken wanneer ze overvloedig aanwezig zijn, op een onjuiste manier verwerkt worden of zich in een verkeerde subcellulaire locatie bevinden. Extracellulaire nucleïnezuren kunnen door immuuncellen worden geïnternaliseerd en endosoom-gelokaliseerde PRRs activeren. Ontregeling van endolysosomale processen en autofagie onder inflammatoire druk kan mogelijk tot crosstalk van de endosoom- en cytoplasma-gelokaliseerde nucleïnezuursignaalroutes leiden. Een mogelijke bijdrage van de RIG-I-like-receptoren en de DNA-receptoren wordt verder ondersteund in ***hoofdstuk 3.2***. De plasmacytoïde dendritische cellen (pDCs) van pSS, SLE en SSc patiënten met systemische type I IFN activatie vertoonde een hogere mate van fosforylering van TBK1 – een component van de nucleïnezuursignaalroute – dan de pDCs van gezonde controles. Bovendien werd de TLR7-gestimuleerde en spontane ISG-expressie in PBMCs van IFN-hoge pSS, SLE en SSc patiënten geremd door de TBK1/IKKε-remmer BX795. In ***hoofdstuk 3.3*** werd het reactievermogen van pSS monocyten en pDCs bij stimulatie van de DNA-signaalroute onderzocht in relatie tot de systemische type I IFN activatie, en vervolgens vergeleken met SLE. Monocyten van pSS patiënten produceerden meer IFNα en vertoonden een hogere mate van STING-fosforylering na stimulatie van STING dan de monocyten van gezonde controles. Diverse positieve en negatieve regulatoren van de DNA-signaalroute kwamen

verschillend tot expressie in monocyten van pSS patiënten, hetgeen mogelijk bijdraagt aan het sterkere reactievermogen van de STING-signaalroute. In tegenstelling tot de pDCs van gezonde controles waren de pDCs van pSS patiënten ontvankelijk voor activatie van STING. Dit verhoogde reactievermogen was niet beperkt tot enkel de patiënten met type I IFN activatie. Dit fenomeen werd ook gezien in SLE.

Stimulatie met type I IFN kan de functionele toestand van cellen veranderen. 'Trained immunity' is een langdurige vorm van cellulaire aanpassing – met verhoogde cytokineproductie, aangepast celmetabolisme en epigenetische herprogrammering – die voortduurt nadat de stimulus is weggenomen. **Hoofdstuk 4** beschrijft een link tussen type I IFN en 'trained immunity'. Type I IFN induceerde een 'trained immunity'-fenotype in monocyten, waaronder verhoogde productie van de pro-inflammatoire en pro-atherogene cytokines CCL2, IL-6 en TNFα, alsmede verhoogde opname van cholesterol. Omgekeerd werd de productie van type I IFN en de transcriptionele respons na her-stimulatie van de type I IFN receptor beïnvloed door 'trained immunity'. Het fenotype van PBMCs van pSS patiënten kwam overeen met dat van cellen met een 'trained immunity'-fenotype. Dit onderlinge verband tussen type I IFN, 'trained immunity' en het cholesterolmetabolisme kan belangrijke implicaties hebben voor pSS en de pathogenese van (subklinische) atherosclerose in deze patiënten.

Hoofdstuk 5 bespreekt de toepassing van type I IFN bepalingen voor onderzoeksdoeleinden en in een klinische context. Het meten van type I IFN activatie kan mogelijk relevant zijn voor de diagnose, prognose of het monitoren van systemische auto-immuunziekten. Klinische validatie van type I IFN bepalingen is echter noodzakelijk. Dit hoofdstuk gaat dieper in op de pathofysiologische mechanismen van de type I IFN activatie in pSS en integreert de bevindingen van dit proefschrift. Daarnaast wordt remming van TBK1 als behandelingsstrategie voor het verminderen van type I IFN activatie besproken. Samenvattend belicht dit proefschrift de betrokkenheid van nucleïnezuursignaalroutes, 'trained immunity' en celmetabolisme bij het reguleren van type I IFN activatie in pSS en enkele mogelijke aangrijpingspunten voor behandeling.

DANKWOORD

Dit proefschrift is niet alleen een product van mijn inspanningen, maar ook het resultaat van de toewijding van velen die mij hierin geholpen en gesteund hebben. In dit dankwoord wil ik mijn waardering uitspreken aan mijn supervisoren, collega's, samenwerkingspartners, patiënten, familie en vrienden die op welke manier dan ook bijgedragen hebben aan de totstandkoming van dit proefschrift. Het onderzoek dat beschreven wordt in dit proefschrift is mogelijk gemaakt door de vrijgevigheid van patiënten. Daarom wil ik allereerst alle patiënten met het syndroom van Sjögren, SLE en systemische sclerose hartelijk bedanken voor het doneren van hun bloed voor dit onderzoek.

Marjan, mijn promotor, ik weet nog goed dat ik in de zomer van 2016 bij jou kwam praten voor een eindstage. Ik had toen niet kunnen denken waar dat me zou brengen. Bij jou gaat het niet alleen om het onderzoek, maar je steekt juist veel energie in het vormen van de onderzoeker. Je vraagt zelfstandigheid van je PhD-studenten, laat ze af en toe wat zwemmen, maar zal ze nooit laten verdrinken. Als ik mezelf weer eens had verstrikt in de chaos van mijn eigen gedachten, stond jouw deur altijd open om mij geduldig te helpen bij het ordenen. Je moedigde mij aan om dingen vooral zo simpel mogelijk te maken. Vaak kwamen we dan ergens in het midden uit, want ik ben wel eigenwijs. Jij wist me te motiveren als ik het even niet meer zag en gaf mij vertrouwen als ik het minste vertrouwen in mezelf had. Je was ook mijn mentor. Je hebt veel oog voor de verdere loopbaan van je studenten. Bedankt voor alle gesprekken over mijn toekomst en de begeleiding bij het maken van de volgende stap. Ik prijs me gelukkig dat ik de afgelopen 5 jaar met jou heb mogen samenwerken!

Wim, mijn copromotor, bij jou kon ik altijd terecht voor een eerlijk gesprek en kritische feedback op mijn manuscripten. Ik moet toegeven dat ik in het begin een beetje bang voor je was. Jij sprak hardop uit wat ik zelf al lang bedacht had, maar erg onzeker over was. Jij leerde me dat je moeilijke vragen niet uit de weg hoeft te gaan, maar dat het in juiste context prima is om beperkingen van projecten bespreekbaar te maken. Ik liet die moeilijke vragen me soms verlammen, maar jij bleef daar niet in hangen en zocht naar een pragmatische oplossing. Ik wil je bedanken voor alle inhoudelijke gesprekken, je input en adviezen, niet alleen over onderzoek, maar ook over carrièrekeuzes.

I would like to extend my sincere appreciation to the members of the thesis Assessment Committee, **prof. Jacobs**, **prof. Hamann** and **prof. Mariette**, for their time and effort to review my thesis and their valuable feedback. Members of the Doctoral Committee, thank you for taking part in the opposition during my defence ceremony.

Corine, wat had ik zonder jou gemoeten? Jij leerde mij dat we niet voor niets twee handjes gekregen hebben. Net als ik heb jij ook maar twee handjes, maar bij jou is 1+1 niet 2, maar 4. Zeker het laatste jaar heb ik je flink bezig weten te houden met mijn uitgebreide

wensenlijsten en onmogelijke planningen. Niets was teveel voor jou en we hebben het voor elkaar gekregen! Je had alles volledig onder controle. Ik kan nog steeds niet helemaal doorgronden hoe jij zo goed functioneert in de chaos die je zelf creëert ;). Soms kon ik je nauwelijks zien vanachter de enorme stapel ijsbakken, bakjes met pipetpuntjes en PBS flessen die je op een ELISA-dag wist te produceren (dat grapje mag ik nu toch wel maken hè?). Maar de kwaliteit heeft er nooit onder geleden, dat kon ik wel aan jou overlaten. Werk was zeker niet het enige belangrijke. Je was ook mijn zorgzame 'moeder' op het lab die zich bekommerde over mijn welzijn en dat van andere collega's en studenten. Bij jou vond ik altijd een luisterend oor. Je hebt meer betekend voor mij dan ik hier goed kan opschrijven en daarvoor ben ik je heel erg dankbaar. Je bent goud waard!

Javad, collega! Jij begon met je PhD toen ik net aan aantal maanden bezig was, maar jij was al veel langer betrokken bij de groep. Iedereen keek uit naar je terugkomst en niet zonder reden. Jouw enthousiasme en creativiteit is aanstekelijk. Met jou kon ik goed brainstormen en jij kwam dan – al ijsberend door het kantoor – op de meest interessante theorieën. Daarnaast ben je erg hartelijk en belangstellend. Het aantal peptalks dat je mij gegeven hebt, is niet te tellen. Die idiote accentjes van jou waren erg vermakelijk. Ik heb veel bewondering voor je presentatievaardigheden (en wie niet?). Onvoorstelbaar hoe overtuigend jij over kunt komen, zelfs wanneer je er zelf geen snars van gelooft. Ik heb ervan genoten om met jou samen te werken! Heel veel succes met het afronden bij jouw PhD, dat gaat ongetwijfeld goedkomen. Je weet zelf.

Astrid, lieve collega! Wat was ik blij toen je bij Wim kon blijven voor je PhD en we echt collega's werden! Ambitieus, goedgemutst, ontzettend aardig en betrokken, zo ken ik jou. Jij en Javad waren de beste kantoorgenootjes die ik me had kunnen wensen. In ons kantoor kon alles gezegd worden en was er oprechte interesse voor elkaar. Hoe druk je het ook had, tijd voor koffie of een ijsje in de zomer kon altijd gemaakt worden. Dank je wel voor die alle mooie en gezellige momenten, lieve collega! Heel veel plezier en succes met de rest van je promotietraject en het bemachtigen van een leuke opleidingsplek daarna.

Iris, mijn voorganger PhD-student. Jij hebt mij opgevoed in het Sjögrenonderzoek toen ik als studentje bij jou kwam stagelopen. Een tijd lang heb ik de kunst van jou kunnen afkijken. Ik heb veel goede herinneringen aan de tijd dat we samen optrokken in de groep van Marjan. Toen jij promoveerde moest ik echt op eigen benen staan, maar jouw advies was altijd maar een appje van mij verwijderd. Bedankt dat je alles zo keurig inzichtelijk achtergelaten hebt, ik stond nooit voor verrassingen.

Alle **studenten** die de afgelopen jaren bij ons in de groep stage hebben gelopen, bedankt voor jullie bijdrage aan het onderzoek. Ook wil ik de medisch studenten die door de jaren heen geholpen hebben bij het informeren en includeren van patiënten hartelijk bedanken. Door jullie inzet zaten de vriezers vol met materiaal waar ik in de coronajaren dankbaar gebruik van heb kunnen maken. Graag noem ik 'mijn' studenten **Mirthe**, **Sander**,

Josje en **Sanne** nog even apart. Ik kijk met veel plezier terug op de periode dat ik met elk van jullie samengewerkt heb. Bedankt voor die leuke tijd en voor jullie vlijtige arbeid. Succes in jullie verdere loopbaan!

Zana, bij jou kon ik terecht voor al mijn klinische vragen en interpretatie van labuitslagen. Nu word ik geacht het lab bij elk mogelijke zuur-basestoornis te doorgronden, maar toen snapte ik geen hout van die dRTAs. Bedankt voor de tijd die jij steeds nam om mijn vragen te beantwoorden, je frisse blik op de projecten en je enthousiasme voor het onderzoek. **Radjesh**, ook jou wil ik hartelijk bedanken voor je originele inbreng tijdens de labbesprekingen en het meedenken bij het maken van protocollen. **Dr. Van Daele**, beste Paul, bedankt voor de hulp bij de patiëntinclusie en de gastvrijheid op de polikliniek, zonder dat was dit proefschrift er nooit gekomen. **Marco,** bedankt voor het nabepalen van auto-antistoffen voor de diverse studies. **Dwin** en **Harmen**, bedankt voor de fijne samenwerking bij het analyseren van de RNAseq-data, jullie expertise is erg gewaardeerd.

Dr. Göpfert, dear Jens, you provided me with the great opportunity to apply the Simoa technology for the measurement of interferon-alpha in systemic autoimmune diseases. Thanks to the data you and your team generated I was able to make myself useful at home during the lockdown period by analyzing the data and preparing the manuscript. Many thanks for a very pleasant collaboration. Graag wil ik de onderzoekers van de Radboud universiteit, **prof. Leo Joosten** , **dr. Siroon Bekkering** en **Vera Mourits** hartelijk bedanken voor het meedenken bij het 'trained immunity'-project. Ik heb veel gehad aan jullie input en heb jullie bereidheid om protocollen te delen en mij op jullie lab te ontvangen erg gewaardeerd. A special thanks to **dr. Jessica Tarn** and **prof. Fai Ng** for your willingness to share data from your dataset on comorbidities in Sjögren's syndrome and the analysis on very short notice. It was very much appreciated! **Prof. Rehwinkel**, thank you very much for the interesting discussions during your visit to the department and the pleasant collaboration that followed. **Dr. Sylvia Kamphuis**, fijn dat je de data van het CHILL-NL cohort wilde delen. I would also like to thank my colleagues **dr. Joel van Roon** and **Ana Lopes** from Utrecht University and **dr. Thomas Mandl** and **dr. Peter Olsson** from Lund University for their enthusiasm to share samples and data from their Sjögren cohorts.

Alle **PhD-studenten** van de afdeling Immunologie, bedankt voor jullie hulp, meeleven en de gezelligheid tijdens de PhD-retreats. Regelmatig werd op vrijdagmiddag rond half 6 nog even bijgekletst in ons kantoor. Vroeg naar huis gaan kon je dan wel vergeten, want het was altijd reuze gezellig. Alle andere **collega's** van de Immunologie, bedankt voor de praatjes in de wandelgangen, adviezen en de gezelligheid bij de lunch, labuitjes en nieuwjaarsdiners. **Harm** en **Annemarie**, ik heb veel goede herinneringen aan de lunches in het park en de paddenstoelenwandelingen. Graag wil ik **Daniëlle, Bibi en Erna** speciaal bedanken voor al het werk dat jullie verricht hebben aan de lay-out van dit proefschrift.

Pietje-precies was niet altijd de makkelijkste klant… Bedankt voor jullie geduld met mij! **Elisa Calamita**, aan jou heb ik de mooie omslag te danken, het is prachtig geworden!

Groeppie, mijn favoriete mensen om de zaterdagavond mee door te brengen. Ik ben altijd in voor een goed gesprek en dat is precies wat ik bij jullie kan krijgen. De avonden met jullie bij de tuinhaard of bij Jasper in het onderhuis kon ik even afstand nemen van het werk en genieten van jullie gezelligheid. Lieve Marije en Janneke, ik heb heel veel aan jullie te danken. Ik heb jullie meeleven tijdens mijn PhD en de zoektocht naar een nieuwe baan enorm gewaardeerd. Zeker de laatste maanden ging ik totaal op de drukte en hebben jullie erg weinig aan mij gehad. Maar jullie stonden paraat om mijn schaduw in de watten te leggen. De nachtjes weg en etentjes werden tot in de puntjes verzorgd, zodat ik alleen maar aan hoefde te schuiven. Bedankt voor jullie onvervangbare vriendschap!

Jenneken, Nelleke, Elisabeth, Johanneke en **Karla,** al sinds de middelbare school blijven we contact met elkaar houden. Elke verjaardag, high-tea of etentje is het weer super gezellig. Karla en Jenneken, het was fantastisch om bij jullie bruiloften aanwezig te zijn. We zijn allemaal iets heel anders met ons leven gaan doen en dat maakt het extra leuk. Het is goed voor mij om er met enige regelmaat aan herinnerd te worden dat er ook nog zoiets als een leven buiten het lab bestaat. En nu Erika gewoon nog even vrolijk verder gaat met een volgende opleiding, hoop ik dat jullie mij daaraan willen blijven herinneren. Ik hoop dat onze vriendschap nog lang voortgezet wordt! **Mariska**, wij begonnen onze onderzoekscarrière samen in Leiden. Vanaf de eerste week waren we dikke maatjes, hoewel we er een sport van maakten om zoveel mogelijk snertopmerkingen te maken naar elkaar. Jij bleek een natuurtalent onderzoeker te zijn en wist zelfs je eigen PhD-beurs binnen te slepen. Ik wist toen allemaal nog niet zo goed wat ik met m'n leven aan wilde. Jij offerde je vrijwillig op om al mijn vermeende verdrietelijkheden van de stageprojecten en twijfels over de toekomst aan te horen. Bedankt voor die goede gesprekken! Succes met de laatste loodjes van jouw PhD en jou kennende, draai jij straks een fantastisch proefschrift in elkaar waar je trots op kunt zijn! **Familie Ruit** en **familie Schouten**, jullie meeleven tijdens mijn PhD en interesse voor mijn onderzoek was bijzonder.

Papa en **mama**, jullie hebben mij van jongs af aan gestimuleerd om te doen waar ik goed in was en altijd gesteund in de keuzes die ik maakte. Mijn doorzettingsvermogen heb ik van jullie geërfd. Als eerste in de familie ging ik een PhD doen en dat was best spannend. Als ik weer eens bedacht had dat het me vast en zeker niet zou lukken, bleven jullie vertrouwen in mij houden. Ik ben jullie veel dank verschuldigd voor alles wat jullie gedaan hebben om mij te brengen waar ik nu ben. Ook nu ik op eigen benen sta, staan jullie nog altijd voor mij klaar wanneer ik dat nodig heb. Bedankt voor jullie onvoorwaardelijke steun en liefde! **Ronald,** ik weet het, jij vindt mij een ongelofelijke nerd. Het zij je vergeven. Nu heb je het product van mijn noeste vlijt in je handen. Niet dat je daar dan iets wijzer van wordt, maar hier was ik dus al die tijd aan bezig. Ik ben je intens dankbaar voor al die

keren dat je mijn brommer hebt gerepareerd, zodat ik door weer en wind nog enigszins veilig in het Erasmus MC kon komen. **Marloes**, als jij nog eens lasagne maakt, houd ik me aanbevolen. Zolang je mijn keuken maar netjes opruimt en ik niets hoef te doen ;).

Annemiek, dear sweet sister en mijn allerbeste vriendin, wie ben ik zonder jou? Jij bent diegene die mij door en door kent. Iedere stap van dit PhD-traject ben jij er geweest om mijn verhalen, klaagzangen en jubelkreten aan te horen. Zonder iets van de inhoud te begrijpen, weet jij precies dat mijn proefschrift over IE-EFF-EN gaat. Jij en ik zijn zo verschillend. Waar jij volledig in paniek raakt van een Excelfile vol getallen, zou ik het geen uur uithouden in een klas vol stuiterende kleuters. Ik mag dan misschien een groot brein hebben, jij hebt een ontiegelijk groot hart. De keren dat ik gebruik mocht maken van jouw stellingskastvoorraad zijn niet te tellen en daar ben ik je oneindig dankbaar voor. Maar houd ook in gedachten dat het niet erg is om af en toe de kastdeuren op slot te doen. Je bent heel speciaal en daar moet je zuinig op zijn. Love you!

CURRICULUM VITAE

Erika Huijser was born in Rotterdam, the Netherlands on the 23rd of March 1994. She pursued her interests in medical biology and laboratory science by studying Biomedical Sciences at Leiden University. She received her cum laude Bachelor's degree in 2015 and continued the Master's research program in Biomedical Sciences. During her studies she became especially interested in immunology and completed several internships focusing on infectious diseases and autoimmunity. In 2017, she carried out her graduation research project at the department of Immunology at the Erasmus MC under the supervision of dr. Marjan Versnel. After attaining her Master's degree cum laude, she proceeded as a research technician at the department of Immunology on a collaborative project on small molecule inhibitors for application in autoimmune diseases. Extending the work on systemic autoimmune diseases, she began her PhD in 2018 concentrating on the immunopathobiology of type I interferon pathway activation in primary Sjögren's syndrome under the supervision of associate professor dr. Marjan Versnel and medical immunologist dr. Wim Dik. In January 2022, she started her residency training in clinical chemistry at LabWest, HagaZiekenhuis in The Hague.

PHD PORTFOLIO

Name PhD student	Erika Huijser
Erasmus MC department	Immunology
Research school	Molecular Medicine (MolMed)
PhD period	March 2018 – December 2021
Promotor	Dr. M.A. Versnel
Copromotor	Dr. W.A. Dik

Courses and workshops

2017	The Monocytes: origins, destinations, functions and diagnostic targets
2018	Advanced course on Applications in flow cytometry
2018	Molecular Medicine
2018	Virology
2018	People in Science: Workshop Career Development for PhD candidates
2018	Personal Leadership and Communication
2018	Photoshop and Illustrator CS6
2018	Scientific Integrity
2018	Follow-up course Photoshop and Illustrator CS6
2018	Basic course on R
2019	Biomedical Scientific English Writing
2019	Advanced Immunology
2019	Next Generation Sequencing for Clinical Genetics
2019	Microbiomics
2020	Microscopic Image Analysis: From Theory to Practice
2020	Advanced Microsoft Excel 2016
2021	Workshop Career Development, CV, LinkedIn
2021	BCF Career Event 2021

(Inter)national scientific meetings and presentations

2018	Regional meeting National Sjögren's Patients Association (NVSP)
2018	22nd Molecular Medicine Day (poster)
2018	14th International Sjögren's Symposium, Washington, DC, USA (poster)
2019	23rd Molecular Medicine Day (oral poster pitch)
2019	NVVI Symposium, Lunteren, the Netherlands
2019	Annual European Congress of Rheumatology, Madrid, Spain (poster)
2019	EULAR IFN-taskforce

2019	4th International Conference on Innate Immune Memory, Nijmegen, the Netherlands (poster)
2019	NVVI Annual Meeting, Noordwijkerhout, the Netherlands (poster)
2020	Research visit Radboud University, Nijmegen, the Netherlands
2020	Annual European Congress of Rheumatology, online (oral poster pitch)
2020	NVVI Annual Meeting, online (oral poster pitch)
2021	NVVI Symposium, online
2021	Annual European Congress of Rheumatology, online (oral poster presentation)

Teaching

2017-2021	Supervision bachelor's and master's students - research internships
2018-2021	Histology Classes Medicine/Clinical Technology students - inflammation and lymphoid organs

Grants and Awards

2018	Travel grant from the National Sjögren's Patients Association (NVSP)
2018	Travel grant from the Erasmus MC Trustfonds
2019	Travel award European Congress of Rheumatology

Other activities

2019-2020	PhD committee
2018-2021	Weekly Journal Club department of Immunology
2018-2021	Seminars department of Immunology
2018-2021	Refereeravonden Allergology/Clinical Immunology
2019-2021	Biosafety Committee

PUBLICATIONS

Manuscripts published

Flessa CM, Zampeli E, Natsis V, Evangelopoulos ME, Bodewes ILA, **Huijser E**, Versnel MA, Moutsopoulos HM, Mavragani CP. Genetic variants of the BAFF gene and risk of fatigue among patients with primary Sjögren's syndrome. Frontiers in Immunology – accepted for publication.

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Schrijver B, Dijkstra DJ, Borggreven NV, La Distia Nora R, **Huijser E**, Versnel MA, Van Hagen PM, Joosten SA, Trouw LA, Dik WA. Inverse correlation between serum complement component C1q levels and whole blood type-1 interferon signature in active tuberculosis and QuantiFERON-positive uveitis: implications for diagnosis. Clinical & Translational Immunology 2020; 9: e1196

Huijser E, van Helden-Meeuwsen CG, Groot N, Bodewes ILA, Wahadat MJ, Schreurs MWJ, van Daele PLA, Dalm VASH, van Laar JAM, van Hagen PM, Waris M, Kamphuis S*, Versnel MA*. MxA is a clinically applicable biomarker for type I interferon activation in systemic lupus erythematosus and systemic sclerosis. Rheumatology 2019; 58: 1302-1303.

Bodewes ILA, **Huijser E**, van Helden-Meeuwsen CG, Tas L, Huizinga R, Dalm VASH, van Hagen PM, Groot N, Kamphuis S, van Daele PLA, Versnel MA. 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.

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Huijser E, van Helden-Meeuwsen CG, Grashof DGB, Tarn JR, Brkic Z, Huisman JMA, Wahadat MJ, van de Werken HJG, Lopes AP, van Roon JAG, van Daele PLA, Kamphuis S, Ng WF, Bekkering S, Joosten LAB, Dik WA, Versnel MA. Trained immunity and pro-atherogenic phenotype induced by type I interferons: potential implications for primary Sjögren's syndrome. Frontiers in Immunology – under review.

* denotes shared authors