Towards stratification of patients with Sjögren's syndrome

Single cell analyses and immune profiling

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I said: What about my eyes?

God said: Keep them on the road.

I said: What about my passion?

God said: Keep it burning.

I said: What about my heart?

God said: Tell me what you hold inside it.

I said: Pain and sorrow.

God said: ...Stay with it. The wound is the place where the Light enters you.

– Rumi

(translated)

Scientific environment

The following doctoral project was conducted at the Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen and within the framework of the Bergen Research School of Inflammation. The work was carried out under the supervision of Silke Appel and Roland Jonsson within a period of 2017 and 2020.

The flow cytometry and mass cytometry experiments were performed at the Flow Cytometry Core Facility, Department of Clinical Science, University of Bergen, Norway.

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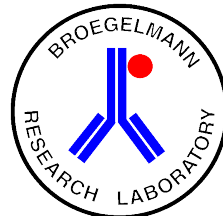
Acknowledgements

I still remember the day of the Skype interview with Silke. The conversation went quite well, but funnily enough, what I remember the most is Silke asking me if I can adjust with the rain in Bergen. I remember explaining to her how much I love monsoon in India. I guess at that time, I did not know what exactly I was signing up for!!! I must admit, over the years, I have complained quite a lot about the rain here. However, despite that, Bergen will always be the most beautiful place I have ever seen. It will always be my 'fairy tale land' where I found myself and made myself better.

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List of Publications

I. Single cell based phosphorylation profiling identifies alterations in Toll-like receptor 7 and -9 signaling in patients with primary Sjögren's syndrome

Richard Davies, **Irene Sarkar**, Daniel Hammenfors, Brith Bergum, Petra Vogelsang, Silje M. Solberg, Sonia Gavasso, Johan G. Brun, Roland Jonsson, Silke Appel. *Front Immunol* (2019) 10:281.

II. Aberrant signaling of immune cells in Sjögren's syndrome patient subgroups upon interferon stimulation

Irene Sarkar, Anders K. Aarebrot, Silje M. Solberg, Aleksandra Petrovic, Richard Davies, Anagha M. Joshi, Brith Bergum, Johan G. Brun, Daniel Hammenfors, Roland Jonsson, Silke Appel

Manuscript

III. Analysis of TAM receptor expression in patients with primary Sjögren's syndrome

Irene Sarkar, Kirsten Lassing, Aleksandra Petrovic, Richard Davies, Magdalena Keindl, Johan G. Brun, Roland Jonsson, Silke Appel

Under revision

Summary

Primary Sjögren's syndrome (pSS) is a systemic, chronic autoimmune disorder that is characterized by progressive lymphocytic infiltration in the exocrine glands i.e., the salivary and lacrimal glands, leading to immune-mediated glandular destruction. It mostly affects middle-aged women, making diagnosis of the disease challenging, as the symptoms (primarily dryness of the mouth and eyes and fatigue) are often confused with the side effects of drugs, other comorbidities or aging. Currently there is no cure, as the exact mechanism of the disease pathogenesis is not known, and treatment strategies mainly aim at alleviating the symptoms. Like most autoimmune diseases, pSS progression and phenotype are complex and multi-faceted, with a wide spectrum of clinical manifestations, ranging from local to systemic, including fatal conditions like B cell lymphoma. Patient heterogeneity is a major obstacle to disease management. Therefore, it is imperative to identify potential disease markers that may help in diagnosis, prediction, stratification of the patients and/or identification of new therapeutic targets.

The overall aim of this thesis was to study the peripheral blood immune system in pSS, to identify disease-specific immune profiles and potential biomarkers that may help in patient stratification. In paper I, phosphoflow cytometry was used to compare basal and TLR7 and -9 stimulated phosphorylation states in immune cells of pSS patients and healthy donors. Both basal and stimulation-induced phosphorylation differed significantly between pSS patients and healthy individuals, and between patient subgroups. Plasma cytokine levels, measured by Luminex assay, also differed significantly between the patients and controls as well as between patient subgroups, and correlated with autoantibody status and other clinical parameters.

In paper II, single cell analysis of peripheral blood immune cells, with special emphasis on intracellular signaling, was done using mass cytometry. We compared the frequencies of different immune cell subsets among the patient subgroups and healthy individuals and analyzed their signaling profiles, upon stimulation with IFNα2b and IFNγ separately. Significant differences in cell frequencies were observed among the

SSA- and SSA+ pSS patients and controls, along with increased activation status in many cell types, particularly in the SSA+ subgroup. Upon IFNα2b and IFNγ stimulation, aberrations in phospho-signaling were detected in the various immune cell subsets of the patient subgroups, which were most prominent in the SSA+ pSS patients.

In paper III, we used flow cytometry and qPCR to analyze the expression of TAM receptors in various immune cells of pSS patients and healthy controls. Significant differences in the mRNA levels of some of the TAM receptors as well as in cell frequencies were observed between the patients and healthy donors. Differential TAM receptor expressions in the immune cells were detected between the pSS patients and healthy controls, with most of them being expressed at slightly lower levels in the patients.

In conclusion, aberrations in cellular compositions, cytokine and TAM receptor levels as well as discrepancies in intracellular signaling pathways were detected in the pSS patients and their subgroups compared to healthy individuals. Further research can shed light on new biomarkers for stratification of patients for personalized treatment. Optimized therapeutic strategies can greatly alter the disease outcome and quality of life.

Abbreviations

ACA	Anti-centromere antibody
ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatism
ADAM10	Disintegrin and metalloproteinase 10
AECG	American-European Consensus Group
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANA	Anti-nuclear antibody
ANOVA	Analysis of variance
Anti-CCP	Anti-citrullinated cyclic peptide
RNP	Ribonucleoprotein
Anti-M3R	Anti-muscarinic acetylcholine M3 receptor
AP-1	Activator protein-1
APCs	Antigen presenting cells
BAFF	B-cell-activating factor
BCRs	B cell receptors
Bregs	B regulatory cells
C/EBP-β	CCAAT/enhancer-binding protein-β
CMV	Cytomegalovirus
CREB	cAMP-responsive-element-binding protein
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein-4
CTLs	Cytotoxic T lymphocytes
CV	Coefficient of variation
CVID	Common variable immunodeficiency
CyTOF	Cytometry by time-of-flight
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
EBV	Epstein-Bar virus
EGF	Epidermal growth factor
EGM	Extraglandular manifestations
ERK	Extracellular-signal-regulated kinase
ESSDAI	EULAR SS Disease Activity Index
ESSPRI	EULAR SS Patient Reported Index
EULAR	European League Against Rheumatism
FasL	Fas ligand
FBS	Fetal bovine serum
FcγR	Fc gamma receptor
FDR	False discovery rate
FMO	Fluorescence minus one
FNIII	Fibronectin type III
FSC	Forward scatter

GAS	IFNγ activated sites
Gas6	Growth-arrest-specific 6
GATEs	IFN-γ-activated transcriptional elements
GC	Germinal center
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome wide association studies
HCQ	Hydroxychloroquine
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HTLV-I	Human T-lymphotropic virus type-I
ICP	Inductively coupled plasma
IFNAR	IFNα/β receptor
IFNGR	IFNγ receptor
IFNs	Interferons
Ig	Immunoglobulin
IKK	Inhibitory kappa kinase
IL	Interleukin
Ir	Iridium
IRF	IFN regulatory factor
ISGF3	Interferon-stimulated gene factor 3
ISGs	Interferon stimulated genes
ISRE	IFN-stimulated response elements
JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
KO	Knock-out
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
LTβR-Ig	Lymphotoxin β receptor IgG fusion protein
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MCP-1	Macrophage chemoattractant protein-1
MdFI	Median fluorescence intensity
MdSI	Median signal intensity
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MST	Minimum spanning tree
MyD88	Myeloid differentiation 88
NAO	Non-Animal Origin
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NSAIDS	Non-steroidal anti-inflammatory drugs
OD	Optical density
ODNs	Oligodeoxynucleotides
OR	Odds ratio

PAMPs	Pathogen-associated molecular patterns
PB	Pacific blue
PC1	First principal component
PCA	Principal component analysis
Pd	Palladium
PD-1	Programmed death-1
pDCs	Plasmacytoid dendritic cells
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PMBCs	Peripheral blood mononuclear cells
PMTs	Photomultiplier tubes
PO	Pacific orange
ProS	Protein S
PRRs	Pattern recognition receptors
pSS	Primary Sjögren's syndrome
PtdSer	Phosphatidylserine
QN	Quantile normalization
RA	Rheumatoid arthritis
RCTs	Randomized clinical trials
RF	Rheumatoid factor
Rh	Rhodium
RIG-I	Retinoic-acid-inducible gene I
ROUT	Robust regression and outlier
SAPK	Stress-activated protein kinase
SBE	STAT3-binding element
SHBG	Sex hormone binding globulin
SLE	Systemic lupus erythematosus
sMer	Soluble Mer
SNPs	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signaling
SOM	Self-organizing map
SS	Sjögren's syndrome
SSA	Sjögren's syndrome-related antigen A
SSB	Sjögren's syndrome-related antigen B
SSC	Side scatter
SSc	Systemic sclerosis
ssRNA	single-stranded RNA
sSS	Secondary Sjögren's syndrome
STAT	Signal transducers and activators of transcription
STING	Stimulator of IFN genes
TBX21	T box 21
TCRs	T cell receptors
Tfh	Follicular T follicular helper
TGF-β	Transforming growth factor β
Th	T helper
TIR	Toll/IL-1 receptor

TLRs	Toll-like receptors
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor- α
TOF	Time-of-flight
Tregs	T regulatory cells
TRIM	Tripartite motif
TYK2	Non-receptor tyrosine kinase 2
USP18	Ubiquitin carboxy-terminal hydrolase 18

Contents

1. Introduction

1.1 The immune system

Living organisms are constantly exposed to myriad pathogens like bacteria, fungi, viruses and parasites. In order to provide protection from diseases caused by them, a complex biological system, called the immune system has evolved across phyla. It can simultaneously discriminate between 'self'-'non-self' and harmful-harmless, thus protecting the host against infectious agents and abnormal 'self', while ignoring harmless substances and normal 'self' [1, 2]. As shown in figure 1, the vertebrate immune system can be broadly divided into two distinct yet functionally interconnected branches: the innate immunity and the adaptive immunity. These differ in terms of reaction time, specificity, diversity and ability to induce memory. In general, the former is non-specific but rapid while the latter is slow but highly pathogen-specific [3, 4] .
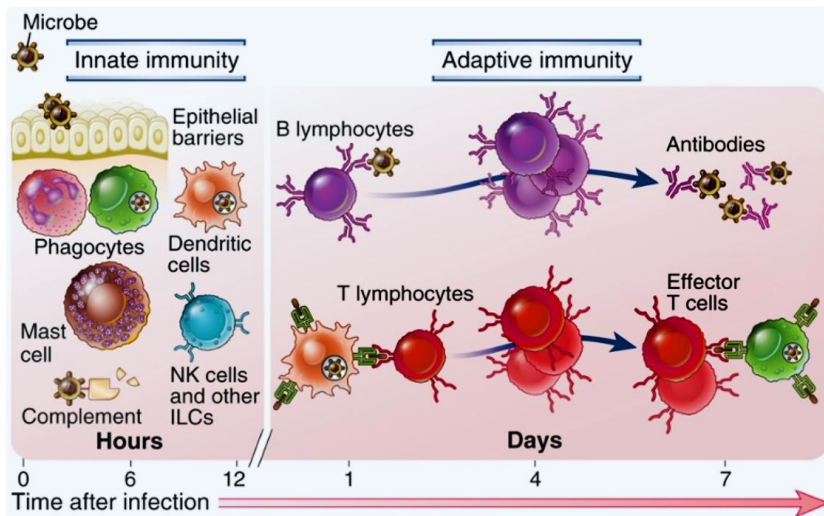
Figure 1. Innate and adaptive immunity. The innate immune system reacts within hours of an infection, while the adaptive immune reactions set in later, after activation of the lymphocytes. Figure from Cellular and Molecular Immunology, Abbas *et al.*, 9th edition [5]. Reprinted with permission from Elsevier.

The innate immune system is the first line of defense against invading pathogens. It is initiated within minutes to hours after infection. The three major mechanisms by which it offers protection to the host are inflammation, antiviral response and stimulation of adaptive immunity [5]. The cells of innate immunity include neutrophils, dendritic cells/DCs, monocytes, macrophages, natural killer/NK cells and NKT cells, amongst others. They express a limited number of germline-encoded receptors, called pattern recognition receptors (PRRs), the most extensively studied being the Toll-like receptors (TLRs) [6]. PRRs recognize evolutionarily conserved, invariant molecular patterns shared by groups of microbes called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), glycoproteins rich in mannose residues, double-stranded RNA and unmethylated CpG DNA [7, 8]. In addition, they can also detect endogenous molecules released from damaged or dying cells called damage-associated molecular patterns (DAMPs) like alarmins [9].

The hallmarks of the adaptive immune system are diversity, specificity and immunologic memory and its cellular components include T and B lymphocytes. Prior to infection, low levels of lymphocytes of each specificity are present in the host. Following an infection, on encountering its cognate antigen, a lymphocyte becomes activated, undergoes clonal expansion and differentiation, producing thousands of progenies, equipped to mount a strong immune response against the intruder [5, 10]. Secondary and subsequent encounters with the same pathogen mount a much more rapid and heightened immune response due to the long-lived memory cells generated from the primary response [5, 10].

Despite their differences, the two arms of the immune system work together for effective host defense. As Beutler, the 2011 Nobel prize winner, has elegantly summarized, "…the roots of adaptive immunity are buried deep in the soil of the innate immune system" [11]. Antigen presenting cells (APCs), specially DCs, play a crucial role in linking the innate and adaptive immune responses. They capture, process and present antigens to T cells in a major histocompatibility complex (MHC)-dependent manner, leading to their stimulation and subsequent activation of adaptive responses [12].

1.1.1 Cytokines as chemical messengers

Cytokines are chemical messengers of the immune system that aid in intercellular communication. These are a diverse group of molecules that bind to receptors on cell surfaces and can induce complex signaling cascades. They may act in an autocrine (acting on the cell secreting it), paracrine (acting on a cell in the vicinity), juxtracrine (between adjacent cells, requiring membrane-anchored proteins) or endocrine (acting on a distant target cell) manner [13]. These pleiotropic proteins are involved in activation, growth and differentiation and migration of immune cells. Those that cause differentiation and proliferation of immune cells are called colony-stimulating factors e.g., granulocyte macrophage colony-stimulating factor (GM-CSF); those that interfere with viral replication are called interferons (IFNs); a special class of cytokines that have chemoattractant property and are involved in leukocyte migration are called chemokines e.g., macrophage chemoattractant protein-1 (MCP-1) [14]. Cytokines can be proinflammatory (e.g., type I IFNs, tumor necrosis factor- α/TNF-α, interleukin 1/IL-1, IL-12.) or anti-inflammatory (e.g., IL-4, IL-10, transforming growth factor β/TGFβ) [15, 16].

Interferons in the immune response

IFNs belong to a diverse family of cytokines that have direct anti-viral effects. They are produced in response to activation of PRRs like TLRs and other cytosolic RNA-DNA sensors (e.g., retinoic-acid-inducible gene I/RIG-I and stimulator of IFN genes/STING) and help to eliminate infected cells and protect uninfected bystander cells [17]. In humans, the IFN family can be divided into three classes: type I, type II and type III. The type I IFN family is the largest and composed of 13 subtypes of IFNα, IFNβ and other variants like IFNϵ, IFNκ and IFNω [18]. While IFNγ is the sole member of type II IFN family, the type III family consists of IFNλ1 (IL29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B) and recently discovered IFNλ4 [19, 20]. IFNs exert their biological activity through the activation of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) signaling cascades [21].

Whereas type III IFN response is mostly restricted to epithelial mucosal surfaces, all nucleated cells can be induced to produce type I IFNs [22-24]. However, plasmacytoid dendritic cells (pDCs) are considered as 'professional' type I IFN producers as they constitutively express IFN regulatory factor 7 (IRF7) and thus account for about 100 to 1000-fold greater IFN production than any other blood cell type upon viral infection [25]. Type I IFNs can effectively inhibit viral replication by degrading viral RNA, inhibiting translation of viral mRNA and apoptosis of infected cells They can also activate NK cells, cytotoxic T lymphocytes (CTLs) and DCs and boost antibody responses [19, 26]. On one hand, their gene products can initiate a feedforward loop inducing production of more type I IFNs, on the other hand they can generate negative feedback resulting in production of negative regulators like suppressors of cytokine signaling (SOCS) proteins that can inhibit uncontrolled inflammation [27-29]. Type III IFNs are involved in blocking viral spread at the site of infection and exerting protective roles to restrict tissue damage [22]. IFNγ is predominantly produced by activated T cells, NKT cells and NK cells and apart from its modest antiviral activity, it plays a pivotal role in stimulation and modulation of immune responses [30]. Recently it has been shown that IFNγ has a number of immunoregulatory functions that optimize the antiviral response and simultaneously limit overzealous responses that may lead to collateral damage [31].

1.2 Signaling pathways

Immune cell signaling is a highly complex and coordinated process that is fundamental in achieving defense against infections while limiting host damage. If the equilibrium is broken due to aberrations in one or more signaling pathways, it may result in serious pathological conditions like autoimmune diseases or cancer. As innumerable signaling molecules are involved in a variety of signaling networks with overlapping and interconnected functions and redundancies, only those that are relevant for this thesis are reviewed in the subsequent sections.

1.2.1 TLR signaling

The human TLR family consists of 10 members (TLRs 1-10) and can be found in many cells like DCs, monocytes, macrophages, B cells, T cells and NK cells [32, 33]. These are integral membrane glycoproteins consisting of extracellular leucine-rich repeats (LRRs), a transmembrane region and an intracellular Toll/IL-1 receptor (TIR) domain. The LRRs are involved in ligand-binding while the TIR domain is responsible for signaling [34]. They can be subdivided into two classes based on their cellular locations: cell surface and intracellular [35]. TLRs 1, 2, 4, 5, 6 and 10 are located on the cell surface whereas TLRs 3, 7, 8, and 9 are localized on endosomal membranes inside the cell [36]. The cell surface TLRs recognize a wide variety of microbial components like LPS and flagellin, while the intracellular TLRs recognize nucleic acids [37-39]. For example, TLR7 recognizes single-stranded viral RNA (ssRNA) and TLR9 mediates the recognition of viral and bacterial CpG DNA [36, 37]. TLRs are pre-assembled dimers that are activated upon ligand-binding and recruit adaptor proteins, like myeloid differentiation 88 (MyD88), for downstream activation of IRFs (like IFR7 in pDCs) or mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NF-κB) [40, 41]. The net result is induction of type I IFNs or proinflammatory responses respectively [36, 41, 42]. In pDCs, TLR7 and -9 can activate both NF-κB and IRFs, leading to both proinflammatory and antiviral states, as shown in figure 2. [36, 43]. The MAPK and NF-κB pathways are further elaborated in the following sections.

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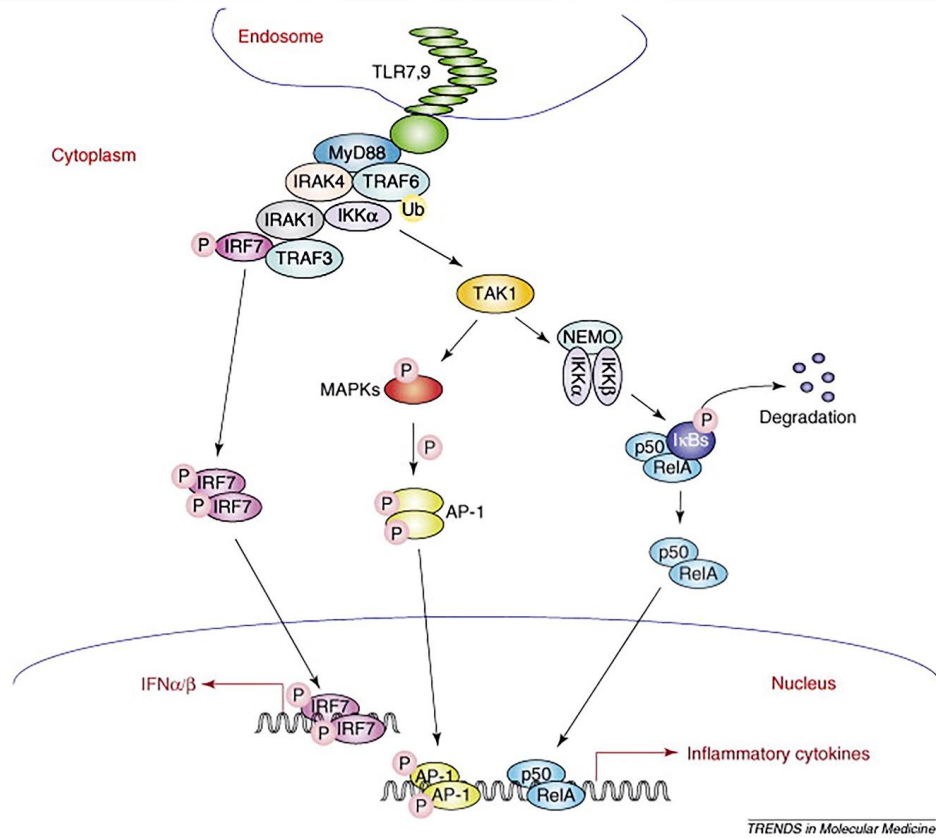
Figure 2. Signaling through TLR7 and -9. TLR7 and -9 reside in endosomal compartments of cells. Following binding of TLR to ssRNA (TLR7) or unmethylated CpG dinucleotides (TLR9), a MyD88-dependent pathway signals through activation of TAK1-mediated NF-κB and MAPK pathways which regulates transcription of genes encoding inflammatory cytokines. In pDCs, IRF7 forms a signaling complex with MyD88 and following its phosphorylation it dimerizes, enters the nucleus and regulates the expression of type I IFNs, including IFN-α and IFN-β. Figure adapted from Kawai and Akira, 2007 [41]. Reprinted with permission from Elsevier.

1.2.2 MAPK cascade

The MAPK cascade is one of the most prevalent and evolutionarily conserved pathways in eukaryotes that controls a wide variety of immune cell functions like proliferation and differentiation, survival, metabolism, cell adhesion and migration, stress response, apoptosis and transformation [44-46]. Each pathway is a three-tiered kinase cascade where extracellular signals are transmitted by sequential phosphorylation and activation of subsequent tiers of signaling molecules. The first kinase in the series is a serine/threonine kinase called MAPK kinase kinase/MAPKKK (e.g., Raf), which upon activation by small GTPases, phosphorylates and activates a MAPK kinase/MAPKK (e.g., Ras), in turn phosphorylating and activating a MAPK. In mammals the MAPK cascade can be divided into three main families – the extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38/stress-activated protein kinase (SAPK). The ERK pathway is activated by various growth factors and mitogens and mainly mediates cell growth and differentiation, whereas the JNK and p38 pathways are induced in response to environmental stress and inflammatory cytokines (figure 3).
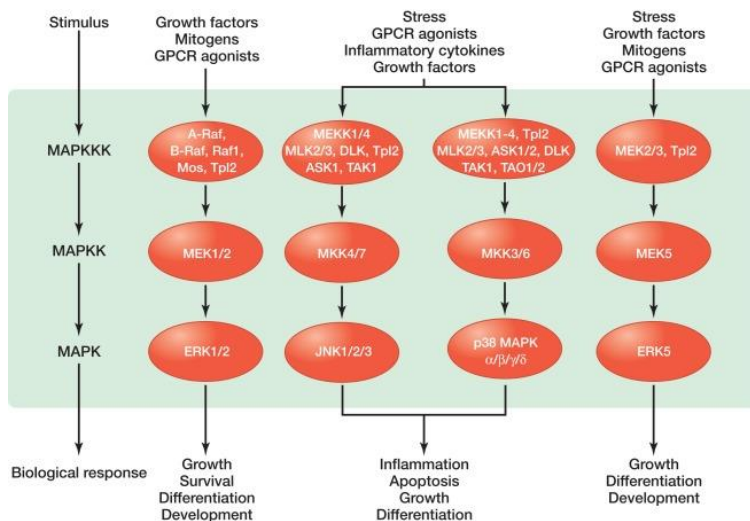
Figure 3. MAPK pathways. The MAPK pathway consists of a three-tiered kinase cascade in which the previous kinase, upon activation by phosphorylation, phosphorylates and activates the subsequent one. Figure adopted from Morrison, 2012 [45]. Reprinted with permission from Cold Spring Harbor.

MAPKs are activated by dual phosphorylation of conserved threonine/tyrosine residues, enabling them to interact with hundreds of different substrates like transcription factors and repressors and chromatin remodeling proteins [44, 45, 47, 48]. The pathway can be inhibited at various levels by phosphatases, that can dephosphorylate the tyrosine and serine/threonine residues on the kinases [49].

The activation of the MAPK pathway in immune cells has been extensively studied in the context of TLRs. As mentioned earlier, TLR signaling can activate both ERK and p38 in a MyD88-dependent manner, leading to activator protein-1 (AP-1) activation (see figure 2) [50]. This ultimately induces the production of proinflammatory cytokines and chemokines like TNF, IL-1β, IL-6 and IL-8 [39]. Both ERK and p38 are involved in type I IFN responses. For example, p38 is required for the transcription of interferon stimulated genes (ISGs) in a STAT-independent manner [17]. There are several studies showing that p38 and ERK are involved in anti-viral responses and p38 also shows growth-inhibitory effects [17]. It has been shown that ERK also participates in an IFNγ-dependent transcription of CCAAT/enhancer-binding protein-β (C/EBP-β), a transcription factor that binds to response elements known as IFN-γ-activated transcriptional elements (GATEs), in the promoters of certain ISGs [51, 52].

1.2.3 NF-κB pathways

NF-κB is a family of proinflammatory transcription factors that can be divided into two subfamilies – the NF-κB proteins and the Rel proteins. The NF-κB proteins consist of two precursor proteins, NF-κB1/p105 and NF-κB2/p100, that are processed into shorter proteins, p50 and p52, respectively; the Rel proteins consist of three members – RelA (p65), RelB and c-Rel. Members of the two subfamilies dimerize to become activators of transcription [53, 54]. The most frequently activated form of NF-κB in TLR signaling is a heterodimer composed of RelA and p50. In quiescent state, these dimers are inactive and are localized mostly in the cytoplasm, bound to inhibitory IκB proteins. Upon receptor activation, IκB is phosphorylated and targeted for proteasomal degradation by the inhibitory kappa kinase/IKK complex (consisting of IKKα, IKKβ and NEMO). The free NF-κB dimer can now enter the nucleus and enhance target gene transcription (figure 4) [55].
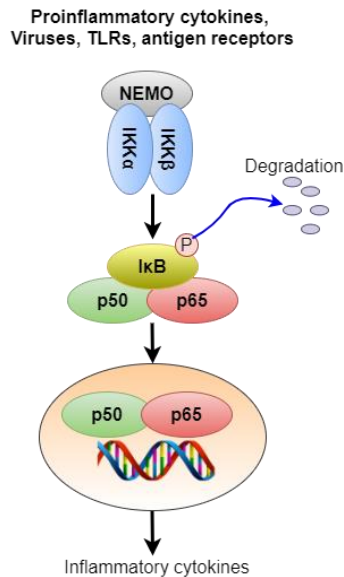
Figure 4. Activation of NF-κB pathway. Upon activation, the IKK complex phosphorylates IκB, sending it for proteosomal degradation. The NF-κB dimer is now free to enter the nucleus to induce transcription of proinflammatory genes. Figure inspired from Luo *et al.*, 2005 [56].

Signaling through NF-κB plays a crucial role in the development of the immune system and innate and adaptive immune responses. As mentioned earlier, signaling through TLR7 and -9 in pDCs leads to NF-κB activation and production of proinflammatory cytokines [41]. NF-κB also plays an indirect role in type I IFN production and response. It generates a positive feedback loop for type I IFN production and IRF7 induction in the early stages of viral infection, when endogenous IRF7 levels are low. Under such circumstances, NF-κB, along with IRF3 and low IRF7, binds to type I IFN genes generating low levels of type I IFNs. This, in turn, promotes more IRF7 production, consequently inducing more type I IFN production [57]. In the past decade it has become evident that NF-κB also has important roles in IFNγ-induced cellular responses in a context-dependent manner [58]. Conversely, it has also been shown that IFNγ can augment NF-κB activity by increasing proteasomal degradation of IκB [59].

1.2.4 JAK/STAT signaling

IFNs function through the JAKs and STATs downstream of their receptors IFNAR1/2 (for type I), IFNGR1/2 (for type II) and IL10R2/IFNLR1 (for type III) [60, 61]. JAKs are tyrosine kinases that are bound to the cytoplasmic domains of the IFN receptors. Members include JAK1, JAK2, JAK3 and TYK2 [62]. STATs are a family of transcription factors comprising seven members (STATs 1, 2, 3, 4, 5a, 5b and 6) that are present in inactive form in the cytosol of resting cells [62]. Binding of the IFNs to their respective receptors cause receptor dimerization and JAK auto- and trans-phosphorylation. Activated JAKs now catalyze the phosphorylation of the receptors on specific tyrosine residues, generating a docking site for the STATs. Once bound, the STATs are phosphorylated by the JAKs on specific tyrosine residues which promotes their dissociation, dimerization and entry into the nucleus to bind to specific DNA sequences called IFN-stimulated response elements/ISRE (for type I and III IFNs) and IFNγ activated sites/GAS (for type I and II IFNs) [60, 63-65], as depicted in figure 5. The canonical type I pathway involves STAT1-STAT2 heterodimers that form a complex with IRF9, called interferon-stimulated gene factor 3 (ISGF3). This complex migrates to the nucleus and binds to ISRE in the promoters of ISGs to induce gene transcription [17, 62, 66, 67]. IFNγ, on the other hand, promotes STAT1 homodimerization and binding to GAS elements in the promoters of ISGs. [17, 65]. Tyrosine phosphorylation is crucial for STAT dimerization and nuclear transport. Additionally, phosphorylation at serine residues are required for complete transcriptional activation of STATs 1 and 3 [66, 68]. Whereas type I signaling through STAT1 is generally proinflammatory, antiproliferative and proapoptotic, type I signaling through STATs 3, 4 and 5 augment survival, proliferation and differentiation [69]. Moreover, STAT3 has been shown to induce both pro- and anti-inflammatory responses [70]. In fact, it is a key mediator in IL-10 signaling leading to anti-inflammatory effects and can also directly inhibit STAT1 activity [17]. Thus, activation of the STATs are highly context-dependent and varies from one cell type to another and the ligand in action [71].
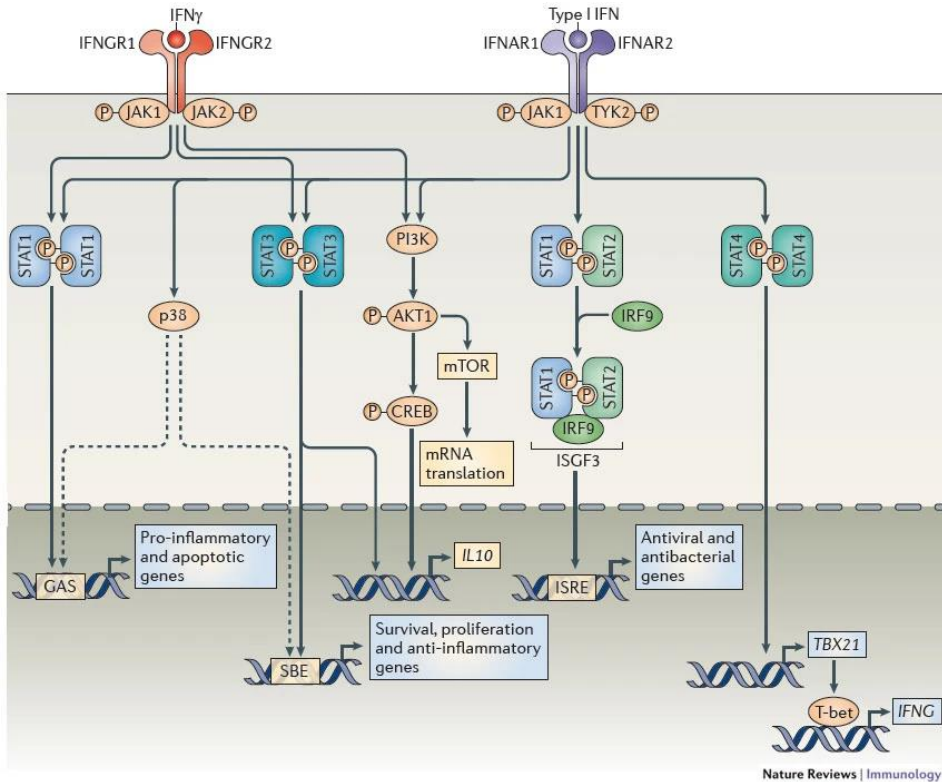
Figure 5. JAK-STAT signaling pathways. Different STAT family members can be activated by IFNs. STAT1 homodimers can be formed in response to both type I and type II IFNs. These homodimers bind to the promoters of ISGs, leading to the induction of genes encoding proinflammatory cytokines and apoptotic factors. Type I and type II IFNs can also activate STAT3 homodimers, that can result in the production of both pro- and anti-inflammatory cytokines (such as IL-10). STAT1-STAT2 heterodimers, following activation by type I IFNs, bind to IRF9 in the cytosol, to form the ISGF3 complex. ISGF3, in turn, translocates to the nucleus, binds to ISREs and activates antiviral and antibacterial genes. In addition, type I IFNs induce IL-10 production either through the phosphoinositide 3-kinase (PI3K)-AKT pathway or through STAT3 homodimers. CREB, cAMP-responsive-element-binding protein; IFNAR, IFNα/β receptor; IFNGR, IFNγ receptor; JAK, Janus kinase; SBE, STAT3-binding element; TBX21, T box 21; TYK2, non-receptor tyrosine kinase 2; Figure and text adapted from Gonzalez-Navajas *et al*., 2012 [17]. Reprinted with permission from Springer Nature.

Some mechanisms by which the JAK-STAT pathway can be modulated include the production of SOCS proteins, microRNAs, internalization of the cell-surface IFN receptors and ubiquitin carboxy-terminal hydrolase 18 (USP18) [67]. STAT3 can also negatively affect signaling through the JAK-STAT pathway by downregulating ISGF3 production and activity and competing with STAT1 for binding to docking sites [72].

1.3 Tolerance and autoimmunity

Unresponsiveness of the immune system to an antigen after repeated exposure to it is called 'tolerance' and non-reactivity to self-antigens is specifically known as 'self-tolerance'. The later ensures that self-reactive lymphocytes that can potentially be a threat to the host are eradicated. Due to the stochastic nature of the generation of B cell receptors (BCRs) and T cell receptors (TCRs), some lymphocytes with self-reactive receptors will be generated, which must be eliminated. Self-tolerance can be divided into central and peripheral tolerance. Central tolerance occurs in immature lymphocytes in the generative lymphoid organs (bone marrow and thymus) whereas peripheral tolerance occurs in mature lymphocytes in the peripheral lymphoid organs [43].

In central tolerance of B cells, the immature self-reactive B cells in the bone marrow can undergo two fates: they can either be subjected to receptor editing, where the self-antigen specific BCR is changed to a new non-reactive one, or undergo negative selection (clonal deletion) when receptor editing fails [73, 74]. For T cells, central tolerance takes place in the thymus where self-reactive clones are either deleted or they develop into T regulatory (Treg) cells. The fate between deletion and Treg generation is most probably determined by the binding affinity between the TCR and self-peptide-MHC complex [75, 76].

However, central tolerance does not ensure that no self-reactive lymphocytes will enter the periphery. Therefore, several mechanisms exist in the periphery (peripheral tolerance) that protect the host from these self-reactive cells. These include anergy, deletion or suppression. Self-reactive mature B and T cells undergo anergy or functional unresponsiveness when they are activated without proper costimulation.

This can be due to absence of innate immune responses, or the engagement of checkpoint molecules, such as cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed death-1 (PD-1) [77, 78]. B cells undergo anergy when they recognize self-antigens without T cell costimulation [79]. Autoreactive B and T cells may also undergo cell death by apoptosis [77, 79]. Apart from the natural Tregs generated by central tolerance, Tregs can also be induced in the periphery (inducible Tregs) from naïve T cells in the presence of IL-2 and TGFβ [80]. Both types of Tregs and B regulatory cells (Bregs) can cause immunosuppression of autoreactive cells by various mechanisms including secretion of regulatory cytokines like IL-10 and TGFβ [79, 81-83].

Failure of self-tolerance results in a condition called 'autoimmunity' where the host's immune system initiates immune responses against self-antigens. Such autoimmune reactions are antigen-specific and involve self-reactive lymphocytes [84]. Often individuals have auto-reactive lymphocytes without any pathological implications. However, sometimes it can cause inflammatory reactions of chronic or acute nature that can be organ-specific or systemic, leading to development of autoimmune diseases [85]. Organ-specific diseases include multiple sclerosis (MS) and type I diabetes while systemic diseases include systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). During their lifetime, about 5-10% of all individuals will develop some kind of autoimmune disorder [86].

The pathogenesis of autoimmune diseases is poorly understood. It is hypothesized that a combination of genetic and environmental factors results in disease development. In genetically predisposed individuals, environmental triggers can lead to the breakdown of tolerance, production of self-reactive lymphocytes, inflammatory responses mediated by innumerable factors and ultimate tissue damage. This sets up a self-perpetuating autoimmune loop. For instance, inflammation-induced tissue damage may lead to the exposure and/or modification of other self-antigens that may result in lymphocyte activation by epitope spreading [87]. Infections are often thought to be the initial driving forces for autoimmunity. Microbial antigens can potentiate autoreactivity by methods like molecular mimicry, release of sequestered antigens and

polyclonal activation. For example, rheumatic fever is a case of autoimmune reactions initiated by streptococcal infections and sustained by cross-reactivity to cardiac myosin [85]. If we look at the genetic aspect, genome wide association studies (GWAS) have identified many risk alleles, amongst which the strongest association has been established with MHC class II/human leukocyte antigen (HLA) locus, especially HLA-DR and HLA-DQ, for multiple autoimmune diseases including SS [88]. Other non-MHC genes involved in diseases like SS include *IRF5*, *STAT4*, *IL-10*, *IL-12A* and *Fas/Fas ligand* (*FasL*) [88-91].

1.4   TAM receptors and autoimmunity

A crucial mechanism for maintenance of immunological homeostasis is apoptosis or programmed cell death. It is a fundamental process of the immune system and mediated predominantly by the phagocytes of the innate immune system like neutrophils, DCs and macrophages. Clearance of dead cells by phagocytosis is crucial for maintenance of self-tolerance and consequently, failure in removing apoptotic debris may lead to autoimmunity [92]. Phosphatidylserine (PtdSer) is almost exclusively located on the inner leaflet of plasma membranes. However, in apoptotic cells it is exposed on the outer leaflets, acting as 'eat me' signals that are recognized by phagocytic cells resulting in engulfment and phagocytosis of the former. Errors in this pathway have been implicated in diseases like SLE, cystic fibrosis and SS [93, 94].

A family of receptor tyrosine kinases, called TAM, has been implicated in efferocytosis, e.g., efficient clearance of apoptotic cells, in the immune, reproductive and nervous systems [95-97]. The TAM family consists of three members- Tyro3, Axl and Mer and their ligands growth-arrest-specific 6 (Gas6) and protein S (ProS). In the context of the immune system, TAMs are mostly expressed on phagocytes but can also be present on some lymphoid populations [95, 98-100]. Impairment of TAM signaling leads to accumulation of apoptotic debris, release of autoantigens, inflammation, making way for breakdown of tolerance and autoimmunity [101]. It has been demonstrated in TAM knock-out (KO) mice that TAM receptors play a crucial role in maintenance of tissue homeostasis and prevention of inflammation, as these mice

develop a plethora of degenerative disorders like sterility and retinal blindness [102, 103]. As TAM triple KO mice mature, they develop chronic inflammation and broad-spectrum autoimmunity [104]. Loss of TAM signaling has been associated with several autoimmune diseases like SLE, SS and MS [105-107].

The extracellular domain of each TAM receptor consists of two tandem immunoglobulin (Ig)-like domains (ligand-binding domain) and two fibronectin type III (FNIII) repeats, followed by a transmembrane domain and an intracellular tyrosine kinase domain. The receptors signal as dimers [108]. Their ligands, Gas6 and ProS are structurally homologous proteins, that act as bridging molecules between the TAM receptors and apoptotic cells (figure 6) [108]. Whereas Gas6 is a potent ligand for all three receptors, ProS can only bind to Tyro3 and Mer [108-111].
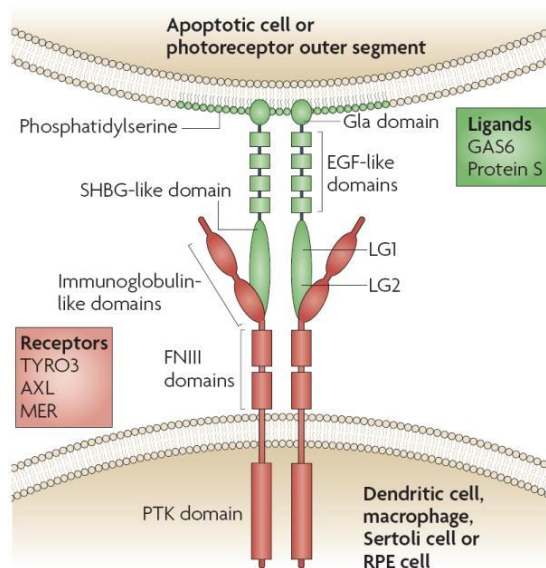
Figure 6. Structure of TAM receptors. Tyro3, Axl and Mer are expressed by several cell types in the immune system, including DCs, macrophages and immature NK cells. TAM receptor dimers bind to their ligands, Gas6 and ProS, through the interaction between the two Ig-like domains on the receptors and the sex hormone binding globulin (SHBG) domain on the ligands. Gas6 and ProS then bind to PtdSer, on the outer leaflet of apoptotic cells, via their Gla domains. EGF, epidermal growth factor, FNIII, fibronectin type III. Figure from Lemke and Rothlin, 2008 [108]. Reprinted with permission from Springer Nature.

Ligand binding causes receptor dimerization, autophosphorylation of the tyrosine residues, recruitment of downstream signaling molecules leading to cytoskeletal reorganization and internalization of the apoptotic cell [112, 113]. The extracellular domains of the receptors can also be cleaved proteolytically by metalloproteinases like disintegrin and metalloproteinase 10 (ADAM10) and ADAM 17 and shed into the plasma [114, 115]. These can act as decoy receptors, competing with the cell-membrane bound receptors for the ligand, thus impeding the removal of apoptotic cells [116]. Soluble TAMs have been associated with disease severity in some autoimmune diseases [117-120]. TAM receptors also play a significant role in the dampening of inflammatory responses by upregulating the production of SOCS1 and SOCS3 proteins via the IFNAR-JAK-STAT pathway, as illustrated in figure 7 [121, 122].
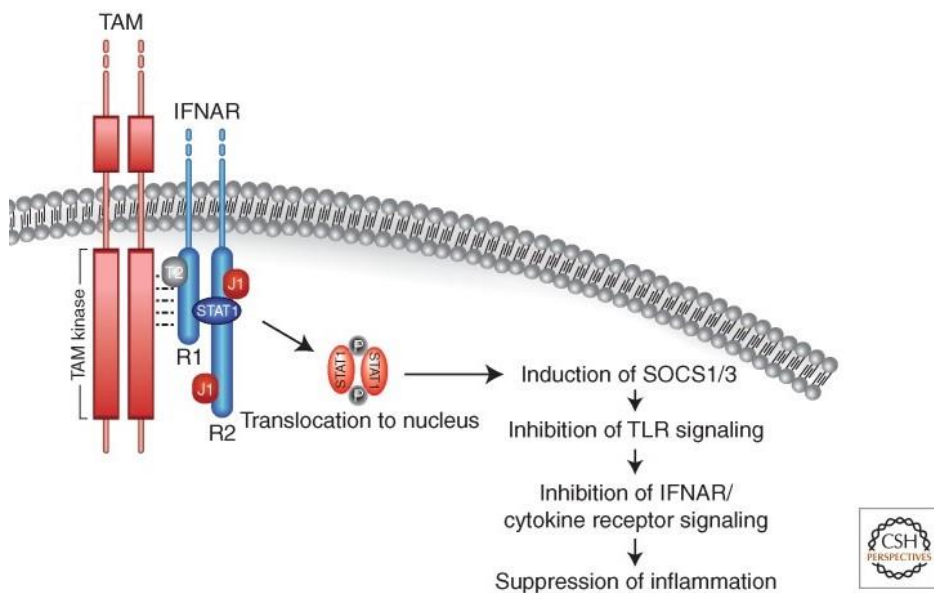
Figure 7. TAM receptors complexed with the IFNAR. In DCs, TAM receptors, when activated by the binding of a TAM ligand, form a complex with IFNAR. Direct activation of the hybrid TAM-IFNAR receptor by Gas6 leads to phosphorylation and activation of STAT1 that dimerizes and enters the nucleus, where it drives the expression of SOCS1 and SOCS3. Figure and text adapted from Lemke. G, 2013 [122]. Reprinted with permission from Cold Spring Harbor.

1.5   Primary Sjögren's syndrome

Sjögren's syndrome (SS) is a systemic, chronic, inflammatory autoimmune disorder, named after its discoverer Henrik Sjögren, a Swedish ophthalmologist [123]. It is characterized by progressive mononuclear cell infiltration in the salivary and lacrimal glands, causing dryness of mouth (xerostomia) and eyes (keratoconjunctivitis sicca) [124]. Persistent focal infiltration causes glandular tissue destruction, loss of glandular function and resultant dryness. It can occur alone (primary Sjögren's syndrome/pSS) or in association with other autoimmune disease like SLE and rheumatoid arthritis (RA) (secondary Sjögren's syndrome/sSS) [125]. Like many other autoimmune diseases, pSS is a female-biased disorder, with female: male ratio being 9:1, mostly affecting post-menopausal women [124, 126]. However, juvenile Sjögren's syndrome has also been reported in children and adolescents, with the mean age at the time of diagnosis being 10 years [127]. The incidence rate of pSS varies in different studies due to discrepancies in the classification criteria and methodologies. Global incidence ranges from 0.1 to 3% with more stringent estimates on using the revised American-European Consensus Group (AECG) criteria [128-132]. Autoantibodies against Ro/Sjögren's syndrome-related antigen A (SSA) and La/Sjögren's syndrome-related antigen B (SSB) are characteristic features of pSS with the presence of anti-Ro/SSA being a classification criterion of the disease [133]. One major problem is patient heterogeneity. Apart from the sicca symptoms, many patients suffer from a wide spectrum of Extraglandular manifestations (EGM) that may point towards unique pathophysiological mechanisms. Hence, it is vital to stratify and target the patient subgroups for effective treatment.

1.5.1   Pathogenesis of pSS

The complete etiopathogenesis of pSS is still unknown. However, it has been established that genetic, hormonal, immunologic and environmental factors work in concert to cause the disease [134]. Often many years pass before the onset of symptoms [124]. Viral infections like Epstein-Bar virus (EBV), Cytomegalovirus (CMV), Hepatitis C, Coxsackie virus and Human T-lymphotropic virus type-I (HTLV-1) have been implicated in pSS [134, 135]. Recently, altered buccal and intestinal microbiome

and chronic bacterial infection from *Helicobacter pylori* have also been associated with it [136]. In genetically predisposed individuals, an initial viral infection of the salivary glands may cause disruption of glandular epithelial cells resulting in an amplified IFN production, especially IFNα by pDCs. This leads to creation of an inflammatory microenvironment and exposure of SSA and SSB autoantigens on the dying cells [135]. These are small ribonucleoprotein particles that are normally localized inside the cells [137]. Exposure of these nuclear antigens in dying cells makes them immune targets. Along with viral antigens, APCs process and present these self-antigens, leading to activation of autoreactive T and B cells and subsequent activation of autoantibody-producing plasma cells. Autoreactive T cells can also enhance tissue damage by secreting cytotoxic granules, further disrupting the epithelium and amplifying the exposure of autoantigens. The autoantibodies produced can form immune complexes with their cognate autoantigens and bind to pDCs via the Fc receptor for IgG (FcγRIIa) and augment type I IFN production. In turn, type I IFNs drive autoantibody production by promoting differentiation and activation of the autoreactive B cells [135]. Recently, T helper 17 and T follicular helper cells, along with their regulatory counterparts, have received considerable attention in the pathogenesis of pSS as their imbalance can cause dysregulation of B cell dynamics and autoantibody production [138]. Stromal cells and glandular epithelial cells have also been implicated in the maintenance of the local inflammatory milieu in the affected exocrine glands [129, 139, 140]. The proposed model for pSS pathogenesis is shown in figure 8.
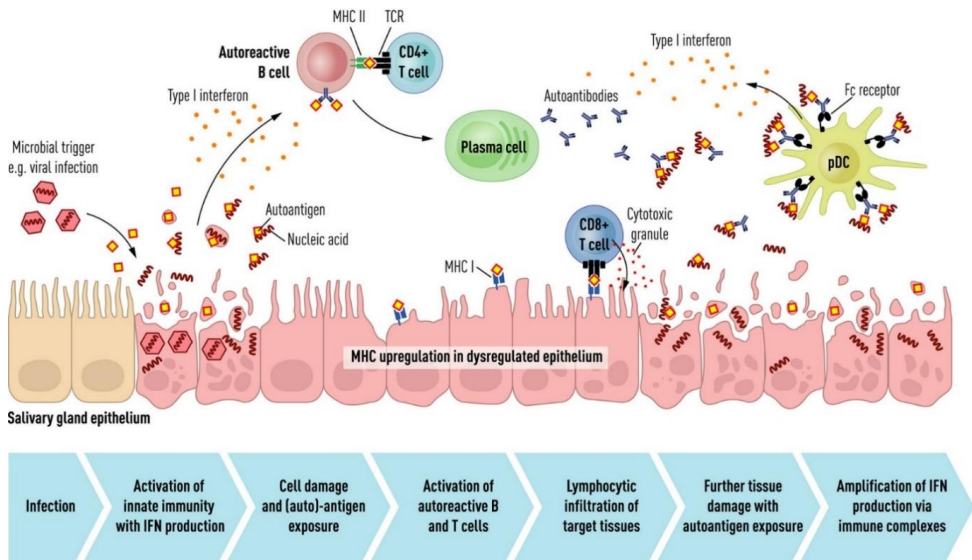
Figure 8. Disease model for pSS. Microbial triggers, such as viral infections, initiate disruption of the salivary gland epithelium and induce production of type I IFNs, thus creating an inflammatory microenvironment and released and exposure of autoantigens on dying cells. APCs then present the self-antigens, which leads to activation of autoreactive T and B cells and subsequent differentiation and activation of autoantibody-producing plasma cells. Autoreactive T cells can also induce tissue damage by secretion of cytotoxic granules, further disrupting the epithelium and causing amplified exposure of autoantigens. Immune complexes formed between autoantibodies and autoantigens bind receptors on pDCs, resulting in enhanced type I IFN production which, in turn, drives further autoantibody production. Through this process, a self-perpetuating cycle of autoimmunity is created. IFN, interferon; pDCs, plasmacytoid dendritic cells; MHC, major histocompatibility complex; TCR, T cell receptor. Figure and text adapted from Björk *et al.*, 2020 [135]. Reprinted with permission from John Wiley and Sons.

As mentioned earlier, susceptibility to pSS has a genetic component, with the strongest association being with HLA, especially HLA-DR and HLA-DQ molecules [141]. Some non-HLA risk genes include *IRF4*, *STAT5*, *CXCR5*, *IL-12A*, *TNIP1*, *IL-1RA*, *IL-10* and *Fas/FasL* [88, 142]. Single nucleotide polymorphisms (SNPs) in two genes, *STAT4* and *IRF5*, have been strongly associated with pSS. These polymorphisms further show an additive effect, with an increase in the odds ratio (OR) for pSS as the number of risk alleles in the carrier increases [143, 144]. It is becoming increasingly evident that environmental triggers often act through cellular pathways containing disease-

associated polymorphisms [145]. Although a genetic predisposition to SS appears to exist, the level of genetic contribution is not known. Large twin studies are lacking. Only a few case reports are available that describe a very similar phenotype with almost identical clinical presentation in pSS twins [141, 146]. Also, there is evidence to suggest the involvement of hormones in the development of pSS. Estrogen seems to play a protective role and lack of estrogen in post-menopausal women may make them prone to the disease [147].

1.5.2 Diagnosis of pSS

The patients used in this study were all diagnosed based on the 2002 AECG classification criteria (Table 1). They consist of a questionnaire for subjective symptoms (oral and ocular), confirmatory tests for oral and ocular dryness as well as histopathological (focus score) and serological (presence of autoantibodies) parameters. To be classified as pSS, patients must display at least four out of the six criteria, including a focus score ≥ 1 (foci are dense aggregates of ≥ 50 mononuclear cells per 4 mm^2 of glandular tissue) or presence of autoantibodies (anti-Ro/SSA and/or anti-La/SSB) [148]. It should be noted that in 2016, a revised version of the previous classification criteria was published following the guidelines of both American College of Rheumatism (ACR) and European League Against Rheumatism (EULAR) [133]. For the assessment of pSS, two disease activity indexes have been developed by the EULAR SS task force: the EULAR SS Patient Reported Index (ESSPRI), completed by the patients, and the EULAR SS Disease Activity Index (ESSDAI), completed by the physicians. The ESSDAI is a systemic disease activity index with 12 domains and is used as a gold standard to evaluate outcome measures in randomized clinical trials (RCTs) [149, 150].

Table 1. The 2002 American-European Consensus Group (AECG) Classification Criteria for Sjögren's syndrome [148]

1. Ocular symptoms: a positive response to at least one of the following three questions: • Have you had persistent feeling of dry eyes for more than three months? • Do you have a recurrent sensation of a foreign body in the eyes? • Do you use tear substitutes more than three times a day?
2. Oral symptoms: a positive response to a least one of the following three questions: • Have you had a daily feeling of dry mouth for more than three months? • Have you had recurrently or persistently swollen salivary glands? • Do you frequently drink liquids while swallowing dry foods?
3. Ocular signs: a positive result for at least one of the following two tests: • Schirmer's test, performed without anesthesia ($\leq$ 5mm in 5 min) • Rose bengal score or other ocular dye score ≥ 4 (according to Bijsterveld's scoring system)
4. Histopathology: a focus score ≥ 1 (50 lymphocytes per 4 mm^2 of glandular tissue) in minor salivary glands
5. Oral signs: a positive result for at least one of the following three tests: • Unstimulated whole salivary flow ($\leq$ 1.5 ml in 15 min) • Parotid sialography showing presence of diffuse destruction without major duct obstruction • Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer
6. Serology: presence of autoantibodies (anti-Ro/SSA or anti-La/SSB or both) in serum

Note: Diagnosis of pSS requires four out of six criteria to be met, including item 4 or item 6.

However, diagnosis of pSS is difficult as the symptoms are often confused with those of ageing and medication side effects. As shown in figure 9, often there is a time gap between disease onset and diagnosis, [124, 151].
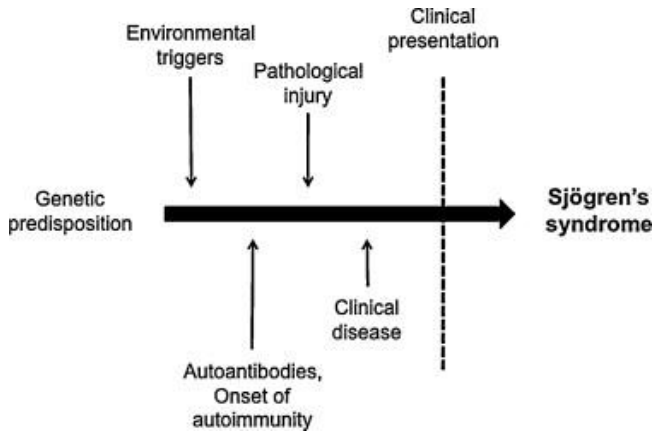
Figure 9. Proposed etiopathogenesis of pSS. Genetic predisposition coupled with environmental factors may lead to pSS development. Often years pass before the disease is diagnosed. Figure adopted from Jonsson *et al.*, 2011 [124]. Reprinted with permission from Elsevier.

1.5.3 Clinical manifestations

The local manifestations of pSS are oral and ocular dryness caused by immune-mediated destruction of the exocrine glands resulting in glandular atrophy. Parotid gland enlargement is often seen in these patients. Oral dryness leads to difficulty in swallowing, speaking and poor oral health (e.g., dental caries, infections, tooth decay, periodontitis) [124, 126]. Ocular dryness can cause irritation, visual impairment, corneal ulcerations and eyelid infections [134]. Overall, these cause a significant reduction in the quality of life of the patients. Histopathologically, the most characteristic feature is the focus score. Moreover, lymphocytic infiltrates, organized into ectopic germinal center (GC) -like structures, are seen in the minor salivary glands of 10-30% of pSS patients [152-154]. They consist of mainly T and B lymphocytes, proliferating cells, follicular dendritic cells and endothelial cells [154]. pDCs have also been reported to be recruited to the salivary glands [155]. About 30-70% pSS the pSS patients develop a variety of EGMs [134]. Fatigue and musculoskeletal involvement (arthralgias and myalgias) are the most common and debilitating symptoms of pSS.

The involvement of other epithelia, e.g., those of the upper airways, renal tubules and gastrointestinal tract are frequently observed. Dry skin, rashes, vasculitis, Reynaud's phenomenon and purpura are often additional complications. Cytopenia, inflammatory bowel disease, chronic liver and kidney problems and peripheral neuropathy are some other associated comorbidities [126, 156-158]. One of the most fatal outcomes of pSS is lymphomagenesis. pSS patients have approximately 16-fold greater risk of developing B cell non-Hodgkin's lymphoma than the general population. This is a classic example of antigen-driven chronic activation of auto-immune B cells in the GC-like structures. In fact, GC-like structures in the salivary glands have been shown to be predictive of increased lymphoma risk in these patients [159-161]. There has also been a recent report on the increased risk of coronary heart disease in pSS patients [162].

Several autoantibodies can be found in the serum of these patients, often years before clinical symptoms arise [163]. Characteristic autoantibodies include the anti-Ro/SSA and anti-La/SSB autoantibodies that are found in approximately 70% of the patients [164]. Anti-SSA can be found alone while anti-SSB is generally found along with anti-SSA antibodies [165]. Other autoantibodies include anti-muscarinic acetylcholine M3 receptor antibodies (anti-M3R), rheumatoid factor (RF) and anti-citrullinated cyclic peptide (anti-CCP) [164, 166, 167].

Although men have a lower risk of developing pSS, the disease presents itself in a more severe form in them [168]. In a population-based study, it was shown that male patients more frequently present with EGM, have more concomitant EGMs and higher autoantibody levels. Enhanced serological responses and higher frequencies of lymphoma-related parameters were also observed in them. These observations may indicate an exaggerated immune activation and a more severe pathophysiological state in male pSS patients compared to female patients [169].

1.5.4 Anti-Ro/SSA and anti-La/SSB autoantibody system

Anti-SSA and anti-SSB autoantibodies are typical serological findings in pSS patients, amongst who approximately 70% are positive for anti-SSA and approximately 40% are

positive for anti-SSB [86]. However, these are also found in other autoimmune diseases like SLE and RA in varying degrees [137].

The Ro/SSA autoantigen is small nucleocytoplasmic RNA-protein complex composed of two different proteins – the 52kDa Ro52 and the 60kDa Ro60 [170]. These associate with small cytoplasmic RNAs forming ribonucleoprotein (Ro-RNP) particles [137]. Ro52/TRIM21 is an IFN-inducible protein that belongs to the tripartite motif (TRIM) family with E3 ligase activity. It can polyubiquitinate members of the IRF family, like IRF3 and IRF7, targeting them for proteasomal degradation [171]. Thus, Ro52 serves as a mechanism to control inflammation. Ro60 antigen binds to misfolded noncoding RNAs (called hY RNA) and targets them for degradation [137]. The 48kDa La/SSB is a nuclear phosphoprotein that acts as a regulator for RNA polymerase III transcription [172]. It binds to nascent small RNAs and protects them from exonuclease digestion and also acts as a transcription factor for mRNAs encoding proteins during stress and apoptosis [173, 174]. Corresponding autoantibody levels for these autoantigens are found to be elevated in pSS patients [175]. Anti-Ro/SSA and anti-La/SSB antibodies have been correlated with earlier onset, more severe dysfunction of the exocrine glands, higher intensity of the lymphocytic infiltrates in the minor salivary glands and more severe extraglandular manifestations [167]. Anti-Ro antibodies may impair the type I IFN negative feedback by inhibiting the E3 ligase activity of Ro52, thus removing the negative regulation of IRF3 and IRF7 by Ro52 [176, 177]. Cross-reactivity between Ro60 and several viral epitopes, like EBV protein EBNA-1 and Coxsackie virus 2B protein in pSS has been suggested [176, 178].

1.5.5 Interferon signature in pSS

In the last few decades, 'signatures' of diseases have been defined using high-throughput techniques, that represent clusters of co-expressed genes, often within a biological network, that may act as biomarkers for diagnosis, classification and drug response prediction [176]. Similar to other autoimmune diseases like SLE, RA and scleroderma, an increased expression of type I IFN stimulated genes, called 'IFN signature', has also been identified in peripheral blood and salivary glands of pSS patients [179, 180]. Over half of the pSS patients exhibit a type I signature that is

correlated with higher ESSDAI scores, higher autoantibody and serum IgG levels and lower lymphocyte and neutrophil counts [181]. Various studies have been carried out to identify the differentially expressed genes between pSS patients and healthy controls, both in peripheral blood and salivary glands, in which a number of IFN inducible transcripts have come up prominently [91, 182, 183]. Several differentially expressed genes were found to be common across multiple studies, such as *IFITM1, IFI44, MX1, IRF7,* and *IRF8* [176]. Only two genes were downregulated, *SOC3* and *CCL18*, which are, in fact, negative regulators of inflammation [155]. Some genes that showed preferential upregulation by type I IFNs include *MxA*, *IFI44* and *OAS1* [181, 184-186]. Many of the upregulated genes also belong to the type II IFN pathway [155]. In a study by Nezos and co-workers, they showed that some of the preferentially inducible IFNγ genes, like *GBP-1* and CXCL9/*MIG-1*, were increased in SS patients compared to controls. They have also reported an upregulation of both type I and II ISGs in pSS patients compared to healthy controls, with a predominance of type I IFN signature in peripheral blood and type II signature in minor salivary glands [187]. Recently, Bodewes and colleagues have shown that pSS patients can be classified into three categories depending on their systemic IFN activity – IFN inactive, IFN-I and IFN-I + II. No one exhibited only IFN-II signature [188]. The increase in systemic type I IFN activity may be important in the development of EGMs like fatigue and joint pain [26].

1.5.6 Treatment

Currently, there is no cure or treatment for pSS except symptomatic care and efforts to prevent further complications. First line of therapy includes sialagogues and topical treatment. Secretagogues, like sugarless candy and chewing gum, and muscarinic receptor agonists, like cevimeline and pilocarpine, can be used to stimulate saliva and tear production. Topical therapies include artificial tears, artificial saliva, nasal saline spray and vaginal estrogen cream. Topical cyclosporine eye drops are effective in some cases [159]. Systemic disease can be controlled with non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) like hydroxychloroquine (HCQ) and corticosteroids like prednisone [189]. Some other

immunomodulatory drugs like azathioprine, methotrexate, cyclosporine, and leflunomide have been tested in clinical trials but showed highly adverse effects [189]. Overall evidence for the success of conventional immunosuppressive therapy is limited. Several biologic therapies are also being tried and have led to variable results till now. For example, rituximab (anti-CD20), that showed promising results in pilot studies, failed to show any significant difference between patients and controls in two larger clinical trials [190]; baminercept, a lymphotoxin β receptor IgG fusion protein (LTβR-Ig), failed to elicit positive impact on any of the clinical measures of disease activity despite evidence of biological effect [191]. Some ongoing trials include those studying effects of belimumab (anti-B-cell-activating factor/anti-BAFF), ianalumab (anti-BAFF receptor), iscalimab (anti-CD40), and abatacept (CTLA4 Ig). However, no drug has shown clear benefits for the treatment of pSS yet [192, 193]. One major reason behind this is patient heterogeneity. Thus, it is imperative to stratify patients into subgroups that can help in differentiating responders from non-responders of a therapy. Identifying and targeting the responders will hugely benefit in achieving the endpoints of clinical trials.

1.6   Biomarkers in rheumatology

Biomarkers in rheumatology can help identify the risk of disease development, improve diagnosis and prognosis, target therapy and assess response to treatment [194]. Genetic markers, gene expression profiles, autoantibodies, cytokines and growth factors, acute phase proteins, tissue abnormalities detected by immunohistochemistry, relative cell frequencies – all act as mechanistic biomarkers for rheumatic diseases [195]. These biomarkers can be measured in serum/plasma, urine, synovial fluid, tissue biopsy and cells from blood or tissue [196]. Unlike in cancer, the development and implementation of new biomarkers in rheumatology has been slow. Very few biomarkers are available currently for disease diagnosis, progression and management. In general, clinical remission is reached in less than 50% of patients and personalized therapeutic approaches are still lacking. For example, some patients receiving anti-TNF treatment show inadequate responses in RA and psoriasis [197, 198]. Use of appropriate biomarkers may help to identify such non-responders before starting

treatment, thereby reducing cost and unwanted effects of a redundant therapy. Thus, a robust biomarker can guide us towards precision medicine, identifying high risk individuals and offering early diagnosis for intervention and disease prevention [194, 199]. Due to the complexity and heterogeneity of rheumatic diseases, and because the mechanism of their pathogenesis is not clearly understood, single biomarker assays may be insufficient. A combination of several markers can be more useful. Some biomarkers used in rheumatology are RF and anti-citrullinated protein antibodies/ACPA (in RA), anti-centromere antibody/ACA (in systemic sclerosis/SSc), anti-SSA and anti-SSB autoantibodies, anti-M3R, Calprotectin and BAFF in pSS [199-202]. High-throughput technologies like flow and mass cytometry, RNA-sequencing and multiplexed functional assays of immune cells show promise in identification of candidate biomarkers in rheumatology, that can be used for establishing a molecular taxonomy of the diseases and disease stratification, successfully guiding patient management in future [203].

2. Aims

The overall aim of this project was to stratify pSS patients based on single-cell-network-profiling and to gain insight into the underlying pathologic mechanisms of the disease.

The specific aims were as follows:

1. To investigate potential aberrations in the intracellular signaling mechanisms of the MAPK/ERK and JAK/STAT pathways in peripheral blood mononuclear cells (PBMCs) of pSS patients compared to healthy individuals by phosphoflow cytometry following stimulation with TLR7 and -9 ligands; to compare the phosphorylation profiles of the stimulated and unstimulated immune cells between the two groups and to correlate them to the clinical parameters of the disease; to study plasma cytokine concentrations in the same and correlate them to the phosphoproteins (paper I).

2. To explore and compare the MAPK/ERK and JAK/STAT signaling networks in unstimulated and IFN-stimulated PBMCs between subgroups of pSS patients and healthy controls using mass cytometry, with the objective of identifying signaling parameters that can be used to stratify the patients (paper II).

3. To compare cell frequencies in peripheral blood of pSS patients and healthy controls that can be instrumental in providing information regarding patient heterogeneity and disease progression (papers II and III).

4. To evaluate the expression of TAM receptors and their ligand Gas6 in immune cells of pSS patients to identify potential impairment of apoptotic cell removal in these patients (paper III).

3. Materials and methods

3.1 Cohort information

Peripheral blood from pSS patients and healthy individuals was used for all the studies incorporated in this thesis. Patients were recruited from the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway. All patients fulfilled the 2002 AECG classification criteria for pSS and did not suffer from any additional autoimmune diseases or lymphoma [148]. Age and sex-matched healthy controls were recruited from the blood bank at the Haukeland University Hospital, Bergen, Norway. The study was approved by the regional ethical committee (#2009/686) and all participants provided written informed consent. Samples from pSS patients and healthy donors were collected in parallel to reduce the influence of seasonal effects.

3.2 Blood collection, PBMC and plasma isolation and cryopreservation

Peripheral blood from all participants was collected in lithium-heparin tubes (BD Diagnostics) and PBMCs and plasma were isolated by density gradient centrifugation on Lymphoprep^{TM} (Axis-Shield, Oslo, Norway). PBMCs were cryopreserved in a freezing mixture composed of 50% X-vivo 20^{TM}, 42.5% ProFreeze^{TM}-CDM (both from Lonza, Switzerland) and 7.5% dimethyl sulfoxide/ DMSO (Hybrid max, Sigma D2650) at approximately 5×10^6 cells/ml. CoolCell® freezing chamber (Biocision; San Rafael, CA, USA) was used to freeze them at -70 °C overnight before moving them to -150 °C for long term storage; plasma was aliquoted and stored at - 80˚C.

3.3 Flow cytometry

Flow cytometry is a powerful tool in basic as well as clinical research in immunology and plays a crucial role in biomarker discovery. It can successfully be used for disease state profiling, pharmacodynamic monitoring and drug screening. It uses a laser-based technology that excites fluorochromes attached to monoclonal antibodies on cells. Using hydrodynamic focusing, the cells are passed through one or several lasers and the resultant florescence and light scatter are detected by photomultiplier tubes (PMTs).

It, thus, allows for identification of cellular subsets in complex populations with precision and rapidity [204]. The basic steps in flow cytometry are depicted in figure 10.
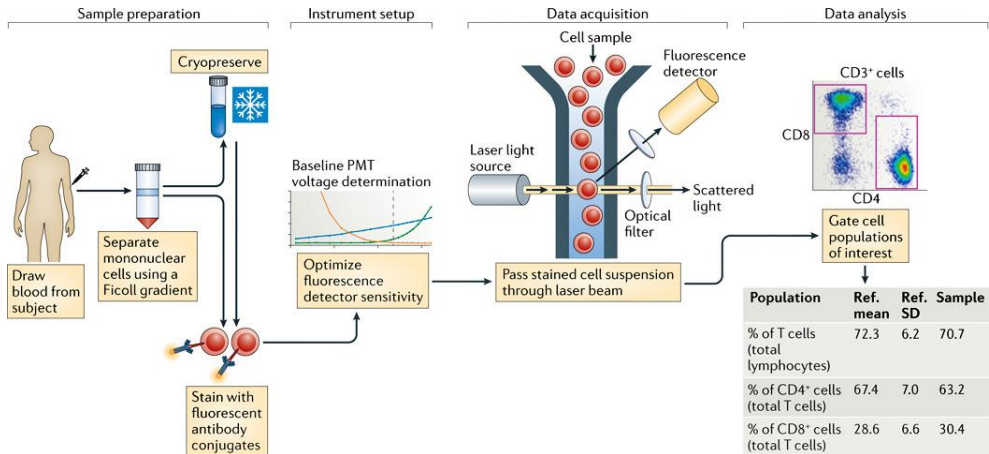
Figure 10. A typical flow cytometry experiment. Sample preparation often involves Ficoll density gradient separation of PBMCs from blood and cryopreservation, before staining with fluorochrome-antibody conjugates. Instrumental setup involves setting the correct voltages for the PMTs to achieve optimal sensitivity. Data acquisition involves passing the stained cells through a laser beam and recording the fluorescence emission from the bound antibody conjugates. This is followed by data analysis. Figure and text adapted from Maecker *et al.*, 2012 [205]. Reprinted with permission from Springer Nature.

Phospho-specific flow cytometry or phosphoflow, is the application of flow cytometry to study phosphorylation states of intracellular proteins. It provides information about the functional responses of cells to stimuli and thereby, the activated intracellular signaling mechanisms. In this technique, antibodies against phospho-epitopes of interest are used to provide insight into the kinetics of signaling pathways that may not be detected by studying surface markers on cells alone [206]. This may lead to the identification of dysregulations in signaling networks. Information about malfunctions in intracellular signaling in patients suffering from autoimmune diseases can help in developing targets for treatment and diagnostic indicators that might act as good biomarkers. The basic steps in phosphoflow cytometry are illustrated in figure 11.
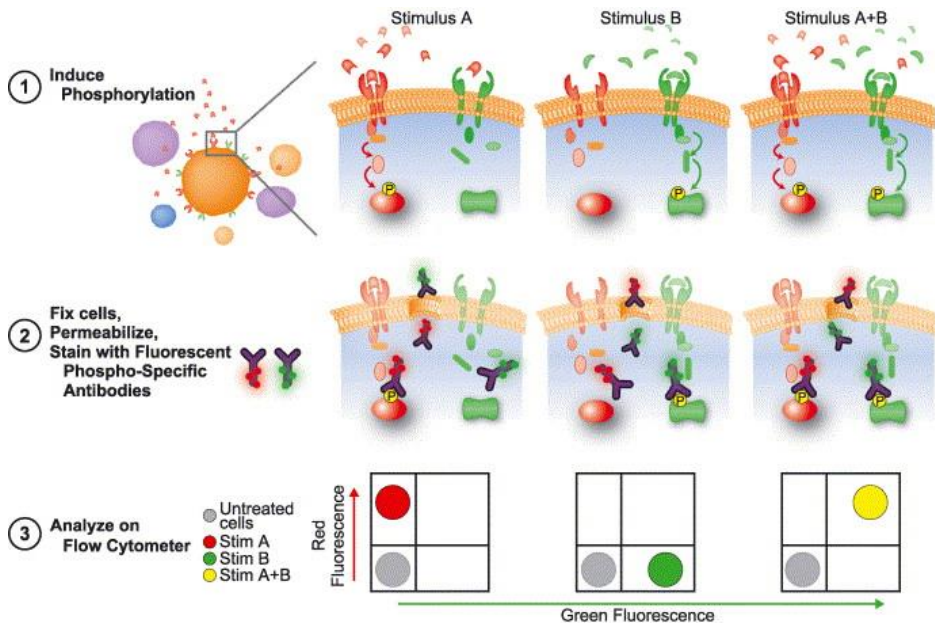
Figure 11. Basic steps in phosphoflow cytometry. (1) A heterogeneous sample of cells is treated with different stimuli, to induce distinct signaling cascades and phosphorylation of target proteins. (2) The cells are then fixed, permeabilized, and stained with fluorophore-conjugated antibodies (that are specific for the phosphorylated forms of the proteins) and surface markers (to identify cell types). (3) The cells are then analyzed on a flow cytometer. Figure adapted from Krutzik *et al.,* 2004 [206]. Reprinted with permission from Elsevier.

Phosphoflow cytometry can detect abnormal signaling in peripheral blood samples from patients and has been successfully used to predict disease outcomes and treatment responses. For example, it has been successfully used for patient classification and prediction of response to therapy in acute myeloid leukemia (AML) [207], for monitoring anti-IFNβ neutralizing antibodies during the treatment of MS [208] and for determining clinical efficacy of DC-vaccinations in glioblastoma patients [209].

3.4   Mass cytometry

Mass cytometry, also called cytometry by time-of-flight (CyTOF), is a platform that couples flow cytometry with mass spectrometry [210]. Ever since its inception in 2009, mass cytometry has ushered in a new era of high-dimensional single-cell analysis, overcoming the limitations of conventional flow cytometry. Instead of fluorophores,

this technique uses stable (non-radioactive), rare earth, non-biological, heavy metal isotopes coupled to antibodies or other target-specific probes for labeling cells. As shown in figure 12, inside the instrument, stained cells are nebulized into single-cell droplets and introduced into an inductively coupled argon plasma/ICP (approximately at 8000 K temperature) where they are vaporized and ionized. The resulting ion clouds are immediately transferred into the high vacuum of the mass spectrometer (as pushes) and are detected by the time-of-flight (TOF) analyzer based on their mass/charge ratios. The low-weight ions (that include biological ions) or ions with multiple charges are filtered out by a quadruple filter [204, 210]. All data in the pushes are integrated over time and subsequently recorded as dual counts (of atoms) for each channel and recorded in the .fsc format [211].
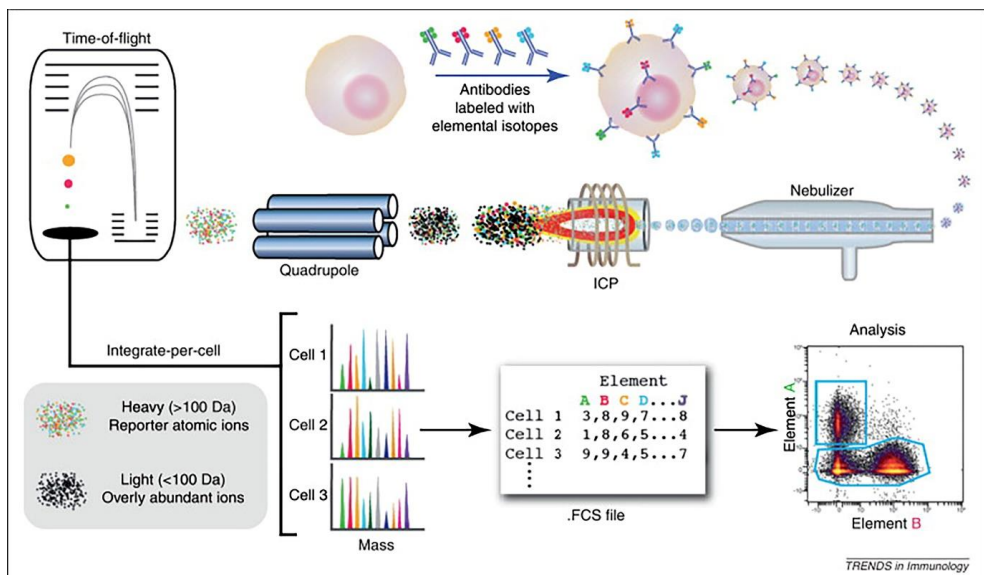
Figure 12. Basic steps in mass cytometry. Cells stained with metal-tagged antibodies, against surface markers and intracellular phospho-markers, are introduce into the ICP by droplet nebulization. Each cell is then atomized and ionized. The lighter and overly abundant ions are removed and the elemental composition of the remaining heavy metals/reporters (that are the ions of interest) are identified. Signals generated are then correlated to their respective markers and analyzed using conventional cytometry platforms. Figure and text adapted from Bendall *et al.*, 2012 [210]. Reprinted with permission from Elsevier.

Currently up to 50 parameters can be measured simultaneously at a single-cell level including cytokines and transcription factors [212, 213]. However, the theoretical limit on the instrument has not yet been reached and is likely between 100 and 200 parameters per cell. CyTOF panels can be designed based on either only surface markers to dissect cellular hierarchy, or a combination of surface and intracellular markers, focusing on the activation states of intracellular signaling pathways. Such multiparametric panels can provide new information about already known cell types and also identify new and/or rare cell populations [214, 215]. For example, mass cytometry has been effective in analyzing surface as well as intranuclear markers, for studying effects of different stimulation conditions and signaling dynamics in PBMCs, immunophenotyping of PBMCs and identification of cell types associated with disease prognosis in AML patients [216-219]. Like phosphoflow, antibodies detecting the phosphorylated states of proteins can be used in mass cytometry to explore *in vitro* activation or treatment effects with drugs [217, 220]. CyTOF is now being used in rheumatological studies as well. For example, it has been used to identify signatures that can differentiate RA patients from healthy controls. A comprehensive understanding of the TNF-mediated signaling patterns may enable more accurate diagnosis, better stratification of patients for appropriate treatment and the identification of candidate targets for treatment [221]. Using mass cytometry, disease-specific signatures and disease endotypes have been identified for SSc, SLE and pSS by cell frequency analysis of circulating immune cells [222]. This technology has also been used to explain the wearing-off phenomenon observed in some patients with relapsing-remitting MS on natalizumab treatment [223]. Extensive panels have been designed to characterize leukocytes in patients suffering from inflammatory and/or autoimmune diseases [224].

The CyTOF technology is rapidly evolving, along with bioinformatics and reagent chemistry, creating a next-generation platform that can be applied in translational research, systems biology and biomarker discovery. Despite its potential, there are multiple challenges to mass cytometry data handling, like determining ways to

visualize and quantify the data optimally. For researchers with little or no computational background, this can be a significant predicament [225].

3.5 Luminex assay

In paper I, cytokine and chemokine concentrations in plasma samples were measured using a 25-plex Luminex panel (Invitrogen, catalog number LHC0009M) following the manufacturer's instructions and run on a Luminex 100 System (Luminex Corporation, Austin, TX). In short, this assay uses polystyrene beads with unique fluorescent signatures, biotinylated detector antibodies and Streptavidin/Phycoerythrin (PE) to capture and detect the analytes under investigation. Finally, the cytokine and chemokine concentrations are calculated from the standard curve. This bead-based multiplex immunoassay is a multistep procedure, as illustrated in figure 13.
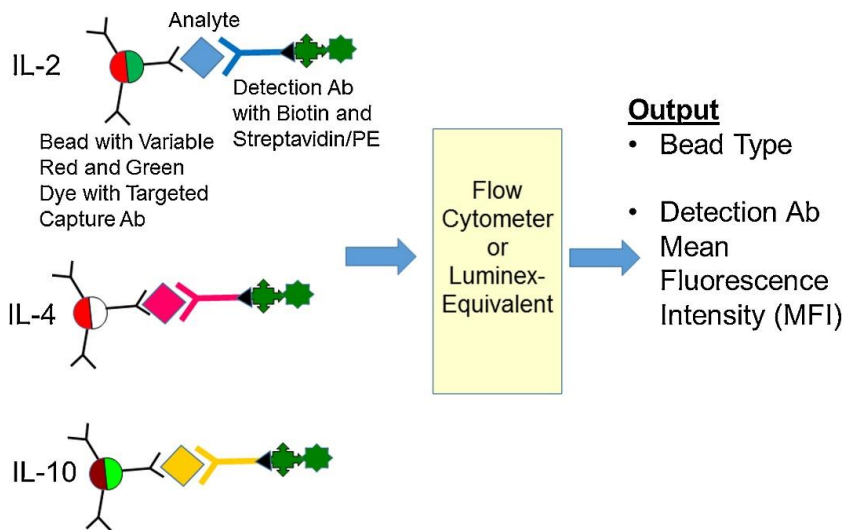
Figure 13. An overview of bead-based immunoassays. Different color-coded beads with dyes that fluoresce either red or green are used. The instrument measures both the bead color intensity and the mean fluorescence intensity (MFI) of the detection antibody which is typically labeled with a Streptavidin/PE conjugate. Figure adapted from Stenken *et al.*, 2015 [226]. Reprinted with permission from Elsevier.

3.6 ELISA

Sandwich ELISA kits were used to measure the plasma levels of Tyro3 (Nordic BioSite), Axl (Nordic BioSite), Mer (Life Technology) and Gas6 (Sigma-Aldrich) following manufacturer's protocols (paper III). In short, this system utilizes a capture antibody and a biotinylated detection antibody to capture the antigen and detect it by a colorimetric reaction. Concentrations are measured using the standard curve. The basic steps in ELISA are depicted in figure 14.
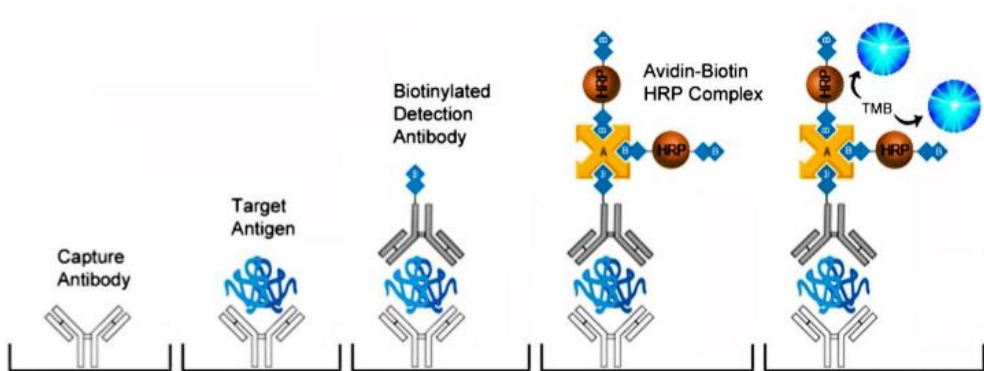
Figure 14. Basic steps in sandwich ELISA. The 96-well plates are precoated with the capture antibodies, to which the samples are added, followed by the addition of biotin-labelled detection antibodies and a horseradish peroxidase (HRP)-conjugate. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) substrate is added for generation of colour. Figure used with permission from LifeSpan Biosciences, Inc. (https://www.lsbio.com/elisakits/human-axl-sandwich-elisa-elisa-kit-ls-f2608/2608)

3.7 Data analysis

Initial visualization and analysis of data was done using Flowjo (Tree Star) (papers I and III). For flow cytometry data, identification of immune cell populations was based on light scatter properties and the relative expression of CD markers; all numbers generated were transferred to Microsoft Excel for further statistical analysis (papers I and III). For mass cytometry data (paper II), initial data visualization was done in Cytobank, based on the DNA-Iridium (Ir) content, event length, live/dead marker and

expression of CD markers; R version 3.5.0 was also used for initial data validation. The Astrolabe Cytometry Platform (Astrolabe Diagnostics, Inc.) was used for final clustering, visualization and statistical analysis of the data.

3.7.1 Principal component analysis

Principal component analysis (PCA) is a mathematical algorithm that reduces the dimensionality of the dataset while retaining most of the variation in it [227]. It identifies new uncorrelated variables that successively maximize variance, and which are linear functions of those in the original data. The new variables are termed as principal components [227]. The largest variability is explained by the first principal component (PC1) and each successive principal component explains less variability then the previous one [228]. Samples can then be visualized in a score plot that helps in finding patterns within the data [228]. The variables are visualized in loading plots and are interpreted in combination with the score plot, to identify the influence of each variable on the spread of the samples in the latter. They are treated as vectors and their positions on the loading plot indicate their relationships to each another [227]. Variables close to the origin contribute little to the clustering of samples on the score plot, while variables on opposite sides are inversely correlated to one another. PCA was performed using Unscrambler® (CAMO) (paper I) and software R version 3.3.1. (http://www.r-project.org/) (paper III).

3.7.2 FlowSOM

FlowSOM is an algorithm that uses self-organizing maps (SOMs), a type of artificial neural network, for hierarchical clustering and dimensionality reduction of complex datasets. It is an unsupervised technique that can reveal how all the markers are behaving on all the cells and identify subsets that might be missed by manual gating [229]. In FlowSOM, all the cells from all the samples are brought together in a big matrix which is then used to train a SOM, resulting in a grid of nodes that correspond to clusters of cells. The nodes/clusters that are closer, are more similar to each other than those at a distance. The resultant clustering is visualized by a minimum spanning

tree (MST). The algorithm also assigns a metacluster to each cluster, effectively grouping them into populations [229].

3.7.3 Statistical analysis (papers I and III)

D'Agostino-Pearson normality test was performed to check for Gaussian distribution of the datasets and since some categories did not pass the test, non-parametric methods were chosen for further analyses of the data. Correlations were performed using Spearman's rank test. Robust regression and outlier removal (ROUT) method with a ROUT coefficient of 1 was used to identify outliers but in most cases the outliers were not removed due to the exploratory nature of the analyses. Repeated measures one-way analysis of variance (ANOVA), with the Greenhouse correction and Holm-Sidak's multiple comparisons test, was used when comparing fold change (arcsinh) in channels measuring phosphorylated proteins in PBMCs for three different isolation methods (paper I). Fold change of the median fluorescence intensity (MdFI) was calculated in Microsoft Excel using the formula [ASINH (MdFI stimulated/cofactor) - ASINH (MdFI unstimulated/cofactor)], with an assigned cofactor of 150 (paper I). Coefficient of variation (CV) values for process triplicates reported in paper I were calculated in Microsoft excel from MdFI values of target phospho-proteins normalized against their respective unstimulated samples. Unpaired Mann-Whitney test was used for analyzing the Luminex assay data (paper I) and TAM expression levels (paper III), in order to compare between the different groups under investigation. Differences were considered statistically significant for $p \leq 0.05$. For papers I and III, all tests and graph generation were done using GraphPad Prism v7.05 (La Jolla, CA, USA).

3.7.4 High-dimensional data analysis (paper II)

Single-cell mass cytometry data was clustered using the *FlowSOM* R [229] and labeled using the Ek'Balam algorithm [230]. Cell subset definitions followed Maecker *et al.* [205], and Finak *et al.* [231] and the subsets identified are given in Table 2. Cluster labeling, method implementation, and visualization were done through the Astrolabe Cytometry Platform (Astrolabe Diagnostics, Inc.). Differential abundance analysis was done using the *edgeR* R package [232]; McCarthy *et al.* [233] following the method

outlined in Lun *et al.* [234]. Differential expression analysis was done using the *limma* R package [235] following the method outlined in Weber *et al.* [236]. Differences were considered statistically significant for false discovery rate (FDR)/adjusted $p \leq 0.05$.

Table 2. Immune cell subsets identified by the Astrolabe Cytometry Platform

Population names	CD markers
B cells	CD3-CD14-CD19+CD56-
B naive	CD27-
B memory	CD20+CD27+
Plasmablast	CD20-CD27+CD38+
T cells	CD3+CD14-CD19-CD56-
Double negative	CD4-CD8a-
CD4+	CD4+CD8a-
Naive	CD27+CD45RO-
Central memory	CD27+CD45RO+
Effector memory	CD27-CD45RO+
Terminally differentiated	CD27-CD45RO-
CD8+	CD4-CD8a+
Naive	CD27+CD45RO-
Central memory	CD27+CD45RO+
Effector memory	CD27-CD45RO+
Terminally differentiated	CD27-CD45RO-
NK cells	CD3-CD14-CD19-CD56+
Natural killer (CD56+CD16+)	CD16+
Natural killer (CD56+CD16-)	CD16-CD56++
NKT-like cells	CD3+CD14-CD19-CD56+
Dendritic cells	CD3-CD14-CD19-CD56-
Conventional dendritic cells	CD11c+CD16-CD123-HLA-DR+
Plasmacytoid dendritic cells	CD11c-CD123+HLA-DR+
Monocytes	CD3-CD19-
Classical monocytes	CD14+CD16-
Intermediate monocytes	CD14+CD16+
Non-classical monocytes	CD14-CD16+CD56-CD123-

4. Summary of the main results

Paper I

Phosphoflow cytometry was used to make quantitative measurements of the phospho-proteins ERK1/2, NF-κB p65, p38, STAT1 (Y701), STAT1 (S727), STAT3 (Y705), STAT3 (S727), STAT4 (Y693) and STAT5 (Y694) in T cells, B cells and NK cells from female pSS patients and age-matched female healthy individuals, at the basal level as well as over a time period of 4 hours following TLR7 and -9 stimulation. Cytokine levels in plasma were determined using a 25-plex Luminex-assay and correlated to the basal phosphorylation levels and clinical parameters in these patients.

Significant increase in basal phosphorylation in T cells (for NF-κB, p38, ERK1/2, STAT5, STAT1 Y701, STAT1 S727), and NK cells (for p38, STAT5, STAT1 Y701, STAT1 S727) was observed in pSS patients compared to healthy donors. B cells showed no significant differences. Following TLR7 and -9 stimulation, stronger responses in B cells for NF-κB, p38 and STAT3 S727, were observed in the EGM-patients, compared to EGM+ patients and healthy controls, which also correlated with the expression of three type I ISGs. 70% of the patients had a positive IFN signature. These patients differed from the IFN signature negative patients, in terms of their phosphrylation profiles and plasma cytokine levels.

When comparing cytokine profiles of patients and controls, 12 out of the 25 cytokines measured were significantly upregulated in patients including IL-1ß, IL-13, IL-6, IL-12, MIP-1α, MIP-1ß, MCP-1, IL-15, IFN-α, TNFα, IL-2, and IL-4. Upon stratification of patients according to the presence or absence of autoantibodies (SSA+/SSA–), significant upregulations were observed in the SSA+ subgroup for IL-1ß, MCP-1, IFN-α, IL-2, and IL-4 compared to SSA- patients. While the basal phosphorylation profiles of all patients showed only moderate correlationsto plasma cytokine concentrations, excluding medicated patients from the analysis resulted in strong to very strong correlations of RANTES to pNF-κB in NK cells, MIP-1ß, MCP-1, IL-2, and IL-4 to pSTAT5 Y694 in B cells and IL-1RA to pSTAT1 Y701 in T cells. Again, on dividing the patients into subgroups based on the presence or absence of SSA and EGM, we

observed strong to very strong correlations in SSA+ patients of MIP-1α, IL-1RA and TNF-α to pSTAT3 Y705 in B cells, TNF-α to pNF-κB in B cells and RANTES to pSTAT4 Y693 and pSTAT1 S727 in NK cells. In SSA– patients, IL-1RA correlated to pERK in B cells and RANTES to pNF-κB in T cells. EGM+ patients showed strong to very strong correlations of several cytokines to pSTAT4 Y693 in NK cells amongst others, while in EGM– patients RANTES, IFN-γ, IL-1RA, IFN-α, and IL-12 correlated with various phospho-epitopes.

Paper II

Mass cytometry was used to immunophenotype different PBMC cell subsets from SSA+ and SSA- female pSS patients and age-matched female healthy donors. Next, expression levels of the phospho-proteins ERK1/2, NF-κB, p38, STAT1 Y701, STAT3 Y705, STAT3 S727, STAT4 Y693, STAT5 Y694 and STAT6 Y641, were analysed in the identified immune subsets, by stimulating them with either IFNα2b or IFNγ.

Differential abundance analysis among the healthy donors and the SSA- and SSA+ pSS patients revealed imbalances in the frequencies of different PBMC subpopulations. Memory B cells were significantly reduced between healthy controls and SSA- patients and between SSA- and SSA+ subgroups, CD8+ T central memory cells were significantly reduced in the SSA+ patients (in groupwise comparison only), CD8+ T effector memory cells were significantly higher between the controls and SSA- patients and CD4+ T EMRA cells showed significant reduction between the donors and SSA+ patient subgroup. Although not statistically significant, a few other cell types showed certain trends. For example, amongst the lymphoid populations, slightly increased frequencies were observed in the naïve B cells and CD8+ T EMRA cells across the three groups, while a decreasing pattern was seen in the CD4+ T effector memory cells, which was most prominent in the SSA+ patients; a slight decrease was also observed in the CD56+CD16+ NK cell subset. Amongst the myeloid cells, the cDCs and pDCs decreased slightly across the three groups while classical monocytes showed a slight increase in both the pSS subgroups compared to healthy donors; the non-classical monocytes revealed a weak decreasing tendency across the three groups. Differential

expression analysis revealed alterations in the levels of activation markers, CD38 and HLA-DR, in many of these cell subsets, with the highest expression being in the SSA+ patients for most cell types.

Upon IFNα2b stimulation, a slightly increased response to pSTAT1 Y701 was observed in the memory and naïve B cells, the CD56+CD16+ NK cell subset, the cDCs and pDCs across the three groups. Various T cell subsets, also showed a similar tendency in the pSS subgroups, with the maximum fold change seen in the SSA+ patients. A slightly decreased pSTAT3 Y705 induction was observed in the memory and naïve B cells, both NK cell subsets, pDCs and classical monocytes for the SSA+ patients compared to the other two groups, whereas the cDCs and the non-classical monocytes showed a decreasing trend across the three groups. We also observed NK- and T cell-specific reductions in STAT4 Y693 phosphorylation in the pSS patients for both the NK cell subsets and the memory T cell subtypes. Slightly reduced STAT5 Y694 phosphorylation was seen in the naive B cells, various T cell subsets, cDCs and classical and non-classical monocytes, which was most noticable in the SSA+ patients. Most cell types also showed slightly reduced pSTAT6 Y641 signaling in response to IFNα2b, which was most pronounced in the SSA+ pSS patients.

The effect of IFNγ stimulation was restricted to mainly STAT1 Y701. A significant increase in pSTAT1 Y701 induction across the three groups was observed between the controls and SSA+ pSS patients in the cDCs, classical and non-classical monocytes. Although not statistically significant, a similar trend was seen in the memory and naïve B cells, and in the pDCs increase in pSTAT1 Y701 was seen only in the SSA+ patients. Again, the SSA+ patients showed the highest fold changes.

Paper III

In this study, we examined the plasma concentrations of the soluble forms of Tyro3, Axl, Mer and free Gas6 from female pSS patients and age-matched healthy controls by ELISA. Next we analyzed their mRNA expression levels in PBMCs from the same cohort by RT-PCR. Finally, to confirm the mRNA data and define subpopulations of PBMCs, we analyzed TAM receptor expression in PBMCs using flow cytometry.

Comparison of the plasma concentrations of soluble TAMs and unbound Gas6 by ELISA revealed no significant differences between the patients and healthy controls.. At the transcriptomic level, the highest mRNA expression was detected for Mer, followed by Axl and Gas6 having similar expression levels, while the lowest expression was observed for Tyro3. The mRNA levels of Tyro3 and Mer were significantly reduced in pSS patients compared to healthy individuals.

Considerable differences were observed in the cell frequencies between pSS patients and healthy donors, with significant decrease in pDC, cDC1 and cDC2 subsets in the patients. At the protein level, surface expression of Mer was the highest. Monocytes and DCs showed the highest surface expression of the TAM receptors, except Axl that was low in all three monocyte sub-populations. The different monocyte populations expressed different levels of the TAM receptors. For example, Mer was highly expressed in intermediate and non-classical monocytes, compared to a much lower expression in classical monocytes. Most of the lymphoid populations showed low TAM expression, except B cells that showed some Tryo3 positivity and T and NKT-like cells that expressed considerable amounts of Mer. Although not statistically significant, all three receptors were expressed at a lower level in patients compared to controls in most cell types analyzed. Exceptions to this were the B cells for Tyro3 and non-classical monocytes and cDC2 for Mer.

5. Discussion

5.1   Methodological considerations

5.1.1 Advantages and limitations of flow and mass cytometry

Although high throughput (measuring up to several thousand events/sec), one major challenge in experiment design and data analysis of flow cytometry is the spectral overlap of fluorescent probes, that requires complicated compensations [210]. Moreover, autofluorescence can also complicate quantitative analysis. Both these obstacles are bypassed by mass cytometry as it uses metal-tagged antibodies instead of fluorescent probes [237]. However, the greatest advantage of mass cytometry is the high number of markers that can be investigated at a single cell level. Detection of >40 targets using a single panel is possible, making broad characterization of the immune system now a reality [238].

However, mass cytometry has a few drawbacks. First, it cannot measure forward scatter (FSC) and side scatter (SSC). So, we do not get any information on the size or granularity of the cells. Second, sensitivities of the lanthanide metals are lower than most fluorophores. Third, measurement is limited to about 1000 cells per second and lastly, the cells cannot be retrieved for downstream analysis [210, 238]. However, DNA intercalators containing Ir or rhodium (Rh) can be used to stain the cells. This ensures the detection of cells as events and discriminates singlets from doublets and debris, when used with event length [237]. Similarly, live-dead stains must also be used. Cisplatin or molecules containing both a chelator and maleimide moiety are used as viability markers in mass cytometry [218]. Finally, at present, the availability of mass cytometers is considerably lower, and the cost is higher than that of flow cytometers.

5.1.2 Common considerations for single cell analyses

Antibody selection: Antibodies against surface markers were chosen based on their ability to distinguish the cell subsets in PBMCs, by their combinatorial expression on cells. Variations in cellular frequencies compared to healthy individuals have been reported in the context of pSS. Lymphopenia is often observed, which is due to the

decrease in CD4+ T cell subsets as well as B memory cells [129]. pDCs and NK cells have also been reported to be decreased in many patients, whereas monocytes and granulocytes have been shown to be increased [239, 240]. Activated T cells and plasmablasts have also been implicated in the disease [129, 240]. In paper I, we chose to look at only the parent populations, i.e., T, B and NK cells, due to limitations in the number of markers that can be analyzed simultaneously in flow cytometry, which was further compounded by the absence of the yellow-green laser when the experiment was conducted. However, in paper II, due to the advantage of mass cytometry, we could investigate the different subtypes of T, B and NK cells as well as NKT cells, monocyte and DC subsets. To check for possible erythrocyte contamination, we also added CD235 to our panel. Studies have demonstrated that TAM receptors are most prominently expressed by the phagocytes, mainly the myeloid cells [95]. Hence, for paper III, we primarily focused on the monocyte and DC subpopulations. As we used PBMCs, we did not expect to see granulocytes and macrophages and hence did not include those markers in our panels.

Phospho-epitopes were selected based on relevant signaling pathways in PBMC subsets: TLR7 and TLR9 for paper I and IFNα and IFNγ for paper II. MAPKs like ERK1/2 and p38 as well as NF-κB are activated downstream of the TLR pathways. Moreover, TLR7 and -9 activation is capable of inducing pathways involved in the production of type I IFNs. IFNs, in turn, activate the STATs in various combinations to drive unique biological functions. Type I IFN can activate STAT1 and STAT3 in almost all cell types whereas STATs 4,-5 and -6 are activated in a cell type and context-dependent manner [67]. For example, while IFNα can stimulate STATs1, -3 and -4 in T cells, a shift from STAT1 to STAT4 activation by inhibition of STAT1 is required for expansion of CD8+ T cells and production of IFNγ during viral infections [69]. A differential STAT1/STAT4 balance is also required for IFNγ production and cytotoxicity functions of NK cells [241]. The major STAT protein activated by IFN-γ is STAT1 [242]. Whereas Type I IFNs can activate p38 to induce the transcription of ISGs in a STAT-independent manner, both type I and type II IFNs can activate ERK

pathways [51]. Hence the MAPK/ERK and JAK/STAT pathways were chosen to be investigated for papers I and II.

Titration: All the antibodies were titrated for their specific experimental conditions to determine their optimum staining concentrations. For all phenotypic markers, the concentrations that gave a clear separation between negative and positive populations were chosen. Barcoding dyes used in phosphoflow cytometry were titrated using a 3 x 3 matrix selected based on their ability to resolve each sample in the matrix, as well as minimizing spillover into critical channels (paper I). Antibodies to phospho-antigens were titrated using unstimulated and stimulated samples to find the concentration required for maximum fold change and minimum noise (papers I and II).

Fixation and permeabilization: Signal transduction and transcription of genes in cells are dependent on intracellular phosphorylation of proteins, and measurement of such phospho-epitopes can therefore describe activity in the cells [206]. However, phosphorylation events are transient and reversible, and the epitopes are inaccessible to antibodies directly because of their subcellular localization (in the cytosol or the nucleus). Hence, the cells must be fixed (by formaldehyde/paraformaldehyde) to stabilize/cross-link the phospho-proteins and then permeabilized (by methanol/saponin) for entry of the antibodies (papers I and II) [206]. Both extra- and intracellular epitopes and antibodies can be sensitive to fixation and permeabilization reagents. So it is important to test the antibody performances for the individual protocols [243].

Effect of freezing and thawing on cells: To optimize recovery and viability of cryopreserved cells after thawing, serum support media, such as fetal bovine serum (FBS), are commonly used for freezing. However, to avoid unspecific stimulation caused by FBS, we made a freezing mixture containing a Non-Animal Origin (NAO) Chemically Defined Freeze Medium, ProFreeze™-CDM, serum-free cell medium X vivo-20™ and cryoprotectant DMSO. The CoolCell® freezing chamber used for freezing the samples ensures a consistent cell freezing rate of -1 °C/minute [244]. Cooling cells at a controlled rate and the use of DMSO minimize damage to the cells

by increasing permeability of the plasma membrane as well as preventing the formation of ice crystals [244, 245]. The cells were thawed rapidly at 37°C to avoid ice recrystallization and osmotic stress, and to maximize cell recovery and viability [244].

Stimulation and stimulants: Before stimulation, the cells were rested in the incubator for 2 hours, to reduce basal signaling levels in them following the thawing process. Instead of FBS, serum-free X vivo-20™ was used for stimulation to ensure that the cells were stimulated only by the IFNs and not by components of FBS as well as to avoid batch to batch variation of FBS. The stimulants used in paper I were TLR7 ligand CL097 and TLR9 ligands ODN 2006 and ODN 2395. CL097 is a derivative of the imidazoquinoline compound R848, mimics viral components (ssRNA) and acts as a TLR7 agonist. ODNs 2006 and 2395 are type B and C CpG ODNs, respectively. These are synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides that act as TLR9 ligands. Unmethylated CpG sequences are more abundant in bacterial and viral DNA compared to human DNA and can stimulate immune responses [246]. While type B and C both stimulate NF-κB mediated signaling from late endosomes resulting in strong B cell activation, type C triggers IRF7 mediated intracellular signaling from early endosomes leading to strong IFNα induction [247]. Since viral infection is a potential trigger for pSS, TLR7 and -9 ligands were used as stimulants to explore the respective pathways. In addition, pSS is often associated with a type I IFN signature, and TLR7 and -9 activation may also lead to type I IFN production. In paper II, recombinant IFNα2b and IFNγ were chosen as stimulants in order to specifically explore the type I and type II IFN signaling pathways, which cause IFN production and upregulation of ISGs through the JAK/STAT and MAPK/ERK pathways [17].

Barcoding: Barcodes label individual samples with a unique signature of dyes/metals, allowing multiplexing of the samples prior to staining and acquisition. Fluorescent cell barcoding and mass-tag cell barcoding have been in used in papers I and II respectively. After barcoding, the samples can be pooled together in one tube for downstream staining and data collection [248, 249]. Barcoding reduces antibody consumption,

increases throughput, shortens instrument measurement time and most importantly, minimizes staining variability.

Pacific orange (PO) and pacific blue (PB), used for barcoding in phosphoflow, are dyes that react to amine groups on protein lysine residues and at the N-terminus, while unbound dyes are subsequently washed off [248]. For mass cytometry, the barcodes were palladium (Pd)-based. Pd has six isotopes, where each sample is either positive or negative for each of the six isotopes. A '6-choose-3' strategy is used in which each of the 20 barcodes are positive for 3 of the 6 possible Pd reagents, as shown in figure 15. A unique combination is, thus, generated for each sample which is used to identify that sample during the process of sample deconvolution [249].

	Pd 102	Pd 104	Pd 105	Pd 106	Pd 108	Pd 110
Sample 1	+	+	+			
Sample 2	+	+		+		
Sample 3	+	+			+	
Sample 4	+	+				+
Sample 5	+		+	+		
Sample 6	+		+		+	
Sample 7	+		+			+
Sample 8	+			+	+	
Sample 9	+			+		+
Sample 10	+				+	+
Sample 11		+	+	+		
Sample 12		+	+		+	
Sample 13		+	+			+
Sample 14		+		+	+	
Sample 15		+		+		+
Sample 16		+			+	+
Sample 17			+	+	+	
Sample 18			+	+		+
Sample 19			+		+	+
Sample 20				+	+	+

Figure 15. Palladium (Pd) barcode scheme used in mass cytometry. Each kit has 20 unique barcodes generated from 6 Pd isotopes, where a combination of 3 isotopes is used to identify each sample. This is called a '6-choose-3' strategy. Figure inspired from Fluidigm (https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/cell-id-20-plex-pd-barcoding-kit-ug-prd023/).

Controls: Using relevant controls is critical for any successful cytometry assay. Controls provide the context based on which one can interpret the samples appropriately. Healthy donors were used as biological controls and in stimulation assays, unstimulated samples were used as the baseline/reference, to analyze the stimulated samples (papers I and II). The phosphoflow and CyTOF assays utilized cryopreserved PBMCs from a single donor (internal control) that were thawed and processed with each experimental run to monitor inter-assay variation.

5.1.3 Flow cytometry: special considerations

Panel design: The choice of fluorophores was a compromise between wavelength of available lasers and filters on the flow cytometer (ideally bright fluorophores used for rare antigens and dim for common antigens), as well as availability of the chosen fluorochrome-conjugated antibody. Minimizing spillover into important and sensitive channels (low abundance markers and phospho-antigens) was also kept in mind in order to reduce compensation. However, some concession had to be made due to availability. For example, we had to use PerCP-Cy^{TM}5.5 for ERK1/2, STAT1 (Y701) and STAT3 (Y705), which is relatively dim.

Controls: BD cytometer setup and tracking beads were used for determining minimum baseline PMT voltages and monitoring cytometer setup and performance (like laser alignment, laser time delay, sensitivity). Single-stained and unstained cell samples were used during experiment setup for optimization of PMT voltages. Voltages were set to achieve minimum spillover into other channels, specially the phospho-epitopes and TAM receptors. A barcoded sample without antibodies (barcode only control) was also used to verify that the MdFI of the other markers did not fluctuate in response to the barcode intensities (paper I). All flow cytometry experiments included single-stained compensation controls (beads or cells) for the measurement and correction of fluorescent spillover before subsequent analysis. Fluorescence minus one (FMO) controls, that included all antibodies in the panel but one, were used to set appropriate gates where distinctions between positive and negative populations were not clear (e.g., the TAM receptors and Gas6).

5.1.4 Mass cytometry: special considerations

Panel design: For mass cytometry, the MaxPar panel designer and panel wheel were used to check for signal spillovers and tolerance for each channel. Although there is no major spillover in this platform, there can be some background due to abundance sensitivity, formation of oxides (+16) and isotopic impurity (+/-1) [238]. Hence, low-abundance antigens were generally chosen for channels that received little/no crosstalk from other channels and high-abundance antigens were assigned to channels that

contributed little/no crosstalk to the channels used for the low-abundance antigens. The instrument's detection efficiency for the isotopes were also kept in mind. Furthermore, some antibodies had to be self-conjugated due to lack of availability in the choice of suitable metals, e.g., pSTAT3 (S727) and pSTAT4 (Y693).

Controls: As mass cytometry overcomes the limitations of spectral overlap associated with flow cytometry, compensation controls are not required in this assay. To monitor changes in instrument performance and signal fluctuations during data acquisition and across batches, polystyrene bead standards, called EQ™ Four Element Calibration Beads (containing 140/142Ce, 151/153Eu, 165Ho and 175/176Lu), were used. These beads help to normalize inter-sample and intra-sample variations.

Contamination/Background noise: The Lanthanide metal isotopes are biologically rare, thus making the endogenous cellular background zero. However, apart from the three sources of spillover mentioned earlier, some other sources of contamination are iodine (127I), tin (120Sn) and lead (209Pb). These can be found in insufficiently purified water but are outside the analytical window (which is 141Pr to 176Yb, 89Y and 209Bi) [218]. However, Barium (137-138 Ba) from soaps and gloves can contaminate samples and interfere with nearby channels.

5.1.5 Luminex assay

The measurement of cytokine levels can be influenced by many factors. When processing samples for storage, time duration between collection and cryopreservation is important [250]. Storage duration and temperature can influence final measurements as well [251, 252]. Detection can also be impacted by the source (e.g., serum or plasma) as well as anticoagulants (e.g., heparin, EDTA, citrate) used in plasma tubes [253, 254]. Some recommendations favor plasma over serum because the coagulation process may have an impact on cytokine release from cells [250]. Hence, we used heparin plasma for our assays. Also, both intra- (like circadian rhythm, infections etc.) and inter-individual variations in cytokine levels can be expected [255, 256]. However, to reduce intra-assay variability related to technical procedures, the samples were analyzed in a random order on the plate. In our final analysis, we did not include Eotaxin, IL-7 and

MIG as they were not recommended to be measured in heparin plasma by the manufacturer. GM-CSF, IL-5 and IL-8 were also excluded from the analysis as they were below detection limit in most samples.

A dysregulated cytokine profile in pSS has been reported by many, with a systemic and local increase of proinflammatory cytokines and a reduction of anti-inflammatory cytokine levels [257]. IL-17, IL-1RA, Il-1β, IL-15, MIP-1α, MIP-1β, IFNα and IL-4 were found to be increased in pSS patients with ectopic GCs [258] Correlations between aberrant cytokine levels and phosphoproteins may suggest a link between the signaling pathways and these chemical messengers.

5.1.6 Quantitative real-time PCR

18S rRNA was chosen as the reference gene because of its invariant expression throughout tissues and cells. It has also been reported to be more reliable than other commonly used housekeeping genes [259]. For paper I, expression levels of *MxA*, *IFI44* and *OAS1* (type I IFN-inducible genes) and *GBP1* (IFNγ-responsive gene) were analyzed because of the known association between type I IFN signature and increased disease activity in pSS [181]. *MxA* has been reported to be a reliable biomarker for identifying systemic type I IFN activity in pSS patients [184]; *IFI44* and *OAS1* are strongly induced by IFNs with *IFI44* being activated by only type I IFN [185, 186]; IFNγ is a stronger inducer of *GBP1* than IFNα [260]. Correlations between upregulation of ISGs and patients with a positive type I IFN score (indicating potentiated intracellular signaling pathways) may point to a mechanistic link between these attributes.

The TAM receptors and Gas6 are involved in efficient phagocytosis of apoptotic cells. In paper III, these were measured because a decrease in their mRNA levels (particularly Tyro3 and Axl) have been previously observed in pSS patients, which is suggestive of defective clearance of the apoptotic burden in them [106]. Thus, any changes in the expression of the TAMs and Gas6 in the pSS patients compared to healthy controls may be linked to the pathogenesis of the disease.

5.1.7 ELISA

Soluble forms of the TAM receptors have been reported to compete with the membrane-bound TAMs and inhibit apoptotic clearance. There are conflicting results regarding their concentration levels in pSS patients. While Qin *et al.* have demonstrated increased soluble Mer in pSS patients compared to healthy donors, Chen *et al.* have shown lower plasma Gas6 concentrations in the former [106, 261]. Aberrations in the levels of soluble TAMs and free Gas6 can imply a role in disease development and progression.

According to the manufacturer, the percentage of recovery of Gas6 was more from plasma than from serum. Also due to reasons mentioned previously (see section 1.5.2) and as all the kits supported heparin plasma as the source material, we used the latter for all the ELISA assays. According to the manufacturer's recommendations, we had stored the plasma samples at -80 °C after isolation, and all reagents and samples were brought down to RT before use. The samples were recommended to be run in duplicates or triplicates. As we had limited availability of plasma and had to run each sample four times (once for each of the TAM receptors and Gas6), we decided to run the samples in duplicates. As for the range of the standard curves, we followed the manufacturer's recommendations for Tyro3 and Gas 6 (125 pg/ml-8000 pg/ml and 0.41 ng/ml-30 ng/ml, respectively), as they included the median concentration values of these proteins found in literature. However, for Axl and Mer we made some minor changes to the standard dilutions to increase the range of the standard curves (13.7 pg/ml-10000 pg/ml for Axl and 6 pg/ml-3000 pg/ml for Mer). As these are not abundant proteins, we wanted to ensure that we can detect them in all our samples by matching our detection range with that from previous studies. As for the sample dilutions, the goal was to get the resulting optical density (OD) values of the samples within the OD values of the standard curve, for them to be accurately calculated. Hence, the sample dilutions were chosen based on information about the antigen concentrations from published literature as well as recommendations from the manufacturer's protocols.

5.1.8 Data analysis

Visualization and interpretation of multivariate data can be difficult due to the sheer number of data points. Therefore, PCA was used to identify the important variables and visualize the datasets generated by phosphoflow cytometry (paper I). Similarly, visualization and analysis of high-dimensional datasets generated by mass cytometry (paper II), using traditional manual techniques like biaxial scatter plots, is not feasible and may be error-prone. The analysis of high-dimensional single-cell cytometry data comes with its own computational challenges in terms of data pre-processing, normalization, dimensionality reduction and clustering [262]. Many of the algorithms circumvent traditional approaches used in flow cytometric analysis, fundamentally changing the way these data are processed and interpreted. However, the large number of available algorithms (like viSNE, SPADE, X-shift, PhenoGraph and Citrus), and the lack of consensus on best practices for data pre-processing and analysis, raise multiple issues [225, 263]. Different pipelines using different tools are being developed constantly, that can be overwhelming, by making it increasingly difficult to comprehend the challenges and considerations for each approach as well as to understand what kind of biological insight each method will reveal.

5.2 Biological implications of the results

Primary Sjögren's syndrome (pSS) is a complex autoimmune disorder, that is difficult to manage due to it's unknow etiopathogenesis and patient heterogeneity. On one hand, new technologies, like mass cytometry, RNA sequencing and multiplexed functional assays, enable the analysis of immune cell composition and function with unprecedented detail and help to elucidate the mechanisms behind pathogenesis of diseases as well as in the discovery of novel biomarkers. On the other hand, blood is a 'gold standard' sample that acts as a good source of potential biomarkers [264]. It has been studied widely, in search of disease-specific signatures and/or immune cell parameters that can differentiate pSS patients from healthy individuals [222, 239, 240, 265-267]. We have used high-throughput single-cell technologies to compare peripheral blood immune cell composition and signaling pathways of pSS patients and

healthy controls. Our studies on PBMCs have revealed differences in immune profiles, in terms of differential cell frequencies, cytokine levels and aberrant signaling potentials, between healthy controls and pSS patients as well as between the SSA- and SSA+ patients. Additionally, we have shown that the phagocytic machinery might be impaired in pSS patients, with slightly decreased expressions of TAM receptors in them. Taken together, all these point towards the complex immune interplay in the disease that should be further investigated for identification of candidate biomarkers.

5.2.1 Altered cell frequencies and activation status of PBMC subsets can differentiate between pSS patients and healthy donors as well as between patient subgroups

Immune cell profiling has been suggested as a tool to guide optimized treatment decisions in rheumatic diseases [203]. In pSS, differential leukocyte counts and lymphopenia have been associated with higher titers of autoantibodies and disease severity [153, 240] . Our flow and mass cytometry analyses reveal differences in cell frequencies between healthy individuals and pSS patients and between the patient subgroups. The decrease in memory B cells, observed by us in our mass cytometry assay, is one of the most well-characterized cellular variation in pSS and has been reported in other diseases as well, like HIV, SLE and common variable immunodeficiency (CVID) [153, 268]. The reduced frequency of memory B cells in pSS patients might be due to a skewing towards plasma cell differentiation [268]. This fits nicely with the findings of Mariette and colleagues, where they have shown increased frequencies of plasmablasts and plasma cells in the peripheral blood of pSS patients [153]. Although we did not include plasmablasts in our final analysis, due to low cell numbers that may give unreliable results, we did see a slight increase in plasmablast frequency in the pSS patient subgroups (data not included in the study). We also observed slightly increased naïve B cells in the patient subgroups, which is in accordance with our previous finding [269]. We found decreased frequencies of the CD4+ T memory subsets, in agreement with previous literature [153, 270]. However, we found opposite results for the CD8+ T memory cell populations, except for the central memory subset. Although Mareitte et al. showed no significant difference in

the CD8+ T lymphocyte populations, there is one report that shows increased cytotoxic T lymphocytes in patients with pSS, that might indicate the presence of persistent viral infections [271]. In line with earlier observations, we also found slightly decreased CD56+CD16+ NK cells, cDCs, pDCs and non-classical monocytes while slightly increased frequencies for the classical monocytes were observed [153, 240]. NK cells play important role in immune regulation by eliminating target cells and autoreactive T and B lymphocytes and reduced numbers and cytolytic activity has been reported previously in pSS patients [272]. Therefore, our finding of reduced CD56+CD16+ NK subset (the cytotoxic NK cells) may be associated with persistent viral infections and development of pSS. Reduced pDCs in circulation is an established fact, that might be due to migration to the affected tissues or due to apoptosis [222]. The increase in classical monocytes may be a cause for the observed increase in proinflammatory cytokines in pSS patients [273]. Moreover, recent microRNA profiling has revealed CD14+ monocyte-specific microRNAs in pSS that suppress TGFβ signaling as opposed to proinflammatory pathways like IL-12, further establishing the role of these monocytes in pSS [274]. As observed by Mariette and colleagues, the changes in cell frequencies were greatest in the SSA+ patients. Our mass cytometry results on DCs were further confirmed by our flow cytometry analysis, where we found significant decrease in pDCs, cDC1 and cDC2 populations in pSS patients compared to healthy controls. Decreased myeloid DCs in blood and corresponding migration to salivary glands has been observed in early SS, that may help to present antigens to infiltrating CD4+ T cells [275].

However, it should be mentioned that lymphopenia can only indicate progressing disease activity but is unlikely to reflect an individual disease or disease subtype unless used in conjunction with other criteria. In addition, medications used by patients could affect cell concentrations. For example, prednisolone has been shown to cause leukocytosis in patients, through increase in monocytes, granulocytes and lymphopenia and even low doses, upon prolonged administration can lead to persistent leukocytosis [276]. Although our mass cytometry cohort consisted of unmedicated patients, the flow cytometry cohorts had patients receiving medication. This may be one reason for some

the discrepancies in our results with earlier studies. Many of the observed differences in cell frequencies did not reach statistical significance but showed slightly increased or decreased tendencies. One major reason behind this is the low sample size of our experiments. Also, various studies have used different sampling methods, like whole blood vs PBMCs, cryopreserved vs fresh, differences in estimation calculations (such as parent population vs grandparent population), which might account for the disparity observed in some of the results. Another important aspect is the EGM status of the patients. EGMs have been associated with increased comorbidities and may indicate a more severe form of the disease [277]. For our mass cytometry study, we had enrolled only EGM- patients, whereas in some of the other studies, a portion of the patients suffered from a variety of EGMs.

While both activated CD4+ and CD8+ T cells have been reported to be upregulated in the periphery, only CD8+ T cells showed significantly upregulated HLA-DR in the salivary glands of pSS patients [153]. Activated T cells may contribute to pSS pathogenesis by producing proinflammatory cytokines and inducing B cell hyperactivation [270]. It can also be hypothesized that activation and subsequent migration of CD8+ T cells to the tissue sites may play a crucial role in tissue damage. We found upregulated trends for CD38 and HLA-DR expression for many cell subpopulations analyzed, including the CD4+ and CD8+ memory T cells, which were most prominent in the SSA+ pSS patients. Enhanced activation status of various cells might make these patients prone to increased secretion of proinflammatory cytokines (e.g., BAFF by pDCs and monocytes), overactive T cell stimulation by cDCs and autoantibody production [124, 278].

5.2.2 Aberrant phospho-signaling can distinguish between pSS patients and healthy individuals as well as between patient subgroups

The connection between type I IFN and development of autoimmunity is well established. It has been observed that patients undergoing IFN therapy developed de novo autoantibodies or increased titers of pre-existing autoantibodies [279, 280]. Continuous activation and dysregulation of TLR and type I IFN signaling have been hypothesized to play a significant role in the pathogenesis and IFN signature of

autoimmune diseases, and IFN signature positive pSS patients have been shown to have increased expression of TLR7 in pDCs and monocytes [281, 282]. Also, increase in TLR7 and -9 mRNA levels have been observed in PBMC of pSS patients by some groups [283]. It has also been proven that genes belonging to both type I and type II IFNs are upregulated in pSS [155]. However, till date, very few studies on the phospho-signaling profiles of pSS patients have been conducted. Additionally, these studies were based on the parent populations, i.e., the T and B lymphocytes, NK cells and monocytes.

Differential signaling through TLR7 and -9 pathways in PBMCs can differentiate pSS patients from healthy individuals

In our phosphoflow assay, due to limitations in the number of markers that can be used in flow cytometry, we analyzed only the T, B and NK cells. We confirmed previously observed upregulation of basal STAT5 Y694 levels in T cells [266]. However, contrary to earlier observations, we found significant increase in STAT1 Y701 in T cells but not in STAT3 Y705 [284]. No differences were observed in B cells. These disparities may be due to the use of cryopreserved PBMCs with long culture period (6 hours) in our study, as the other studies used freshly isolated cells. Following stimulation with TRL7 and -9, increased induction of NF-κB, p38 and STAT3 S727 were observed in B cells, even after exclusion of medicated patients, and was strongest in the EGM- patients. It is difficult to speculate the cause or the relevance of this finding, as B cells do not differ in TLR7 and -9 expressions between pSS patients and healthy individuals [285]. However, increased secretion of several cytokines and chemokines have been observed in them upon stimulation with TLR7 and -9 ligands, like IFNα by TLR7 ligands [286]. Additionally, NF-κB can bind to the promoters of proinflammatory genes, such as TNFα and IL1β, which can be secreted by B cells upon antigenic stimulation [287, 288]. Increased numbers of TNFα and IL1β secreting cells have been shown to be present in the peripheral blood of pSS patients [289]. Induction of NF-κB and STAT3 S727, following TLR7 and -9 stimulation in B cells, was stronger in SSA+ patients, justifying the need for patient stratification. Strongest signaling in the EGM- patients was regardless of glucocorticoid (prednisone) usage, which inhibits NF-κB activation

[290], and HCQ that inhibits TLR7 and -9 signaling [291]. The reason behind low response of EGM+ patients is unclear, but interestingly it was observed that the patients with high TLR signaling all showed lower responses to IFNα (data not included in the study). This suggests a negative regulatory mechanism or a different disease trajectory. SOCS1 mRNA expression levels are elevated in PBMCs of pSS patients [292] and can be induced by TLR ligands like CpG-DNA [293]. As SOCS1 can suppress several cytokine signaling pathways like IFNα, lower responses of these patients to IFNα could be due to a TLR-driven upregulation of SOCS1 [294]. The observed correlations between the type I ISGs and TLR7 and -9 stimulated phospho-proteins can be due to multiple reasons. For example, promoters of early type I IFNs genes have NF-κB response elements that are essential for constitutive and early expression of IFNβ following viral infections [287]. Consequently, increased responsiveness of NF-κB could potentially drive increased early type I IFN expression. Moreover, as many of the same components function in the NF-κB, STAT3 S727 and p38 TLR-driven responses, it is possible that higher levels of type I IFN gene expression is indirectly associated with the increased responses of these phospho-proteins and vice versa.

Differences in type I and type II IFN signaling in PBMC subsets may point towards progressively increasing disease severity in pSS patient subgroups

Due to the advantage of mass cytometry over flow cytometry, in our CyTOF study, we could subdivide the PBMCs into their subpopulations and analyze many cell types simultaneously. Confirming our previous results, in response to IFNα, slightly increased STAT1 Y701 activation in B, T, NK and NKT-like cells were seen in the pSS patient subgroups that was reflected in several subsets of these parent populations, as well in the DC subsets [295]. IFNγ stimulation also resulted in slightly increased pSTAT1 Y701 induction in the memory and naïve B cells as well as significant increase in the classical and non-classical monocytes. This confirms previous findings, where B cells and monocytes were shown to exhibit significantly increased STAT1 Y701 phosphorylation following stimulation with IFNγ [292]. They had also reported that the greatest difference in STAT1 phosphorylation between patients and controls was observed for IFN-γ stimulation, which was also observed by us in the cDCs and

classical and non-classical monocytes, that showed significant increase in the SSA+ subgroup. pDCs also showed slightly increased pSTAT1 Y701 induction by IFNγ in our analysis. DCs are vital for initiation and maintenance of immune responses as well as for self-tolerance. Thus, defects in their functions can lead to aberrant immune activation [124]. STAT1 signaling is crucial for the anti-proliferative effects of IFNα and IFNγ [296]. The increased activation of STAT1 may partially explain the low counts of some of the cell subsets observed by us in the patient subgroups. Thus, an enhanced response to both IFNα and IFNγ can play a vital role in pSS pathogenesis through its antiproliferative effects. In contrast to STAT1, IFNα-induced repression of phosphorylation of the other STAT proteins were observed in our study, like STAT3 Y705, STAT4 Y693, STAT5 Y694 and STAT6 Y641, that partially confirms our previous results. This reciprocal nature of the STATs has been observed by others [297]. This has also been previously observed for SLE with reduced responses of STAT3 and STAT5 to IFNα in T and B cells [298]. A similar pattern of increased pSTAT1 induction and reduced induction of other pSTATs, was observed at 120 minutes following TLR7 and -9 stimulation (paper I), further indicating a preferential STAT1 activation over other STATS in pSS patients. Interestingly, majority of the T memory subsets also showed an impaired STAT1: STAT4 ratio, with increased STAT1 and deceased STAT4 levels upon IFNα stimulation. Low STAT1 has been associated with antigen-specific CD8+ T cell proliferation and reduced STAT1 responsiveness coupled with enhanced STAT4 responses has been reported in LCMV infections [299, 300]. It was reported that type 1 IFN preferentially activates STAT1 on day 0 and STAT4 on 8 in CD8 T cells in vivo and that STAT4 blocks the STAT1-mediated anti-proliferative effects in these cells [300]. Hence, a higher STAT1: STAT4 ratio might fail to protect CD8+ T cells from the effects of STAT1 inhibition on their expansion and function. However, in contrast to our earlier result, we did not observe any noticeable difference in STAT3 S727 phosphorylation upon IFN stimulation.

A dysregulated NK cell signaling profile was observed in pSS patients and patient subgroups. In paper I, we found increased basal signaling by NK cells through STAT1 Y701 and STAT1 S727 in pSS patients. Following stimulation with IFNα (paper II),

pSTAT1 Y701 induction was slightly increased in the CD56+CD16+ subset, while pSTAT4 Y693 reduced slightly in both the NK cell subsets. A similar pattern was also observed after 120 minutes of TLR7 and -9 stimulation (paper I). Such a profile has been observed in hepatitis C infected patients receiving IFNα treatment, and may polarize NK cells towards low IFNγ production and increased cell cytotoxicity [301]. However, no difference in NK cell killing ability has been observed on a per-cell basis between healthy individuals and pSS patients [272]. No such patterns in phosphorylation responses were observed upon IFNγ stimulation. This is in accordance with previous literature where we find reports of selective NK cells hypo-responsiveness to IFNα and normal responses to IFNγ, in pSS patients compared to heathy controls [302]. Whether this aberrant profile has any links to the known STAT4 polymorphism is unclear [303]. On the other hand, a polymorphism in NKp30 (rs11575837 (G>A), a NK cell activating receptor that regulates the cross talk between NK cells and DCs and IFNγ production, has been associated with pSS pathogenesis [304]. STAT3 was also found to be reduced in NK cells after 120 minutes of TLR7 and -9 stimulation (paper I). STAT3 has anti-apoptotic effects, enhancing cell survival and proliferation [305]. Hence, reduced STAT3 activation may be associated with decreased NK cell numbers and increased percentage of apoptotic NK cells observed in pSS patients [272]. It should be highlighted that the SSA+ pSS patients showed the most prominent changes for many of our observations, while the SSA- subgroup was in between the healthy controls and SSA+ patients. This might indicate a progressively increasing disease severity in the patients, again underscoring the necessity to stratify them for optimum treatment.

5.2.3 Altered TAM receptor expression may indicate potentially impaired phagocytosis in pSS patients

Increased apoptosis of the glandular epithelial cells is partially be responsible for glandular dysfunction in pSS. Apoptosis of salivary gland epithelial cells can be mediated by the Fas/FasL system, the TRAIL/ caspase 9 system, inflammation induced by TLRs as well as by cytotoxic T cells [306, 307]. Recently the co-stimulatory molecule B7-H3 has also been implicated [308]. Clearance of apoptotic debris is

crucial in maintaining tissue homeostasis and tolerance and defective apoptosis has been linked to inflammation and autoimmune diseases [309]. TAM receptors (Tyro3, Axl, Mer), a major phagocytic machinery of our cells, are tyrosine kinase receptors that have been implicated in autoimmune diseases like SLE and MS as well as cancers and have emerged as potential targets for therapy [310]. The importance of TAMs in phagocytosis is evident in TAM KO mice models that develop infertility and retinal blindness [122]. As expected, we found highest expression of Mer and lowest expression of Tyro3 in the PBMCs of pSS patients [113, 311]. mRNA levels of Tyro3 and Mer were significantly reduced in pSS patients compared to controls, which is partly in line with the previous study by Qin *et al.[106]*. Although not significant, we found slightly increased levels of soluble Mer (sMer) and sAxl in the patients. This is in accordance with earlier reports where cleavage of the extracellular domain of the receptors by proteases have been reported in inflammation-driven conditions [312]. There are reports on soluble Mer inhibiting macrophage clearance of apoptotic cells and soluble Axl inhibiting tyrosine phosphorylation of membrane-bound Axl [115]. All these might point towards impaired phagocytosis in the pSS patients. However, functional assays are required for further confirmation. As observed by others, we found highest expression of the TAMs in the monocytes and DCs, with differential expression of the receptors in these two populations- Mer was highest in monocyte populations while Axl was highest in the DC subsets [113]. Seitz *et al*. observed that phagocytosis of apoptotic cells by macrophages took 60 minutes while it took 6 hours by DCs [313]. Different usage of the TAM receptors may cause different apoptotic efficiencies in macrophages and DCs, justifying their roles as phagocytes vs professional APCs [313]. We also found different monocyte populations showing different Mer expressions, with the intermediate and non-classical subsets having highest Mer levels. CD16+ monocytes have been reported to have the highest Mer expression among the monocytes, that can be related to their preferential role in phagocytosis [311]. Among the lymphoid populations, we found T cells and NKT-like cells expressing Mer while B cells expressed Tyro3. T cells were long thought to be TAM deficient. However, recent studies have reported expression of Mer on TCR-activated T cells that is involved in proliferation and cytokine secretion, acting as a late

costimulatory molecule [314]. There is evidence for the emerging role of Tyro3 dysregulation in tumorigenesis and has been shown to be expressed in B and T-cell acute lymphoblastic leukemia (ALL) cell lines and chronic lymphocytic leukemia patients. Initial data suggest that TYRO3 expression is ectopic. However, a thorough analysis of TAM expression in B and T cells has not yet been published and it is possible that TYRO3 is expressed in specific subsets or during specific phases of the immune response [315]. Although we did not find any significant TAM expression on NK cells, there are reports that all three receptors are required for their differentiation and cytotoxic functions [108]. NKT-like cells have also been shown to express Mer in mouse models and negatively regulates cytokine production in them [316]. However, these observations highlight the role of Mer in signaling events, as opposed to phagocytosis, in these cells. Our general observation of slightly lower expression of the TAM receptors in most cell types in the pSS patients, once again emphasizes the possibility of compromised phagocytotic activities in them, compared to healthy individuals. However, stratification of patients based on autoantibodies must be done to see if there are any differences in TAM receptor expression in the patient subgroups. Glucocorticoids, like prednisone, act by increasing Mer expression on macrophages and stimulating their phagocytic abilities [122]. Patients with low TAM expressions can thus be benefitted from these drugs.

5.2.4 Differential plasma cytokine levels may potentially help to identify responders to cytokine-based therapies in pSS patient subgroups

Dysregulated cytokine signaling network is a central player in pSS pathogenesis. Our finding of differential expression of several cytokines and chemokines in the pSS patients, mostly proinflammatory, partially confirms previous reports by us and other groups [257, 317-319]. When the patients were subdivided based on presence/absence of autoantibody, IL-1β, MCP-1, IFNα, IL-2 and IL-4 levels were significantly elevated in SSA+ pSS patients compared to SSA- patients and healthy donors. Majority of these are have been implicated in pSS. IL-1β is a key cytokine in chronic inflammation and has been associated with disease duration in SS patients [318]. It was previously reported to be increased in pSS patients by us [319]. In contrast to our previous finding,

here we observed increased levels of MCP-1 in pSS patients compared to healthy individuals. Elevated IFNα levels have been reported by some, while others have reported lower levels of circulating IFNα [318]. 70% of our patients showed activated type I IFN system, that might be a consequence of the observed increase in IFNα levels or vice versa. In accordance with our earlier findings of elevated IL-2 levels in EGM+ pSS patient and in patients with high ESR, our current analysis also revealed increased plasma IL-2 levels [319]. Although IL-4 is an anti-inflammatory cytokine, it is involved in the Th2-mediated humoral responses [318]. Consequently, it may be involved in B cell activation and autoantibody production. This might partly explain the increased levels of IL-4 in the SSA+ pSS patients. Basal phosphorylations could be correlated to some of the cytokines in the patients that become stronger on exclusion of medicated patients. Stratifying the patients based on SSA and EGM further yielded significant correlations to various phospho-epitopes. Several biologics are being developed targeting the different cytokine families, like anti-IFN and anti-TNF [320]. However, cytokine-directed therapies have shown variable efficacies till date [257]. Patient selection is an important criterion for clinical studies and differential cytokine levels in the subgroups of pSS patients may help in choosing patients that might benefit from these therapies.

5.3   Limitations of the study

In all three studies, sample size was a compromise between our research question and feasibility, i.e., use of expensive reagents and technologies as well as laborious methods. Hence, the cohort sizes were not optimal. This particularly affects high-dimensional and big datasets (paper II) as corrections for multiple testing are needed for optimal statistical analysis of such data and a low sample size negatively affects the statistical significance of individual parameters.

We used healthy individuals as controls for all the studies in this project. The use of non-pSS sicca patients would have been a more relevant choice, particularly in the context of clinics where the likelihood of having to distinguish between pSS patients and healthy individuals is extremely rare. The more practical scenario would be to

accurately differentiate pSS patients from non-pSS sicca patients. However, we were restricted by the unavailability of such patients at the time of inclusion.

As the samples were collected over time and we wanted to analyze them together, cryopreservation/freezing was required. This might have an impact on cell recovery and intracellular signaling as well as the cytokine levels. However, the procedure was identical for all patients and controls and hence, any error arising due to this would be a systematic one.

For the cytometry assays, there are a few noteworthy limitations. First, some rare cell populations could not be analyzed due to low cell numbers (e.g., intermediate monocytes and plasmablasts in paper II); some cellular subsets that were analyzed had quite low numbers (e.g., cDC1, Tyro and Axl positive subsets in paper III), which might affect the analysis negatively. In paper II, in spite of having CD25 and CD127, we excluded Tregs from our final analysis due to absence of FoxP3 in our panel, without which Tregs cannot be identified with confidence. Also, high inter-individual variations were observed, particularly for the TAM positive cell subsets in paper III, and accounted for the wide data spread. The addition of live-dead markers (PO for papers I and III and cisplatin for paper II) enabled us to exclude dead cells from our analysis easily. However, as they do not differentiate between the different cell subsets, there are chances of higher levels of apoptosis in some subsets, particularly the rarer ones. Therefore, we cannot exclude the effects of apoptotic cells in signaling profile alterations, especially in the rare populations. For example, increased percentages of apoptotic NK cells are seen in pSS patients [272]. Therefore, differences in signaling observed in NK cells (papers I and II) may be a consequence of that. Another factor affecting the interpretation of signaling levels can be cell frequency. The phospho-epitopes were measured in terms of median expressions. However, differences observed in them could be due to shifts in the cell frequencies, rather than actual potentiation of signaling pathways. One practical limitation for the phosphoflow cytometry experiment (paper I) was the absence of the yellow-green laser (561 nm) at the time of experiment designing and execution. This laser can nicely separate FITC and PE signals and in its absence, we had certain constraints on the choice of antibodies

for our panel. As we were using PE-conjugated antibodies for CD56 and PE-Cy7^{TM} for three phospho-epitopes, we could not use FITC. Finally, the signal intensities of the phosphoproteins detected by CyTOF were in general quite low (paper II). Also, we observed a clear batch effect, specifically in batch 2 samples, that showed lower q95 values for some of the phospho-markers compared to the other three batches. It is worth mentioning that the batch effect was not consistent and was more prominent for some phospho-markers in some cell types and less/absent in others. We considered quantile normalization (QN) using the internal controls. However, artifacts can be introduced by this method [321]. Moreover, as mentioned earlier, since the batch effect was inconsistent for the different cell types and phospho-proteins, we decided to report the results as it is.

Some other limitations include the lack of information regarding some clinical parameters like, Ig levels, C-reactive protein (CRP), complements, anti-nuclear antibody (ANA) and focus score for some patients. Furthermore, we did not have any knowledge regarding the dosage or time of administration of medication with respect to the sampling time (for the medicated patients in paper I). Finally, all our experiments were performed using PBMCs. Although peripheral blood is a good indicator of pSS pathogenesis and severity, the main site of disease activity i.e., the salivary glands, must be studied in order to get a comprehensive picture of the disease.

6. Conclusions

The three studies included in this thesis identify single-cell analysis as a useful approach towards understanding the complex immunological interplay in primary Sjögren's syndrome. Patient stratification, based on unique immune profiles, may enable development of personalized treatment strategies.

Study I

TLR7 and -9 stimulation results in increased response of B cells through STAT3 S727, NF-κB and p38 in pSS patients, which also correlate with the type I IFN signature. This suggests that the type I IFN signature may either induce an enhanced NF-κB, p38 and STAT3 S727 signaling upon TLR7 or -9 activation, or partially be derived in response to it, thus, facilitating increased production of interferons. Plasma cytokines also correlate with the basal phosphorylation levels of several phospho-epitopes in the patients.

Study II

Various PBMC subpopulations show altered cell frequencies between the healthy donors and the SSA- and SSA+ pSS patients. Many cell subsets show an enhanced activation status and increased response to type I and type II IFNs through pSTAT1 Y701. These, coupled with reduced pSTAT3 Y705 and pSTAT5 Y694 signaling, may predispose the pSS patients, particularly the SSA+ subgroup, to an increased expression of IFN-induced genes and autoantibody production.

Study III

The mRNA levels of Tyro3 and Mer are significantly reduced in the plasma of pSS patients compared to healthy controls. Altered cell abundances as well as differential TAM receptor expression in the PBMC subsets are observed between healthy individuals and pSS patients. Reduced TAM receptor levels in the patients may point towards defective phagocytic clearance.

7.   Future perspectives

We analyzed the phospho-signaling potentials of the TLR7 and -9 pathways in the T, B and NK cells only. Signaling profiles of the different subsets of these cells as well as the myeloid subsets, upon TLR7 and -9 stimulation, should be explored in the future using mass cytometry. Reasons behind the enhanced TLR7 and -9 responses in the EGM- pSS patients through NF-κB, p38 and STAT3 S727 in B cells, compared to the EGM+ patients, should be further investigated. In addition, TLR7 and -9 ligands were used in combination due to limited availability of PBMC from each patient. It will be informative to study these pathways separately in the future.

The observed phospho-signaling responses in PBMC subsets, upon IFNα2b and IFNγ stimulation, should be correlated with clinical parameters as well as IFN scores and ESSDAI. This will be useful in understaning the relationship between the observed changes and disease progression and severity. Additionally, we only incorporated EGM- patients in this study. Presence of EGM has been associated with higher comorbidities and risk of mortality in pSS patients [277, 322]. Therefore, studying patients with EGM is of future interest.

The underlying cause for the abberant responses to TLR and IFN stimulations should be examined. As mentioned earlier, several polymorphisms have been associated with pSS, which play a role in signal transduction. Dividing the patients by polymorphisms and associating them with the phospho-signaling may help to identify unique genetic profiles, thus facilitating patient stratification.

Although differential expressions of the TAM receptors were detected in pSS patients, the implications of such findings must be investigated by performing functional assays, e.g., phagocytosis assays. In addition, it will be interesting to explore whether TAM receptor expression levels vary according to presence or absence of autoantibodies in the pSS patients. Also, TAM receptor signaling is negatively regulated by the production of SOCS1 and SOCS3 proteins that establish a negative feedback loop via the IFNAR-STAT1 cascade [121]. While Mer acts mainly in steady-state conditions and induced tolerance, Axl is specialized for inducing feedback inhibition of

inflammation [312]. In DCs, Axl is induced by TLR and type I IFN stimulation, which binds to IFNAR and converts the proinflammatory signaling into an immunosuppressive type through expression of the SOCS proteins [122]. Therefore, transcriptomic analyses of the SOCS proteins and correlation studies between the expression levels of the TAM receptors and SOCS proteins is an exciting future prospect.

Some cell subtypes were not included in our studies, like the T helper 1 (Th1)/Th2 cells, Th17 and T follicular helper (Tfh) and T follicular regulatory (Tfr) cells. Tregs could not be analyzed with confidence due to lack of FoxP3 in our panel. Also, leukocyte migration markers were not included. Some of these cells types have been reported to be increased in blood (e.g., Th17 and Tfh cells) while some others show contradictory results (e.g., Tregs) [323, 324]. Several Th cells have been reported to be selectively localized in the labial salivary glands and the expression of Th2 and certain Tfh-related molecules have been associated with lymphocytic accumulation and ectopic GC formation [325]. Thus, Tfh cells in pSS may be a suitable target for peripheral blood analysis, if they migrate from the blood to the salivary glands during these processes. Tfh and Tfr cells have been recently suggested as biomarkers for ectopic lynphoid activity in pSS [326]. Therefore, it will be useful to study these cell populations in the future. However, it should be mentioned that anaylsis of these cell subpopulations is challenging as the chemokine receptors used to identify them are sensitive to freezing/thawing and fixation procedures. Additionally, sicca patients should be included as conrols instead of healthy donors for future experiments.

Finally, all our studies were based on peripheral blood. In order to fully understand the pathophysiological mechanisms and reasons behind the observed patient heterogeneity, it will be important to study the active disease site, i.e., the salivary glands. In this regard, imaging mass cytometry is of particular interest, as it allows visualization of multiple markers in tissue sections simultaneously and can also perform neighborhood analyses. It can, therefore, reveal the complex interplay among the various cell subsets involved in glandular destruction. Germinal center (GC)-like structures have been proposed to be strong predictors of lymphoma development in

pSS patients [327]. Consequently, it will be of great relevance to study the cellular interactions in the salivary glands using imaging mass cytometry.

8. References

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Single Cell Based Phosphorylation Profiling Identifies Alterations in Toll-Like Receptor 7 and 9 Signaling in Patients With Primary Sjögren's Syndrome

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Primary Sjögren's syndrome (pSS) is associated with polymorphisms and mRNA expression profiles that are indicative of an exaggerated innate and type I IFN immune response. Excessive activation potential of signaling pathways may play a role in this profile, but the intracellular signaling profile of the disease is not well characterized. To gain insights into potentially dysfunctional intracellular signaling profiles of pSS patients we conducted an exploratory analysis of MAPK/ERK and JAK/STAT signaling networks in peripheral blood mononuclear cells (PBMC) from 25 female pSS patients and 25 female age-matched healthy donors using phospho-specific flow cytometry. We analyzed unstimulated samples, as well as samples during a 4 h time period following activation of Toll-like receptor (TLR) 7 and 9. Expression levels of *MxA*, *IFI44*, *OAS1*, *GBP1,* and *GBP2* in PBMC were analyzed by real-time PCR. Cytokine levels in plasma were determined using a 25-plex Luminex-assay. Principal component analysis (PCA) showed that basal phosphorylation profiles could be used to differentiate pSS patients from healthy donor samples by stronger intracellular signaling pathway activation in NK and T cells relative to B cells. Stimulation of PBMC with TLR7 and −9 ligands showed significant differences in the phosphorylation profiles between samples from pSS patients and healthy donors. Including clinical parameters such as extraglandular manifestations (EGM), we observed stronger responses of NF-κB and STAT3 S727 in B cells from EGM-negative patients compared to EGM-positive patients and healthy controls. Plasma cytokine levels were correlated to the basal phosphorylation levels in these patients. In addition, 70% of the patients had a positive IFN score. These patients differed from the IFN score negative patients regarding their phosphorylation profiles and their plasma cytokine levels. In conclusion, we here report increased signaling potentials in peripheral B cells of pSS patients in response to TLR7 and −9 stimulation through STAT3 S727

and NF-κB that correlate with a type I IFN signature. Induction of these pathways could contribute to the generation of a type I IFN signature in pSS. Patients displaying elevated potentiation of STAT3 S727 and NF-κB signaling could therefore benefit from therapies targeting these pathways.

Keywords: Sjögren's syndrome, extraglandular manifestations, autoantibodies, phosphoflow, Toll-like receptors, type I interferon

INTRODUCTION

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by lymphocytic infiltrates of the salivary and lacrimal glands. The hallmarks of the disease are dryness of the mouth (xerostomia) and the eyes (keratoconjunctivitis sicca) (1, 2). This dryness and other clinical manifestations result in a significant decrease in quality of life. Currently there is no cure or effective disease modifying treatment for SS, with management of the disease based on the relief of symptoms. The lack of effective treatments is linked to the pathogenic complexity of the disease, with genetic predisposition, hormonal, and environmental factors all contributing to disease etiology and pathogenesis. While almost all SS patients display abnormal tear and/or saliva secretion (3), there is significant heterogeneity in the disease manifestations, pathology and clinical course. This heterogeneity may reflect distinct patient subgroups with unique pathophysiologic mechanisms (4). For example, Sjögren's syndrome can present with a wide range of extraglandular manifestations (EGM) including fatigue and constitutional, musculoskeletal, articular, cutaneous, pulmonary, liver, and kidney involvement, as well as neuropathies and lymphomas (5). B cell hyperactivity is also a common feature of SS. It can manifest as hypergammaglobulinaemia and presence of autoantibodies including anti-Sjögren's syndrome A (SSA) and anti-Sjögren's syndrome B (SSB) (5) often preceding clinical symptoms (6).

Aspects of SS pathogenesis that have gained considerable attention during recent years are abnormal cytokine production and genetic associations. Of prominent interest are features associated with type I interferon (IFN). The type I IFN family consists of multiple members including IFN-α and β, and they are involved in various biological functions including defense against viral or bacterial infection, immune-modulation, and negative regulation of proliferation (7). An activated type I IFN system known as the interferon signature plays an important role in different autoimmune diseases, amongst them pSS (7, 8). In pSS patients, the interferon signature is associated with higher disease activity index scores (9).

It has been speculated that the initiating factor in the activated type I IFN response is a genetically determined exaggerated innate immune response against inappropriately overexpressed endogenous or exogenous danger signals. Extracellular nucleic acids present during viral infections, for example, can induce type I IFN production through interactions of extracellular nucleic acid with endosomal receptors, including TLR3, TLR7, and TLR9 (7). In SS it has been speculated that expression of danger signals resulting from transient or persistent viral infection of

epithelial cells leads to continuous activation of TLR signaling eventually contributing to SS pathogenesis (7). Interestingly, a number of infectious agents including Epstein-Barr virus, human T-lymphotropic virus type 1, hepatitis C virus and enterovirus have been reported as potential initiators of glandular lesions in SS patients (7).

Dysfunctional intracellular signaling mechanisms may influence the immunological response of a cell to a given stimulus, affecting transduction of a given signal and resulting in aberrant gene expression. We have previously shown that patients with pSS have an altered response of PBMC to IFN stimulation (10). Interestingly, several genetic variants associated with SS function in downstream signaling from TLRs or their regulation, including *IRF5* (11, 12), *IL-10* (13), *IκBα* (14), TNFAIP3 interacting protein 1 (*TNIP1*) (12), and *OAS1* (15). Potentiation, chronic activation or dysregulation of TLR signaling pathways could lead to exaggerated production of type I IFN and contribute to the type I IFN signature and disease pathogenesis. However, not much is known about TLR signaling in patients with pSS.

In this study, we characterized intracellular signaling pathways including those downstream from TLR7 and −9 receptor activation in PBMC by phospho-specific flow cytometry (phosphoflow) (16). We focused here on direct targets of TLR signaling such as ERK/MAPK as well as epitopes activated upon IFN signaling such as JNK/STAT. Increased induction of phosphorylation of STAT3 S727 and NF-κB was observed in B cells from pSS patients following TLR7 and −9 stimulation compared to B cells from healthy donors. The activation was shown to be increased in patients with SSA autoantibodies and patients without extraglandular manifestation. The increased responses following TLR7 and −9 stimulation through STAT3 S727 and NF-κB in B cells were associated with increased expression of three genes upregulated in response to type I IFN (*MxA*, *IFI44*, *OAS1*) but not type II IFN inducible genes (*GBP1* and *GBP2*). Plasma cytokine levels were different in SSA+ and SSA− patients and correlated with basal phosphorylation levels of several phospho-epitopes in patient subgroups. In conclusion, this study provides support that enhanced responses through TLR7 and −9 may play a role in the induction of a type I IFN signature observed in pSS patients indicating viral infections as potential trigger of the disease. Alternatively, induced expression of type I IFN inducible genes may potentiate TLR7 and −9 responses. Patients displaying elevated potentiation of these pathways may therefore benefit from therapies targeting these pathways.

MATERIALS AND METHODS

Blood Sampling

Peripheral blood from patients with pSS was collected in Lithium-heparin tubes (BD diagnostics) at the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway. Blood from healthy age- and gender-matched donors was collected at the blood bank at the Haukeland University Hospital in Bergen, Norway. PBMC were isolated by density gradient centrifugation with lymphoprep^{TM} (Axis-Shield, Oslo, Norway) and cryopreserved as described previously (17). Plasma was aliquoted and stored at $-70°C$, and PBMC were stored at $-150°C$ for $\sim$12–16 months. All patients fulfilled the pSS American-European Consensus group (AECG) criteria (18) and displayed no additional autoimmune diseases or lymphoma. An overview of the cohort is shown in **Table 1**. The study was approved by the regional ethical committee (#2009/686). All participants provided written informed consent.

Routine Laboratory Assays

Identification of anti-Ro/SSA and anti-La/SSB, other antinuclear antibodies (ANA), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and extraglandular manifestations were obtained as part of routine clinical investigation at time of blood sampling. SSA, SSB, and ANA were reported as either present or absent, while other serum and blood parameters were reported as continuous values. Extraglandular manifestations were defined as disease features outside surface exocrine glands.

TABLE 1 | Characteristics of patients and controls used in study.

	Sjögren's syndrome	Healthy controls
COHORT CHARACTERISTICS		
Females/males	25/0	25/0
Age, median (range) years	56 (33–73)	54 (42–70)
CLINICAL FEATURES (PATIENTS)		
SSA antibodies (%)	19 (76)	
SSB antibodies (%)	12 (48)	
SSA and SSB antibodies (%)	12 (48)	
ANA (%)	19 (76)	
Positive Schirmer's test (tear flow <5 mm/5 min) (%); $n = 24$	14 (58.3)	
Focus score† $\geq$ 1 (%); $n = 14$	10 (71.4)	
ESR, high levels††	5 (20)	
CRP high levels ($\geq$5 mg/L)	2 (8)	
Extraglandular manifestations (%)	14 (56)	
Treatment		
DMARDs	8 (32)	
Corticosteroids	2 (8)	

Continuous data is expressed as median. Categorical data is expressed as frequency and percentage. †Focus score indicates the number of inflammatory foci containing more than 50 mononuclear cells per 4 mm^2 biopsy tissue; ††Age and gender dependent. DMARDs, disease-modifying anti-rheumatic drugs; ANA, anti-nuclear antibodies; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Real-Time Quantitative PCR

Total RNA was isolated from PBMC of 20 pSS patients and 17 healthy controls and transcribed into cDNA as described previously (10). The following Taqman gene expression assays were utilized: Hs00895608_m1 (*MxA*); Hs00973637_m1 (*OAS1*); Hs00951349_m1 (*IFI44*); Hs00977005_m1 (*GBP1*); Hs00894837_m1 (*GBP2*); Hs03928990_g1 (18S rRNA) (all Thermo Fisher Scientific, Waltham, USA). All PCR reactions were run in duplicates on a Light Cycler 480 (Roche Diagnostics, Oslo, Norway). 18S rRNA was used as reference gene, and relative expression levels were calculated as $2^{-\Delta Ct}$. The IFN score was calculated according to Feng et al. (19) by standardizing expression levels using mean and SD of the healthy controls for the respective gene and using the following formula:

$$\sum_{i}^{3} = \frac{gene\ i_{pSS} - mean\ gene\ i_{Ctr}}{SD\ (gene\ i_{Ctr})}$$

where $i =$ each of the 3 type I IFN-inducible genes (*MxA, IFI44, OAS1*), *gene i_{pSS}* = the gene expression level in each pSS patient, and *gene i_{Ctr}* = the gene expression in controls. To set a threshold, $3 \times$ SD of healthy controls was utilized.

Antibodies Used for Flow Cytometry

The following phospho-specific monoclonal antibodies were used in 3 different panels during the flow cytometry protocol described previously (17): Alexa Fluor$^{®}$647 conjugated anti-STAT4 (pY693, clone 38/p-STAT4, panel 1), anti-STAT 1 (pS727, clone K51-856, panel 2), and anti-STAT3 (pS727, clone 49/p-STAT3, panel 3); PerCP-Cy^{TM} 5.5 conjugated anti-ERK1/2 (pT202/pY204, clone 20A, panel 1), anti-STAT1 (pY701, clone 4a, panel 2), and anti-STAT3 (pY705, clone 4/P-STAT3, panel 3); and PE-Cy^{TM}7 conjugated anti-p38 MAPK (pT180/pY182, clone 36/p38, panel 2), and anti NF-κB p65 (pS529, clone K10-895.12.50, panel 1), anti-STAT5 (pY694, clone 47/STAT5(pY694), panel 3) (all from BD Biosciences, San Jose, CA, USA). Cell surface markers incorporated in the assays were BV786 conjugated anti-CD3 (clone SK7, BD Horizon^{TM}), Alexa Fluor$^{®}$ 488 conjugated anti-CD20 (clone H1 (FB1), BD Biosciences) and PE conjugated anti-CD56 (clone N901, Beckmann Coulter, CA, USA).

Cell Culture and Stimulation

Before stimulation, cryopreserved PBMC were rapidly thawed using a water bath set to $37°C$ and washed once in prewarmed X-vivo 20^{TM} by centrifugation at 300 g for 7 min. The cells were then resuspended in prewarmed X-vivo 20^{TM} and rested at $37°C$ at 5% CO_2 for 30 min before the cell concentration was adjusted to 3×10^6 cells/ml in X-vivo 20^{TM}. Two hundred microliters were dispensed into 7 wells of a Megablock$^{®}$ 96 well plate (Starstedt, Nümbrecht, Germany), along with 2 wells of a reference sample. The cells were rested at $37°C$ with 5% CO_2 for 2 h. Following, the cells were either left unstimulated or stimulated according to a reverse time course for 15, 30, 60, 120, 180, or 240 min with a combination of TLR7 (CL097; Invivogen) and −9 ligands (CpG type B ODN 2006 and type C ODN 2395; Invivogen,

Carlsbad, California, USA) at 2 µg/ml each. Due to limited cell numbers and samples, time points were excluded in 2 patients, both for 60 and 120 min, and 1 healthy control for 180 and 240 min.

Fluorescent Cell Barcoding and Phospho-Epitope Staining for Flow Cytometry

PBMC were fixed by adding 16% PFA (Electron Microscopy Sciences (Hatfield, PA, USA) warmed to 37°C directly into the PBMC cultures resulting in a final PFA concentration of 1.5%. The samples were mixed thoroughly by pipetting. The cells were fixed at RT for 10 min before pelleting at 1,000 g for 5 min. The PBMC were then vigorously resuspended by vortexing in 50 µl PBS before drop wise addition of 1 ml ice cold methanol and incubation on ice for 30 min. The permeabilized cells were kept overnight at −80°C. After washing with PBS, the PBMC were stained according to a 3 × 3 barcoding grid (9 stimulation conditions) using 3 levels of pacific orange (PO) and pacific blue (PB) succinimidyl ester dyes (PB 100, 25, and 6.3 ng/ml; PO 250, 70, and 0 ng/ml; Life Technologies, Grand Island, NY, USA) for 30 min in the dark at 4°C in a volume of 1 ml. Barcoded PBMC were then washed once with staining media (PBS containing 1% BSA), and the 9 different dye concentration/combination samples were combined into one sample. The sample was washed and incubated with 2 µl Fc receptor block (Miltenyi Biotec, Bergisch Gladbach, Germany) per 1 × 10⁶ cells for 10 min on ice. Following, the sample was subdivided into 3 parts and incubated for 30 min at RT in the dark with the 3 different antibody staining panels. An aliquot of the barcoded cells was collected before addition of antibody as a barcoding only control. The samples were then washed twice and re-suspended in staining medium containing 2 mM EDTA (Sigma-Aldrich) prior to analysis.

Flow Cytometry Data Analysis

Samples were acquired on a LSRI Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA) with BDFACSDiVa™ Software (BD Biosciences) at the Bergen Flow Cytometry Core Facility, University of Bergen, Norway. The flow cytometer was equipped with 407, 488, 561, and 635 nm lasers, and emission filters for PerCP-Cy5.5 (LP: 685, BP: 695/40), Alexa Fluor-488 (LP: 505, BP: 530/30), PE-Cy7 (LP: 750, BP: 780/60), PE (LP: –, BP: 582/15), APC (LP: –, BP: 670–/-14), Pacific blue (LP: –, BP:450/50), Pacific orange (LP: 570, BP: 585/42), and BV 786 (LP: 750, BP: 780/60). The cytometer was routinely calibrated with BD cytometer setup and tracking beads (BD Biosciences). A minimum of 200,000 events in the intact cell gate was collected for each sample, giving a minimum of 2,000 events per analyzed cell population (T cells, B cells, NK cells). Flow cytometry data were analyzed in FlowJo (Tree Star) and Cytobank (http://www.cytobank.org). A representative gating strategy and phosphorylation profile for a single donor is shown in **Supplementary Figure S1**. Cryopreserved PBMC from a single donor with unstimulated and stimulated samples were run in each assay as a positive control for inter-assay

normalization and assessing assay to assay variability. Median fluorescence intensities (MdFI) for gated populations were exported to Microsoft excel. The raw flow cytometry data for which this article is based can be found at the flow data repository of the International Society for Advancement of Cytometry (20), FR-FCM-ZYED. The robustness of the flow cytometry assay used was previously established and published, see "An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells" (17). Relevant information for repeating the experiment as presented in "The minimum information about a Flow Cytometry Experiment (MIFlowCyt)" (21) are provided in **Supplementary Table S1**.

Cytokine Determination

Cytokine and chemokine concentrations were determined in plasma samples using a 25-plex Luminex assay cytokine and chemokine panel (Invitrogen, catalog number LHC0009M) and run on a Luminex 100 System (Luminex Corporation, Austin, TX) according to the manufacturer's instructions.

Statistical Analysis

Generation of graphs and comparisons between categories were done using an Unpaired Mann-Whitney test using Graphpad Prism (version 6.05). Differences were considered statistically significant when $p \leq 0.05$. The analysis was exploratory in nature hence no correction was made for multiple comparisons. Principle component analysis (PCA) using Unscrambler® X software (Camo software) was used to reduce dimensionality of the dataset and find clusters of patients with similar signaling profile which could be used to differentiate between disease status, presence of SSA autoantibodies, EGM and medication (DMARDs and corticosteroids). PCA was performed using the algorithm NIPALS, the data was mean centered and run with no weighting for change of MdFI, and weighted for absolute MdFI by dividing by standard deviation. Two methods were used to remove "redundant" variables to simplify interpretation and focusing subsequent analysis. First variables that described <50% of the variation were removed from the initial PCA, than if appropriate stepwise reduction of less significant variables with low variable leverage was performed. Correlations were assessed by the Spearman's rank test, with outliers removed using robust regression and outlier removal (ROUT) method and a ROUT coefficient Q of 1 used.

Since most of the cytokine data did not follow a normal distribution, Mann-Whitney U test was performed to study significant differences between the groups, and Spearman's correlation was used to find any significant relationships between the cytokines and the phosphoproteins. Degree of correlation was determined according to the recommendation of the British Journal of Medicine (https://www.bmj.com/about-bmj/resources-readers/publications/statistics-square-one/11-correlation-and-regression)—$r = 0.4$–0.59 (moderate), $r = 0.6$–0.79 (strong), and $r = 0.8$–1.0 (very strong). Analysis was done using GraphPad Prism 7 and $p \leq 0.05$ was considered to be statistically significant.

RESULTS

PBMC From pSS Patients Display Shifts in Phosphorylation States of Proteins Involved in Signaling Pathways

In order to reveal possible dysfunctional intracellular signaling mechanisms upon TLR stimulation in pSS patients, we here analyzed MAPK/ERK and JAK/STAT signaling networks in peripheral blood cells from female pSS patients and female age-matched healthy donors in unstimulated cells and upon stimulation with a combination of TLR7 and −9 ligands. In this pilot study, we limited our analyses to the main lymphocyte populations (T cells, B cells, NK cells) as all have been shown to be affected by TLR7/9 stimulation (22–24). An overview of unstimulated and TLR stimulated measurements can be found in the **Supplementary Table S2** (T cells), **Supplementary Table S3** (B cells), and **Supplementary Table S4** (NK cells).

Significant increases of basal phosphorylation in cells from pSS patients were observed in T cells for NF-κB, P38, ERK, STAT5, STAT1 Y701, STAT1 S727, and NK cells for P38, STAT5, STAT1 Y701, and STAT1 S727 compared to healthy donors. B cells showed no significant differences in basal phosphorylation (**Figure 1A**).

PCA was used to concurrently relate multiple basal signaling states to various clinical parameters such as production of SSA autoantibodies, presence of extraglandular manifestations (EGM) and medication (DMARDs and corticosteroids) within the patient cohort (**Figures 1B–F**). Using basal phosphorylation levels, pSS patients could be separated from healthy donors (**Figure 1C**). Spatial groupings indicated closer similarities within the pSS and healthy donor cohorts than between the groups. Separation of pSS and healthy donor samples was primarily along PC2 which explained 29% of the variation. Examination of the loading plot (**Figure 1B**) indicated differences between basal signaling phenotype of pSS patients and healthy donors, with pSS patients showing weaker basal pathway activation in B cells relative to NK and T cells compared to healthy donor cells. No groupings were shown along PC1 which explained 42% of the variation. Including clinical parameters in the analysis, patients without autoantibodies against SSA grouped closer to the healthy controls (**Figure 1D**), while patients with EGM (**Figure 1E**), and patients prescribed DMARDs or corticosteroids (**Figure 1F**) grouped throughout the pSS cluster.

We next analyzed MAPK/ERK and JAK/STAT signaling networks upon TLR7 and −9 stimulation of PBMC. Initial responses (15–60 min) were weak relative to respective basal measurements in both T and NK cells, with little or no change observed in phosphorylation of the measured epitopes (**Figure 2**). The strongest initial responses were seen in B cells for NF-κB, P38, STAT1 S727, and STAT3 S727. The induction of phosphorylation of STAT1 S727 and STAT3 S727 in B cells from pSS patients was significantly stronger than healthy donor cells. In order to exclude effects of the medication on the analyses, we removed medicated

patients from the analyses. This resulted in an even more pronounced difference between pSS patients and healthy donors (**Supplementary Figure S2**).

After 60 min of TLR7 and −9 stimulation, many epitopes of pSS patients displayed altered phosphorylation pattern compared to healthy donors, independent of medication (**Figure 2**, **Figure S2**).

Next, we included phosphorylation profiles of TLR7 and −9 stimulated T, NK and B cells in the PCA. Phosphorylation levels after 15 min showed the strongest clustering of subgroups, while extended time course (>15 min) gave no additional resolution (**Supplementary Figure S3**), hence we focused on induced MdFI at 15 min (MdFI^{15min}-MdFI^{basal}) after stimulation with TLR7 and 9 ligands (**Figure 3**). PCA visualization showed a positive shift along PC1 for approximately half the pSS samples away from healthy donor samples (**Figure 3B**). The pSS samples that were distributed away from the healthy donors were largely composed of EGM-negative (**Figure 3D**) and unmedicated patients (**Figure 3E**). PC1 explained 83% of the variation with positive movement along PC1, strongly influenced by phosphorylation of NF-κB and STAT3 S727 in B cells (**Figure 3A**). PC2 explained 9% of the variation and was influenced primarily by induced phosphorylation of NF-κB in B cells in a positive direction and negatively by STAT3 S727 in NK, T and B cells (**Figure 3A**).

Further comparisons of variables used in the final PCA were conducted by Mann-Whitney U tests (**Figure 3F**). Comparisons between groups and subgroups (pSS patients, healthy donors, EGM+/−, SSA+/−) were analyzed with and without exclusion of medicated patients, in order to exclude that the effects seen were merely due to medication used by patients. EGM− patients had a significantly increased response to stimulation by TLR7 and −9 ligands in B cells through NF-κB compared to EGM+ patients. T cells from EGM-negative patients exhibited a significantly decreased response in STAT3 S727 compared to those from EGM+ patients. B cells showed a significantly increased response in STAT3 S727 in pSS patients compared to healthy controls, SSA+ compared to SSA− and EGM− patients compared to EGM+ patients. Upon removal of medicated patients, in particular the B cell phospho-epitopes for NF-κB, pP38, and STAT3 S727 resulted in stronger and significant differences between healthy donors and pSS patients (**Supplementary Figure S4**).

To summarize, after omitting patients prescribed DMARDs or corticosteroids from the analysis, B cells from pSS patients showed an increased response to TLR7 and −9 stimulation through NF-κB.

Phosphorylation Profiles of Immune Cells Allow for Stratification of Patient Subgroups

A subgroup of pSS patients is characterized by a so-called type I IFN signature that correlates with increased disease activity (9). We therefore calculated an IFN score using three type I IFN inducible genes (*MxA*, *OAS*, *IFI44*) according to Feng et al. (19). As control, two type II IFN inducible genes (*GBP1*, *GBP2*)
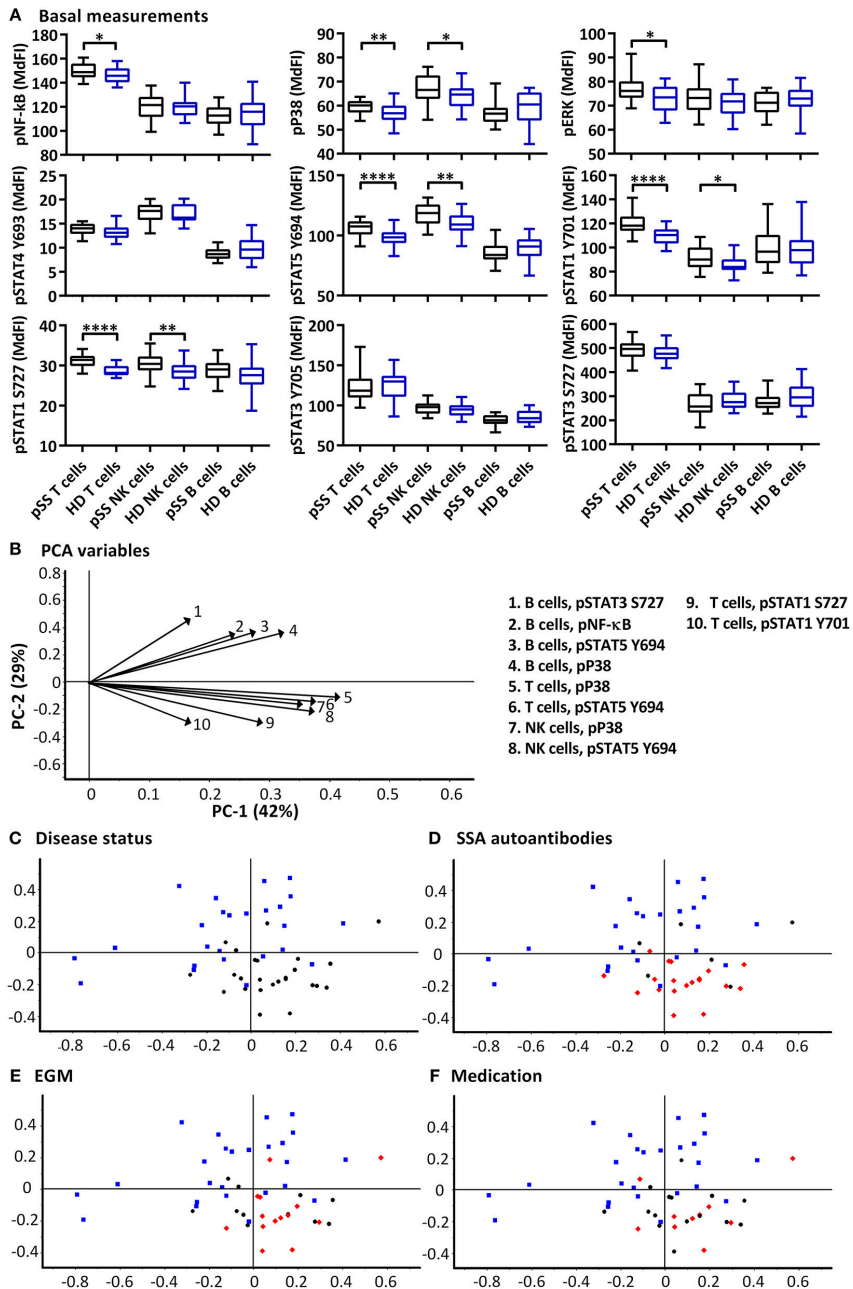
FIGURE 1 | Basal phosphorylation profiles in B cells, T cells and NK cells of pSS patients differ compared to healthy controls. Basal phosphorylation levels of NF-κB, P38, ERK1/2, STAT4 Y693, STAT5 Y694, STAT1 Y701, STAT1 S727, STAT3 Y705, and STAT3 S727 were analyzed by flow cytometry in T cells, B cells and NK cells are given in **(A)**. Comparisons of phosphorylation levels (MdFI) between healthy donor (blue) and pSS patient (black). Comparisons between pairs were done using an
(Continued)

FIGURE 1 | Unpaired Mann-Whitney test. Graphs show the median, 25–75 percentiles and minimum and maximum. Differences were considered statistically significant when $p \leq 0.05$, with significance indicated as * ≤ 0.05, ** ≤ 0.01, and **** ≤ 0.0001. PCA analysis of the profiles is given in **(B–F)**, disease status is highlighted in **(C)**, SSA autoantibody positivity in **(D)**, presence of EGM in **(E)**, and medication (DMARDs or corticosteroids) use in **(F)**, with healthy donors, blue squares; pSS patients, black circles; SSA+, EGM+, or medicated patients, red diamonds **(D–F,** respectively). The loading plot, which contains information about the variable for the corresponding PCA is given in **(B)**, with variable indicated by vectors and the key to the right. Variables contributing little to the PCA are plotted around the center as denoted by the gray axis, while variables that have high contributions are plotted further from the axes. After initial calculation of principal components, the model was recalculated with only variable explaining >50% of the variance retained. The data represents 25 healthy controls and 25 patients pooled from 13 independent experiments.

were included. The threshold was set to 8.8 based on $3 \times$ SD of healthy controls. A type I interferon signature was found in 70% of patients and 0% of controls (**Figure 4A**). Medicated patients tended toward reduced expression compared to unmedicated patients (**Figure 4B**).

To investigate whether type I IFN activation was reflective of phosphorylation levels of intracellular signaling proteins, the gene expression was correlated to signaling profiles (**Figures 4, 5**). Comparisons of patients subdivided into type I IFN signature positive (IFN+) and negative (IFN–) patients and healthy donors were made for each intracellular signaling molecule and cell type, with basal phosphorylation variables that showed the strongest associations with type I IFN gene expression shown in **Figure 4C**. Both, IFN+ and IFN– patients displayed increased phosphorylation of STAT1 S727 in T cells. Interestingly, an increased phosphorylation of STAT1 Y701 was detected in NK cells of IFN+ patients compared to IFN– patients.

Basal phosphorylation levels in B cells from pSS patients were generally negatively correlated with type I IFN inducible gene expression, while positively correlated in NK and T cells, with weaker associations found with type II IFN inducible gene expression. The strongest significant correlations between type I IFN regulated genes were observed in NK cells for pERK, pSTAT1 Y701 and pSTAT3 Y705, and T cells for pSTAT1 S727 (**Figure 4D**). No significant associations were observed against type II IFN regulated genes for the aforementioned epitopes (**Figure 4D**).

We next analyzed the phosphorylation status upon TLR7 and –9 stimulation in correlation to IFN inducible gene expression in pSS patients against variables identified previously by PCA (**Figure 5A**). IFN+ patients showed increased phosphorylation of NF-κB and STAT3 S727 in B cells, and these differences remained significant following the removal of medicated patients. No difference was seen between the patient groups for phosphorylation of STAT3 S727 in T or NK cells.

Especially in B cells a prevalent positive correlation of phosphorylation levels and IFN inducible gene expression were detected. The strongest correlations were seen for phosphorylation levels of NF-κB and STAT3 S727 (**Figure 5B**). STAT4 Y693, NF-κB, P38, and STAT3 Y705 in NK cells and NF-κB in T cells showed positive associations, but only NF-κB and P38 reached statistical significant correlations. Of the other two variables identified by PCA, STAT3 S727 in T and NK cells, no significant correlation with gene expression was observed (**Figure 5B**).

Plasma Cytokine Levels Correlate With Presence of Autoantibodies and Signaling Responses in Patient Subgroups

The role of cytokines in pSS has been a matter of great interest over the past few years (25, 26). Our aim was to examine the plasma cytokine concentration of our pSS patient cohort, compare them to healthy controls, and possibly correlate them to clinical parameters and phosphorylation pattern of the epitopes included in this study.

GM-CSF, IL-5, and IL-8 were excluded from the analyses as they were below detection limit in most samples analyzed. Eotaxin, IL-7, IP10, and MIG were not included in the analysis as they were not recommended to be measured in heparin plasma by the manufacturer.

When comparing patients with controls, 12 out of the 25 cytokines measured were significantly upregulated in patients including IL-1ß, IL-13, IL-6, IL-12, MIP-1α, MIP-1ß, MCP-1, IL-15, IFN-α, TNFα, IL-2, and IL-4 (**Supplementary Figure S5, Supplementary Table S5**). When dividing the patients into subgroups based on the presence or absence of autoantibodies (SSA+/SSA–), extraglandular manifestations (EGM+/EGM–), IFN score, medication and Focus score, the only significant differences were seen in SSA+ patients, where IL-1ß, MCP-1, IFN-α, IL-2, and IL-4 were significantly upregulated compared to SSA– patients (**Figure 6**, **Supplementary Table S6**).

Correlation analysis of the individual cytokines of the patients to the phosphoproteins yielded significant results. While the basal phosphorylation profiles of all the patients showed only moderate correlations (<0.6) to the plasma cytokine concentrations, excluding medicated patients from the analysis resulted in strong to very strong correlations of RANTES to pNF-κB in NK cells, MIP-1ß, MCP-1, IL-2, and IL-4 to pSTAT5 Y694 in B cells, and IL-1RA to pSTAT1 Y701 in T cells (**Figure 7**).

We next explored the correlation of the plasma cytokine levels to basal phosphorylation pattern depending on presence or absence of SSA and EGM. The exclusion of medicated patients in the subgroup analysis resulted in too few patients per group for reliable data, therefore all patients were included in this part of the analysis. We observed strong to very strong correlations in SSA+ patients of MIP-1α, IL-1RA, and TNF-α to pSTAT3 Y705 in B cells, TNF-α to pNF-κB in B cells, and RANTES to pSTAT4 Y693 and pSTAT1 S727 in NK cells (**Figure 8A**). In SSA– patients, RANTES correlated to pNF-κB in T cells and pERK in T cells (**Figure 8B**). Patients with EGM had strong to very strong correlations of several cytokines to amongst other pSTAT4 Y693 in NK (**Figure 9A**), and RANTES, IFN-γ, IL-1RA,
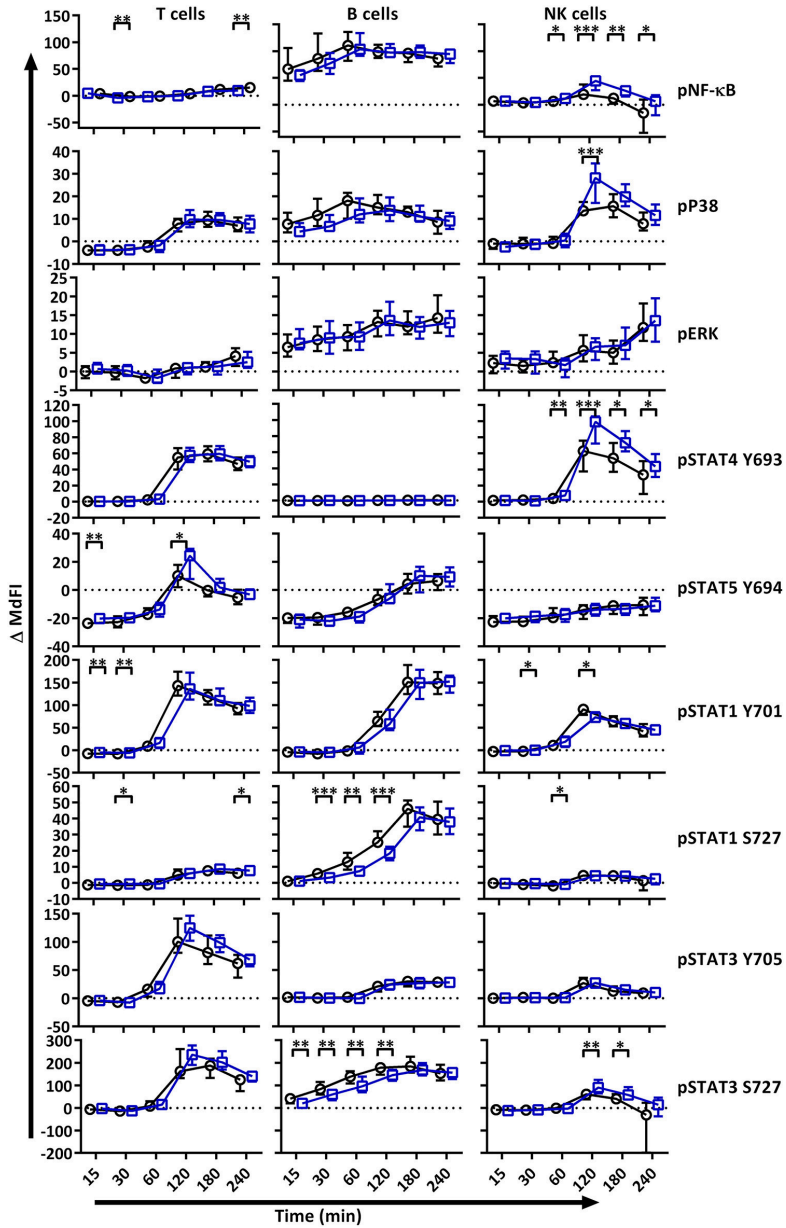
FIGURE 2 | TLR stimulation results in different phosphorylation profiles in B cells, T cells, and NK cells of pSS patients compared to healthy controls. Phosphorylation levels of NF-κB, P38, ERK1/2, STAT4 Y693, STAT5 Y694, STAT1 Y701, STAT1 S727, STAT3 Y705, and STAT3 S727 were analyzed by flow cytometry at different time points after stimulation with TLR7 and −9 ligands. Comparisons of change of phosphorylation levels (ΔMdFI) between pSS patient (black) and healthy donors (blue) are given. Comparisons between pairs were done using an Unpaired Mann-Whitney test. Line graphs show the median and 25–75 percentiles. Differences were considered statistically significant when $p \leq 0.05$, with significance indicated as * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001. The data represents 25 healthy controls and 25 patients pooled from 13 independent experiments.

FIGURE 3 | PCA analysis of induced phosphorylation in PBMC at 15 min following stimulation with TLR 7 and −9 ligands. Groupings of samples by PCA are shown by disease status **(B)**, SSA autoantibody positivity **(C)**, EGM presence **(D),** and medication use (DMARDs or corticosteroids) **(E)**. Healthy donor samples are indicated by blue squares, pSS patients as black circles, and pSS patients with SSA autoantibodies, EGM or using prescribed DMARDs or corticosteroids (**C–E,** respectively) displayed as red diamonds. The loading plot, which contains information about the variable for the corresponding PCA is shown in **(A)**, with variables contributing to the PCA given as vectors. Variables contributing little to the PCA are plotted around the center as denoted by the gray axis, while variables that have high contributions are plotted further from the axes. After initial calculation of principal components the model was recalculated with only variable explaining >50% of the variance retained, stepwise reduction of less significant variables with low variable leverage was then performed. Scatter box plots of variable used in PCA for TLR7 and −9 ligand induced responses are given in **(F)**. Figures show change in MdFl from 0 to 15 min (Y axis) following addition of TLR7 and −9 ligands to PBMC cultures. Measured phospho-protein and responding cell type are labeled above each figure. Groups are identified at the base of each figure (X axis), initially with pSS patients (pSS) and healthy donors (HD Ctrl), pSS patients are further divided into SSA autoantibody positive and negative patients, patients with EGM (EGM+) or without EGM (EGM–), and unmedicated and medicated patients. Statistical comparisons were made between each of these pairs as indicated by dashed lines, with black bars representing medians. The data represents 25 healthy controls and 25 patients pooled from 13 independent experiments. Comparison between pairs were conducted using an Unpaired Mann-Whitney test with significance indicated as * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

FIGURE 4 | Associations of the basal phosphorylation profile with type I IFN inducible gene expression in pSS patients and healthy controls. IFN score was calculated based on standardized expression levels of three type I IFN inducible genes (MxA, OAS1, IFI44). A threshold was set to 8.8 based on 3 × SD of healthy controls as seen in (A). Association between IFN score and the use of DMARD or corticosteroids is seen in (B). Unpaired Mann-Whitney test comparisons of

(Continued)

FIGURE 4 | basal phosphorylation levels for IFN+ patients ($n = 14$), IFN– patients ($n = 6$), and healthy controls ($n = 17$) with the strongest associations are given in (C), with medians indicated by black bars. Comparisons were conducted using an Unpaired Mann-Whitney test. Correlations between three type I IFN inducible genes (MxA, OAS1, IFI44) and two type II inducible genes (GBP1, GBP2) with basal phosphorylation levels in pSS patients ($n = 20$) epitopes, as given in (C), is shown in (D). Correlations were assessed with Spearman's rank test, with outliers removed using robust regression and outlier removal (ROUT) method and a ROUT coefficient Q of 1 was used. Significant values are indicated as * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, and **** ≤ 0.0001. The flow cytometric data represents 17 healthy controls and 20 patients pooled from 13 independent experiments, with real time qPCR data representing a single experiment incorporating the 17 healthy controls and 20 patients.
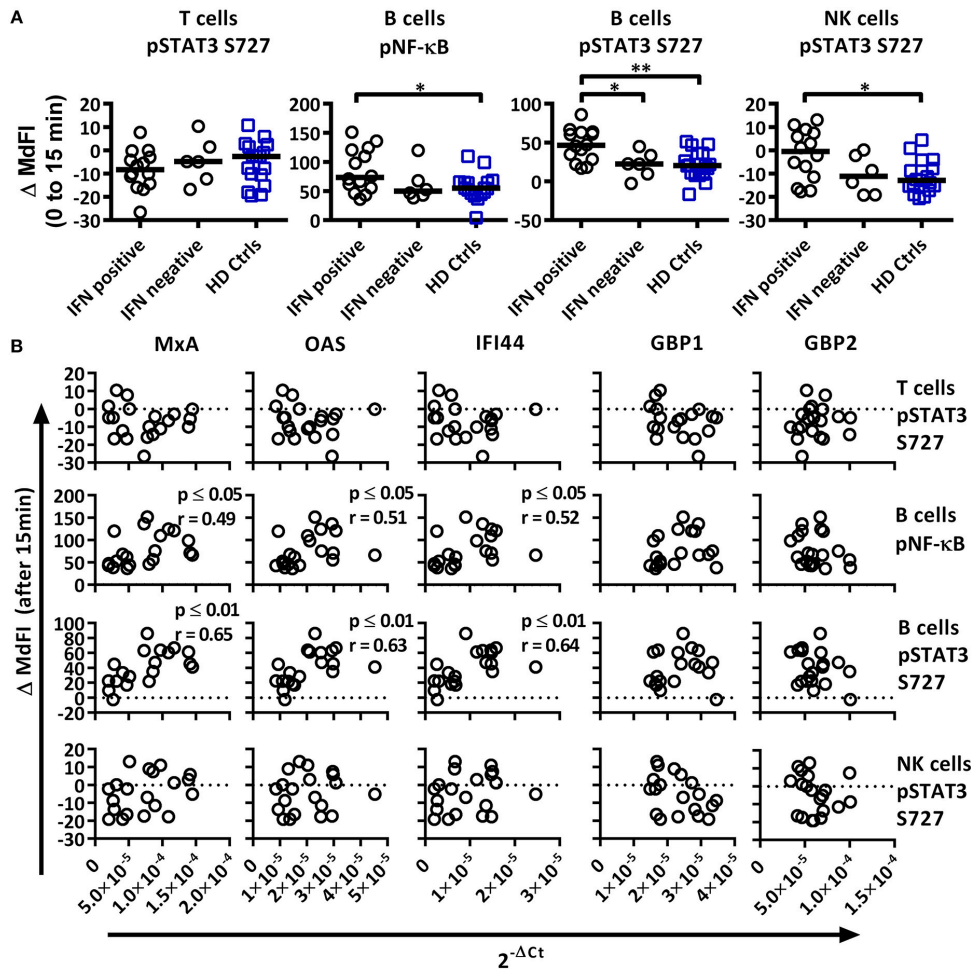
FIGURE 5 | Associations of the phosphorylation profile 15 min after TLR7 and −9 stimulation with IFN inducible gene expression in pSS patients and healthy controls. (A) Unpaired Mann-Whitney test comparisons of phosphorylation levels after TLR7 and −9 stimulation for IFN signature positive patients ($n = 14$), IFN signature negative patients ($n = 6$) and healthy controls ($n = 17$), medians are given by black bars. (B) Correlation plots of variable identified by PCA and three type I IFN inducible genes (MxA, OAS1, IFI44) and two type II IFN inducible gene (GBP1, GBP2) for pSS patients ($n = 20$). Correlations were assessed with Spearman's rank test, with outliers removed using robust regression and outlier removal (ROUT) method and a ROUT coefficient Q of 1 was used. Significant values are indicated as * $\leq$ 0.05 and ** $\leq$ 0.01. The flow cytometric data represents 17 healthy controls and 20 patients pooled from 13 independent experiments, with real time qPCR data representing a single experiment incorporating the 17 healthy controls and 20 patients.

FIGURE 6 | Differential expression of cytokines between autoantibody positive (SSA+) and autoantibody negative (SSA-) pSS patients. Cytokine profiles were measured by 25-plex Luminex assay in plasma. Cytokine levels (pg/ml) showing significant differences between SSA+ patients (red) and SSA– patients (black) are shown. Healthy controls (HD Ctrls) are shown in blue. Medicated patients are shown as diamond. Comparison between pairs was done by unpaired Mann-Whitney test between the two patient subgroups. Differences were considered statistically significant for $p \leq 0.05$, with significance being indicated as * ≤ 0.05, ** ≤ 0.01. The median is indicated. The data represents pSS patients ($n = 25$) grouped into SSA+ patients ($n = 12$) and SSA– patients ($n = 13$) (except for IL-4, $n = 11$ in each category).

IFN-α, and IL-12 correlated with various phospho-epitopes in EGM– patients (**Figure 9B**).

DISCUSSION

Autoimmune diseases often exhibit skewed cytokine and gene expression profiles. Elucidating mechanisms that contribute to these profiles are crucial in understanding the pathogenesis of autoimmune disease. Of prominent interest in autoimmunity is an increased expression of type I IFN regulated genes known as the "interferon signature" which has been observed in various autoimmune diseases (8, 9, 27, 28). Continuous activation and dysregulation of TLR and type I IFN signaling have been speculated to play a part in this signature and pathogenesis of autoimmune disease (29), and IFN signature positive pSS patients have been shown to have increased expression of TLR7 in certain cell types (30). In addition, we have previously shown that PBMC of pSS patients have an altered response to IFN-α stimulation (10). Hence we investigated cell signaling profiles in PBMC of pSS patients upon stimulation via TLR7 and −9, determined the gene expression profile of several IFN inducible genes and correlated these findings to plasma cytokine levels.

In accordance with a previous observation we found increased basal STAT5 Y694 phosphorylation in T cells from pSS patients compared to healthy donors (31). However, in contrast to our

study, the authors also found significant differences in B cells for basal phosphorylation of STAT5 Y694 and no differences in phosphorylation of STAT1 Y701 in T cells (31). Another study also reported on significant differences in basal phosphorylation levels of STAT3 Y705 in T cells (32), which also is in contrast to our findings. However, these differences are likely the result of the use of cryopreserved PBMC and long culture period (6 h) in our study, as both other studies used freshly isolated cells.

Although PCA using basal measurements allowed for grouping of pSS patients and healthy donors, its use to identify important variables of basal measurements for subgrouping of the patient cohort was largely unsuccessful. The majority of B cell associated variables largely correlated with each other, as did T and NK cells with the grouping of pSS patients being a consequence of higher basal phosphorylation in T and NK cells. Even though some differences were seen when dividing the pSS patients according to medication, the small sample size limits how much we can speculate on the pathophysiological significance of this.

Basal STAT1 Y701 in NK cells was increased in type I IFN+ patients, but the difference was no longer significant when excluding medicated patients. However, the sample size following exclusion was relatively low, while the data spread remained similar. Further, phosphorylation of many of the measured phospho epitopes in NK and T cells from pSS patients, in particular STAT1 Y701 in NK cells, were positively correlated

FIGURE 7 | Correlations of basal phosphorylation profiles with plasma cytokine levels in pSS patients without medication. Correlations were assessed by Spearman's rank test. Strong ($r = 0.6$–0.79) to very strong ($r = 0.8$–1.0) associations are shown with respective p and r-values given at the side of each graph. Correlations were considered statistically significant for $p \leq 0.05$. X-axis denotes concentration of cytokines (pg/ml) and Y-axis denotes phosphorylation levels (MdFI). The data represents pSS patients ($n = 16$) without medication (except for IL-4, $n = 14$, and RANTES, $n = 15$).

with the expression levels of the three type I IFN inducible genes, while in B cells a negative correlation was observed. In contrast, little relationship was observed for the type II IFN induced genes. Not much is known about NK cells in Sjögren's syndrome, so the correlation with basal phosphorylation of ERK, STAT1 Y701, and STAT3 Y705 in NK cells is especially interesting. Moreover, levels of several plasma cytokines also correlated with basal phosphorylation in NK cells. Further studies are required to confirm these correlations.

Following stimulation with TLR7 and −9 ligands, B cells from pSS patients showed a significantly increased STAT3 S727 response compared to healthy donors. After excluding medicated patients, phosphorylation of NF-κB and P38 was also significantly elevated in B cells from pSS patients compared to healthy donors. These findings support the notion that pSS patients display a hyperactive B cell response and are in line with our previous study showing increased expression of IFN-α in B cells from pSS patients after incubation with TLR7 ligands compared to B cells from healthy donors (33). The increased response to TLR7 and −9 ligands through these pathways may play a role in the increased expression of IFN-α from B cells of

pSS patients, and may also contribute to the observed IFN signature in some pSS patients. Thereby, it opens for speculations regarding the importance of viral infections for pSS patients. Further, a number of polymorphisms associated with pSS and the presence of autoantibodies in pSS could potentially affect signaling through NF-κB, P38, and STAT3 S727. If the potentiation of these signaling profiles are associated with polymorphisms in negative regulators of TLR signaling, including A20 (antiapoptotic signaling protein) which deubiquitylates TRAF6 (tumor-necrosis-factor-receptor-associated factor 6), and affects both MyD88-dependent and MyD88-independent pathways (34), these differences will also likely be reflected in other cell types using the same pathways. Alternatively, the increased response through these pathways may be attributed to the cellular effects of induction of type I IFN gene expression.

Interestingly, 70% of the patients included in this study had an activated type I IFN system. This is somewhat higher than previously reported for pSS patients [around 55%; (9)] and SLE patients [around 50%; (27)]. This might be due to limited sample size and differences in patient inclusion criteria. However, also the plasma levels of IFN-α were elevated in our cohort of pSS

FIGURE 8 | Correlation of basal phosphorylation status with plasma cytokine levels in pSS patients grouped according to autoantibody status. Autoantibody positive pSS patients (SSA+) **(A)**, autoantibody negative pSS patients (SSA–) **(B)**. Correlations were assessed by Spearman's rank test. Medicated patients are shown in red while un-medicated patients are shown in black. Strong ($r = 0.6$–0.79) to very strong ($r = 0.8$–1.0) associations are shown with respective p and r-values given at the side of each graph. Associations were considered statistically significant for $p \leq 0.05$. X-axis denotes concentration of cytokines (pg/ml) and Y-axis denotes phosphorylation levels (MdFI). The data represents pSS patients ($n = 25$) grouped into SSA+ patients ($n = 12$) (except for RANTES, $n = 11$) and SSA– patients ($n = 13$).

patients, especially in SSA+ patients, which might explain the high percentage of IFN+ patients.

Induced phosphorylation of STAT3 S727, NF-κB, and P38 correlated significantly with type I IFN inducible gene expression. Type I IFN has been shown to enhance B cell responses to TLR7 ligands and upregulate TLR7 and MyD88 expression in naïve B cells (35, 36). Increased type I IFN gene expression may therefore act to potentiate these signals.

Systemic autoimmune diseases are associated with the production of autoantibodies and have an important role in

FIGURE 9 | Correlation of basal phosphorylation profiles with plasma cytokine levels in pSS patients grouped according to the presence of extraglandular manifestations (EGM). Extraglandular manifestation positive pSS patients (EGM+) **(A)** and extraglandular manifestation negative pSS patients (EGM–) **(B)**. Correlations were assessed by Spearman's rank test. Medicated patients are shown in red while un-medicated patients are shown in black. Strong ($r = 0.6$–0.79) to very strong ($r = 0.8$–1.0) associations are shown with respective p and r-values given at the side of each graph. Associations were considered statistically significant for $p \leq 0.05$. X-axis denotes concentration of cytokines (pg/ml) and Y-axis denotes phosphorylation levels (MdFI). The data represents pSS patients ($n = 25$) grouped into EGM+ patients ($n = 14$) (except for IL-4, $n = 11$) and EGM– patients ($n = 11$).

the immunopathogenesis of various autoimmune diseases (29). Animal models have indicated links between TLR recognizing nucleic acids and the production of nucleic acid recognizing antibodies (37). Additionally, type I IFN inducible gene expression has been observed to positively correlate with titers of SSA and SSB autoantibodies in SS (38). We showed increased responses of B cells from SSA+ pSS patients through phosphorylation of STAT3 S727 in response to TLR7 and −9 stimulation compared to SSA– patients. Our study thereby links all three observations, enhanced TLR7 and −9 responses, increased type I IFN gene expression and autoantibodies, further highlighting their importance in autoimmunity.

Principal component analysis suggests that it is possible to subdivide pSS patients based on presence of EGM. EGM negative patients displayed enhanced TLR responses through NF-κB, P38, and STAT3 S727 in B cells compared to EGM+ patients. This was also seen after removal of patients prescribed the glucocorticoid prednisone, which has been reported to inhibit NF-κB activation (39), and hydroxychloroquine (Plaquenil®) inhibiting TLR7 and −9 signaling (40). However, it is still surprising that it was the EGM negative patients that had an enhanced response in B cells, as a number of EGM in SS are associated with high prevalence of hyperreactive B-cells as well as SSA and SSB autoantibodies (41). One possible explanation might be that the lower responses of B cells from EGM+ patients represent movement of more reactive B cells from the periphery to other compartments not being analyzed in this study.

Several plasma cytokines correlated significantly with basal phosphorylation levels of various phospho-epitopes in T-, B-, and NK cells. However, even though presence of outliers was tested using ROUT's method, most outliers detected by the test were not excluded from the analyses except a few very obvious ones, as Rout's method is not very reliable for non-parametric data. The low number of patients per subgroup further requires caution concerning interpretation of the data. A larger number of patients has to be analyzed before a more reliable correlation between phosphorylation pattern, cytokine profile, presence of autoantibodies and EGM might be found. This might also help clarifying the pathophysiological relevance of our findings.

This study has a number of limitations, for one, small sample size, which is further affected by the heterogeneity of the patients, and in particular the number of medicated patients. Second, as this was a pilot study, the analysis was limited to the three main subsets of lymphocytes (T, B, and NK cells). As these cell subsets are made up of numerous other subtypes, differential responses and shifts in their relative frequency in the peripheral blood may affect cellular responses. Immunophenotyping studies have shown altered distribution of various cell types in peripheral blood (42, 43). We can therefore not be certain that the changed signaling profiles

are not caused by these alterations rather than potentiated or repressed signaling. Moreover, certain subpopulations might be more prone to apoptosis upon longer stimulation with TLR7 and −9 ligands, which we did not address in this study. Finally the type I and II IFN regulated gene expression was assessed in PBMC, and assessment for each cell type might have strengthened associations and be more informative in determining origin of the signature. In addition, some of the statistically significant differences were rather small. Future studies will have to address the biological relevance in more functional assays.

In conclusion, we have identified increased responses by B cell from pSS patients to TLR7 and −9 stimulation through STAT3 S727 and NF-κB. The increased response was found to correlate to a type I IFN signature. The results suggest that the type I IFN signature may either induce or in part be derived in response to increased activation of NF-κB and STAT3 S727 upon TLR7 or −9 activation, facilitating increased production of interferon.

AUTHOR CONTRIBUTIONS

PV, RJ, and SA conceived of study. RD, PV, and SA designed the study. RD and BB processed PBMC samples and conducted flow cytometric analysis. SA conducted real-time quantitative PCR. IS and SMS performed cytokine assays. RD, BB, SG, IS, and SA analyzed and processed the data. DH and JGB selected patients and collected patient data. RD and SA drafted the manuscript. All authors revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu. 2019.00281/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
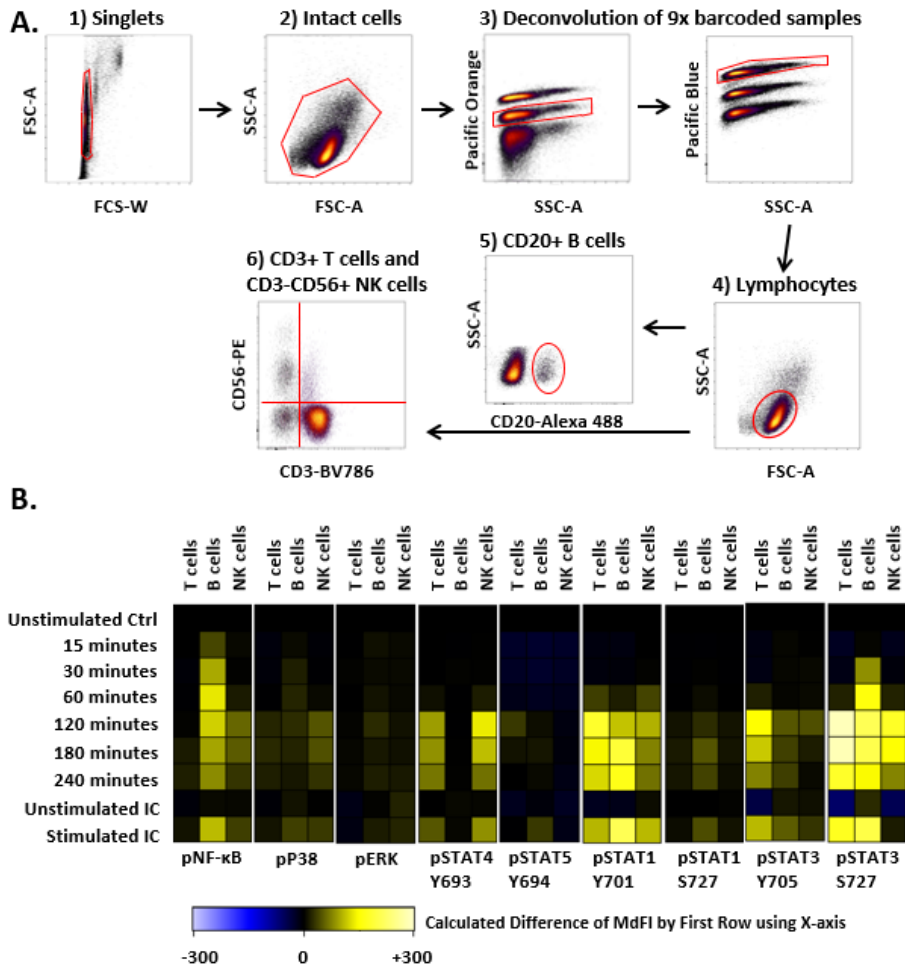
Figure S1. A. Representative gating strategy used in the analysis of intracellular signaling pathways in PBMC. Single cells were gated based on their forward scatter area (FSC-A) and forward scatter width (FSC-W), followed by intact cells based on side scatter area (SSC-A) and FSC-A. The different stimulation conditions were then identified through the intensities of their pacific orange and pacific blue stains. Lymphocytes were identified based on their FSC-A and SSC-A scatter properties. Lymphocytes were then subtyped as B cells (CD20+), T cells (CD3+CD56-) or NK cells (CD3-CD56+) based on surface antigen expression. **B. Cell type specific signalling profile for a single donor following stimulation with TLR7 and -9 ligands over a 4 hour time course**. The figure indicates the change of MdFI compare to an unstimulated sample for each phospho-epitope.
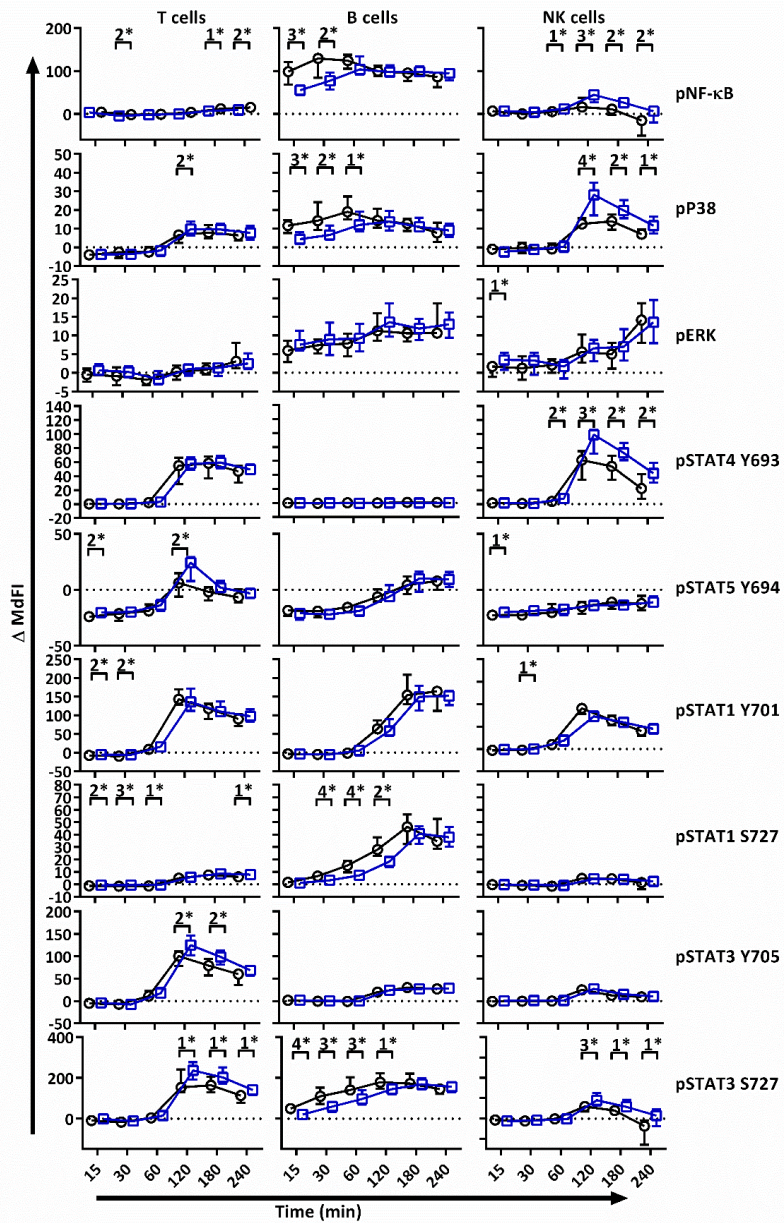
Figure S2. **Phosphorylation profiles in B cells, T cells and NK cells of unmedicated pSS patients compared to healthy controls** (see also Figure 2). Phosphorylation levels of NF-κB, P38, ERK1/2, STAT4 Y693, STAT5 Y694, STAT1 Y701, STAT1 S727, STAT3 Y705 and STAT3 S727 were analysed by flow cytometry at different time points after stimulation with TLR7 and -9 ligands. Comparisons of change of phosphorylation levels (ΔMdFI) between unmedicated pSS patient (black) and healthy donors (blue) are given. Comparisons between pairs were done using an Unpaired Mann-Whitney test. Line graphs show the median and 25 to 75 percentiles. Differences were considered statistically significant when $p \leq 0.05$, with significance indicated as $1^* \leq 0.05$, $2^* \leq 0.01$, $3^* \leq 0.001$ and $4^* \leq 0.0001$.
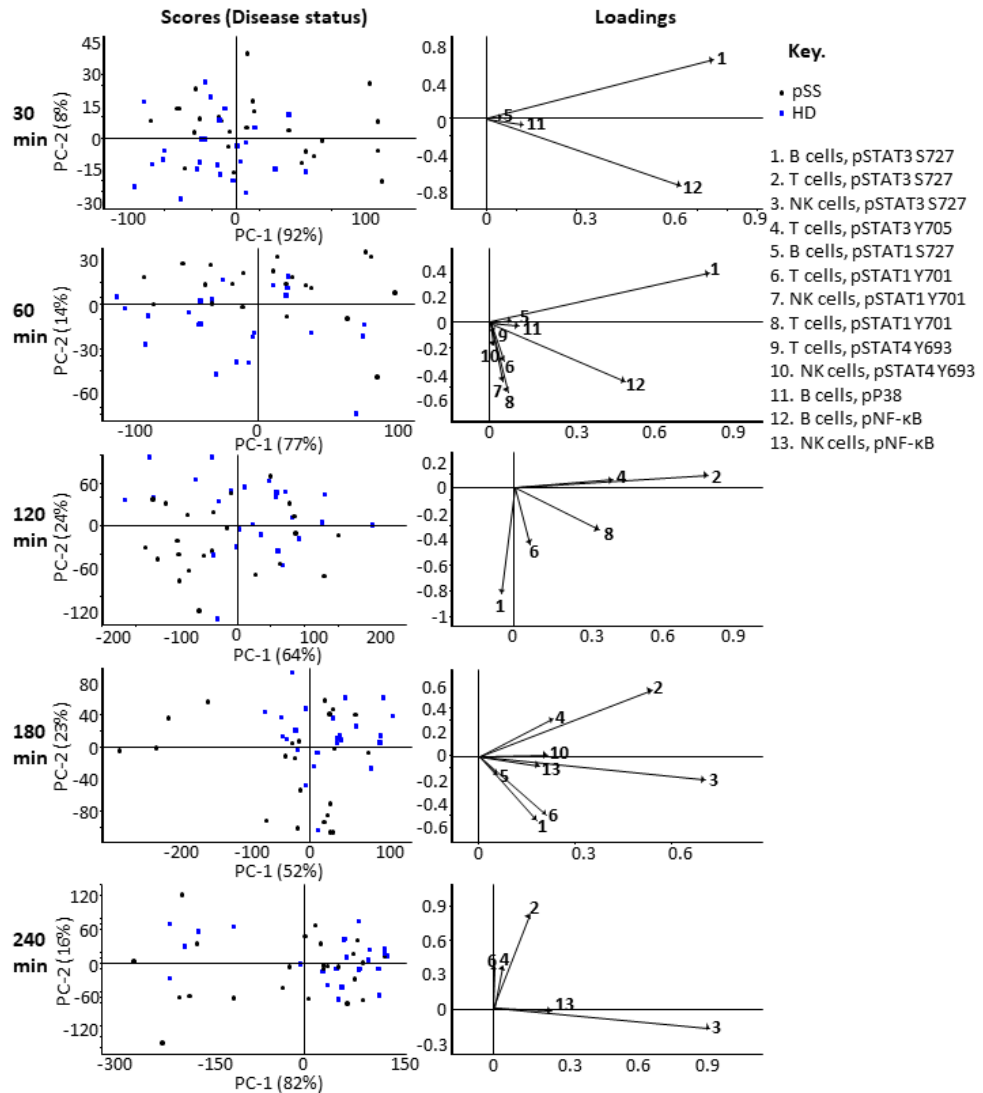
Figure S3. **Groupings of samples by PCA for time points 30, 60, 120, 180 and 240 min**. Black circle: pSS patient, blue square: healthy donor. The figure key is shown on the right side. The loading plot containing information about the variables for the corresponding PCA is shown on the right, with variables given as vectors. Variables contributing little to the PCA are plotted around the center as denoted by the axis, while variables having high contributions are plotted further from the axes.
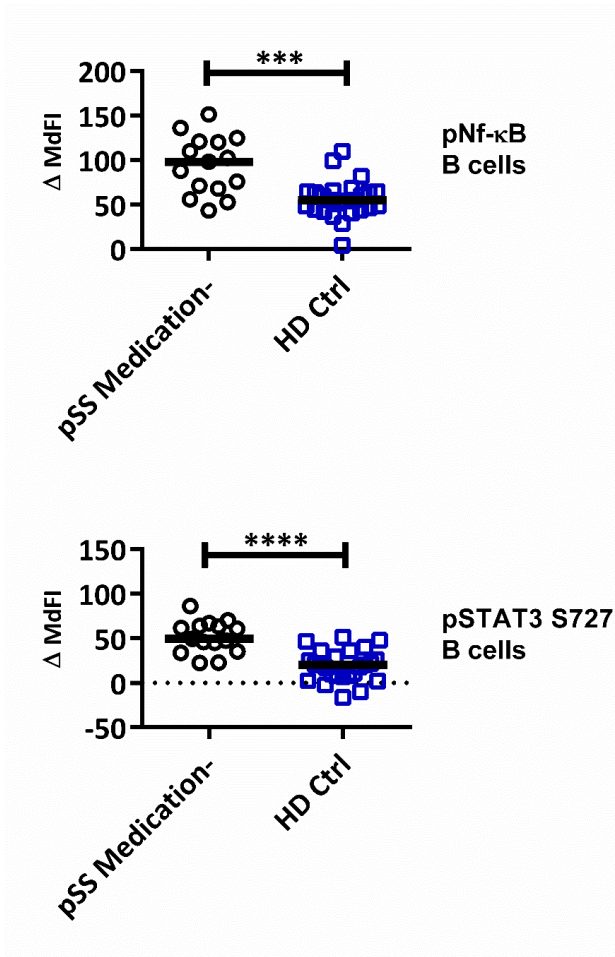
Figure S4. **Comparison of unmedicated patients to healthy controls as shown in Figure 3F**. Unpaired Mann Whitney tests between healthy donors and un-medicated patients give significant differences for both change of pNf-κB and STAT3 S727 in B cell after 15 minutes with p-values of 0.0002 (median: pSS- 98.22, HD 55.09; 95% CI: 16.04 to 59.98) and > 0.0001 (median: pSS- 49.39, HD 19.97; 95% CI: 20.15 to 44.88) respectively. Black circle: unmedicated patients; blue square: healthy controls.
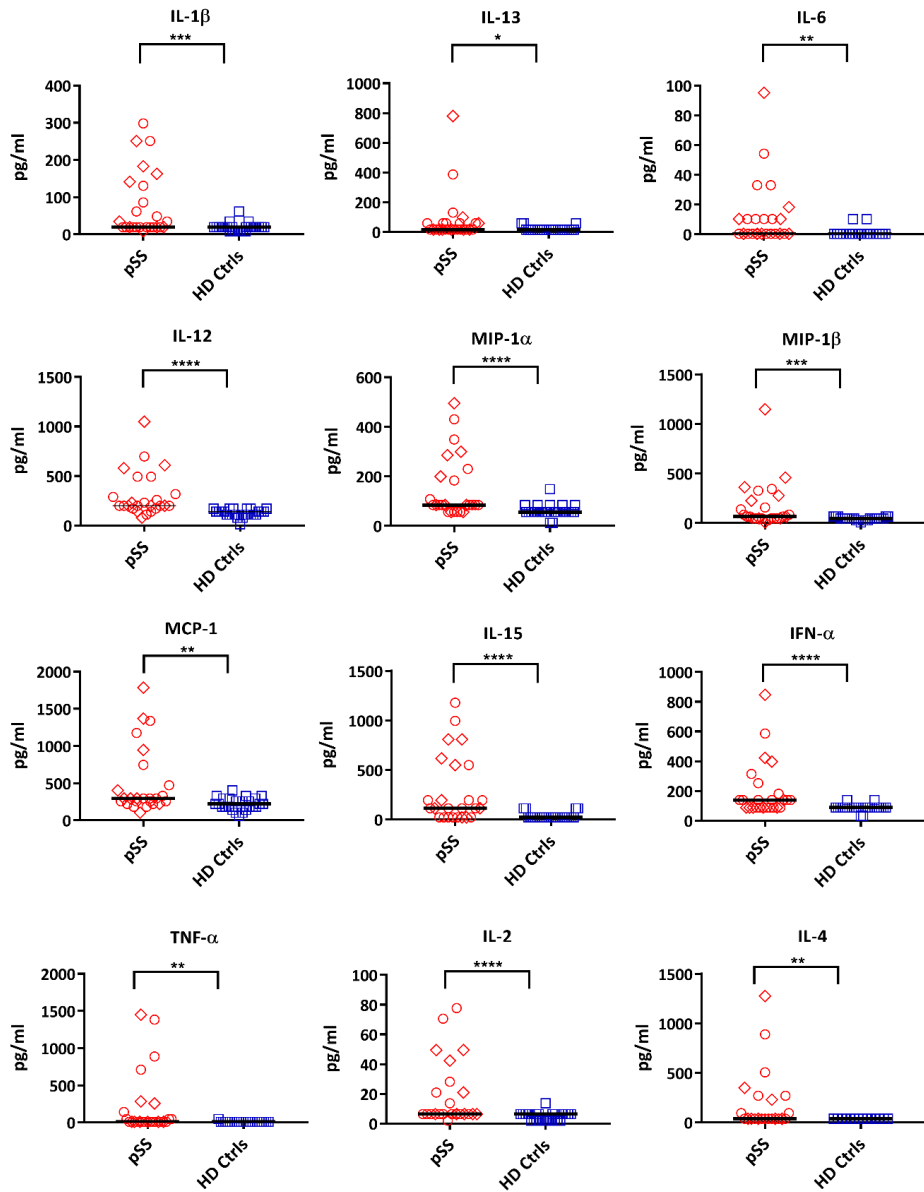
Figure S5. Variation in plasma cytokine concentrations between pSS patients and healthy controls. Cytokine levels (pg/ml) were measured by 25-plex Luminex assay in plasma and significant differences between patients (pSS; red) and healthy controls (HD Ctrls; blue) are shown. Medicated patients are shown as diamond. Comparison between pairs was done by unpaired Mann-Whitney test. Differences were considered statistically significant for p values ≤ 0.05, with significance indicated as * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 and **** ≤ 0.0001. Medians are shown by line graphs. The data represents pSS patients (n=25) (except for IL-15 and IL-4 where n= 21 and 22 respectively) and HD Ctrls (n=25) (except for IL-6, TNF-α and IL-4 where n=24, 23 and 16 respectively).

Table S1. Relevant information for repeating the experiment as presented in "The minimum information about a Flow Cytometry Experiment (MIFlowCyt)".

Data set	Sample/Reagent/ Controls/Instrument	Details
Samples/Specimens	Patient samples	*Species: Homo Sapiens, Sex: female, Median age (range): 56 (33-73), Phenotype: pSS, Location: Department of Rheumatology, Haukeland University Hospital, Bergen, Norway. Collection methodology: Venule puncture*
	Healthy donor samples	*Species: Homo Sapiens, Sex: female, Median age (range): 54 (42-70), Phenotype: Unknown, Location: Blood bank, Haukeland University Hospital, Bergen, Norway. Collection methodology: Venule puncture*
	Single donor control	*Species: Homo Sapiens, Sex: Unknown, Age: Unknown, Phenotype: Unknown, location: Bergen, Norway. Collection methodology: Venule puncture*
Sample treatment	Blood collection	*Lithium-heparin tubes for patients and healthy donor samples, and a citrate phosphate dextrose adenine blood collection bag for the single donor control*
	PBMC isolation	*PBMC isolated within 1hr of blood collection by density gradient centrifugation with lymphoprep (Axis-Shield, Cat#07861)*
	Cryopreservation	*Immediately following isolation, PBMC samples were washed 2x with PBS (Lonza, Cat# 17-516F) and frozen at 5x10^6 cells/ml in 7.5% DMSO (Sigma-Aldrich, Cat#D2650), 50% X-vivo 20^{TM} (Lonza, cat#BE04-448Q), 42.5% Profreeze^{TM} CDM NAO media (Lonza, cat# 12-769E) and at a cooling rate of 1 Celsius/min(CoolCell LX, Biocision, Cat#BCS-405) at -70°C overnight before long term storage in -150°C freezer*
	Thawing	*Cells were thawed rapidly at 37°C in a water bath, 1ml of 37°C X-vivo 20^{TM} was added dropwise to each vial prior to addition to 9ml X-vivo 20^{TM} and washed (300g) prior to culturing*
	Culturing	*The cells were cultured at 37°C with 5% CO2 at 3 x10 cell/ml in a Megablock® 96 well plate (Starstedt, Cat#82.1972.002). The cells were rested for 2 hours prior to stimulation.*
	Stimulation	*50µl of X-vivo 20^{TM} (at 240 minute time point) or X-vivo20^{TM} with TLR7 (CL097; Invigogen, cat#tlrl-c97-5) and -9 ligands (CpG type B ODN 2006 and type C ODN 2395; Invivogen, cat#tlrl-2006-1 and cat#tlrl-2395-1 respectively) at 2 µg/ml each according to a reverse time course for 15, 30, 60, 120, 180, or 240 minutes*
	Fixation	*Following stimulation cells were immediately fixed at room temperature for 10 minutes with 37°C 16% paraformaldehyde (Electron Microscopy Sciences, cat#15710) for a final concentration of 1.5%.*
	Permeabilization	*After fixation PBMCs were washed at 1000g for 5 min 4°C with PBS, and resuspended in 50µl. 1ml ice cold methanol (Sigma Aldrich, cat#32213-2.5L-M) was added dropwise. Cells were then incubated on ice for 30min, and then kept overnight at -80°C.*
	Barcoding	*Prior to barcoding cells were washed 1x with PBS. Cells were then stained for 30 minutes at 4°C with 3 levels of pacific orange and pacific blue succinimidyl ester dyes (PB 100, 25 and 6.3 ng/ml; PO 250, 70 and 0 ng/ml; Life Technologies, cat#P30253 and #10163 respectively) diluted in PBS.*
	Antibody staining	*Barcoded cells were then washed 1x in PBS containing 1% BSA (Sigma-Aldrich, cat#A7906-500g) before being combined into a single sample. The sample was washed (PBS with 1% BSA) and incubated with 2µl Fc receptor block (Miltenyi Biotec, cat#130-059-901) per 1x10^6 cells for 10 minutes on ice. The sample was then divided into 3 and incubated for 30 minutes at room temperature stained with 3 different antibody panels.*
	Antibody panels and staining concentrations	*Phospho-specific monoclonal antibodies- Alexa Fluor® 647 conjugated anti-STAT4 (pY693, clone 38/p-STAT4, panel 1, cat#558137, dilution- 1:10), anti-STAT1 (pS727, clone K51-856, panel 2, cat#560190, dilution- 1:10) and anti-STAT3 (pS727, clone 49/p-STAT3, panel 3, cat#558099, dilution- 1:20); PerCP-Cy^{TM}5.5 conjugated anti-ERK1/2 (pT202/pY204, clone 20A, panel 1, cat#560115, dilution- 1:10), anti-STAT1 (pY701, clone 4a, panel 2, cat#560113, dilution- 1:10) and anti-STAT3 (pY705, clone 4/P-STAT3, panel 3, cat#560114, dilution-1:6.5); and PE-Cy^{TM}7 conjugated anti-NF-κB p65 (pS529, clone K10-895.12.50, panel 1, cat#560335, dilution- 1:20), anti-p38 MAPK (pT180/pY182, clone 36/p38, panel 2, cat#560241, dilution- 1:10) and anti-STAT5 (pY694, clone 47 / STAT5(pY694), panel 3, cat#560117, dilution- 1:10) (all from BD Biosciences). Cell surface markers BV786 conjugated anti-CD3 (clone SK7, BD Horizon™, cat#563799, dilution- 1:100), Alexa Fluor® 488 conjugated anti-CD20 (clone H1 (FB1), BD Biosciences, cat#558056, dilution- 1:20) and PE conjugated anti-CD56 (clone N901, Beckmann Coulter, CA, USA, cat#A07788, dilution- 1:50)*
	Acquisition	*Following staining samples were washed 2x in PBS with 1% BSA and resuspended in PBS containing 1% BSA and 2mM EDTA (Sigma-Aldrich, cat#E7889-100ml) and then immediately analyzed on the flow cytometer.*

Controls	Flow cytometer calibration	*Flow cytometer calibrated daily with BD cytometer setup and tracking beads (BD Biosciences, cat#655051). Standardized consistent fluorescence intensity target values across experiments were obtained with "Application Setting" on the BD FACSDiva software*
	Single donor control	*Cryopreserved PBMCs from a single donor were included in each assay (n = 13) as a positive control, for inter-assay normalization and assessing assay to assay variability.*
	Biological control	*For each donor an unstimulated PBMC sample was analyzed to assess donor specific changes in the phosphorylation of phospho-epitopes follow IFNα stimulation*
	Compensation	*Single stained compensation controls were analyzed for each assay to assess and control for spectral spillover. Unstained / single stained beads (BD Biosciences, cat#552843) were used for compensation for antibody-fluorochrome conjugates; for amine reactive dyes (Pacific blue and Pacific Orange), unstained and single stained cells were used.*
Instrument	Instrument type	*BD LSRFortessa™ (BD Biosciences)*
	Software	*BD FACSDiva software version 8.0.1 (BD Biosciences)*
	Measurement parameters	*See flow data repository of the International Society for Advancement of Cytometry*
Data Analysis	Compensation	*Automatic compensation was conducted through FlowJo (Tree Star) prior to importing compensated datafiles into cytobank*
	Gating	*Gating and analysis was performed in cytobank, gating strategy was assessed through backgating of identified populations*

Table S2. Comparison of phosphorylation of ERK, Nf-κB, p38, STAT1 Y701, STAT1 S727, STAT3 Y705, STAT3 S727, STAT4 Y693, STAT5 Y694 (median MdFI and 95% confidence interval of median MdFI) in T cells from pSS patients (n=25*) and healthy donors (n=25**), by time following TLR7 and -9 stimulation of PBMC cultures. * 2 patients not included at 60 and 120 minutes, ** 1 healthy donor not included at 180 and 240 minutes.

Phospho epitope	Time (minutes)	pSS patients		Healthy donors		Comparison
		Median	95% CI*	Median	95% CI*	P**
NF-κB	0	148.7	147.3 to 153.5	145.7	141.5 to 148.7	**0.0128**
	15	4.7	3.3 to 6.4	4.0	2.5 to 5.9	0.5601
	30	-4.0	-5.1 to -2.8	-1.6	-2.6 to 0.8	**0.0032**
	60	-1.7	-2.5 to -0.3	-0.8	-3.3 to 2.5	0.5904
	120	0.2	-2.5 to 4.6	3.8	0.8 to 5.4	0.1735
	180	8.0	5.1 to 10.3	11.8	8.7 to 14.4	0.0503
	240	9.4	5.8 to 11.0	15.3	10.7 to 17.3	**0.0040**
P38	0	60.1	57.8 to 61.0	56.8	54.9 to 57.8	**0.0016**
	15	-4.0	-4.7 to -2.6	-3.8	-4.3 to -1.9	0.3233
	30	-4.0	-5.6 to -2.4	-3.7	-4.4 to -2.4	0.4048
	60	-2.5	-3.8 to -0.5	-1.7	-4.1 to -0.6	0.9134
	120	7.8	5.3 to 8.9	9.7	7.4 to 11.4	0.0984
	180	9.4	7.3 to 12.0	9.5	7.8 to 12.3	0.8149
	240	7.0	4.6 to 10.4	7.7	4.3 to 11.3	0.5955
ERK1/2	0	76.1	74.4 to 79.1	73.4	70.1 to 75.2	**0.0199**
	15	0.0	-1.2 to 1.1	0.7	-0.2 to 1.8	0.1748
	30	-0.3	-1.7 to 0.8	0.2	-0.8 to 1.6	0.2868
	60	-1.8	-2.6 to -1.2	-1.7	-2.8 to -0.2	0.8963
	120	0.8	-0.8 to 2.0	1.0	-0.4 to 1.9	0.6776
	180	1.2	0.1 to 2.2	1.3	-0.6 to 2.4	0.6228
	240	4.1	2.3 to 5.6	2.5	1.1 to 5.2	0.2945
STAT4 Y693	0	14.0	13.4 to 14.3	13.1	12.4 to 13.7	0.0539
	15	0.3	0.1 to 0.7	0.4	0.0 to 0.6	0.7391
	30	0.2	0.0 to 0.6	0.4	0.1 to 0.5	0.4157
	60	2.1	1.5 to 3.0	3.1	1.9 to 5.2	0.1429
	120	54.9	40.4 to 64.0	57.6	54.4 to 63.9	0.3536
	180	59.1	51.3 to 66.0	59.3	52.2 to 66.7	0.8302
	240	46.8	42.9 to 53.5	49.6	43.3 to 55.3	0.4538
STAT5 Y694	0	107.5	103.6 to 110.2	98.5	96.2 to 101.0	**<0.0001**
	15	-23.7	-25.1 to -21.3	-20.3	-21.9 to -19.7	**0.0042**
	30	-22.9	-26.0 to -20.4	-19.8	-22.3 to -18.6	0.0910
	60	-17.3	-19.8 to -13.0	-13.8	-17.8 to -9.8	0.2162
	120	10.2	3.6 to 16.8	24.3	16.9 to 28.4	**0.0147**
	180	-0.3	-2.0 to 2.0	1.9	-1.3 to 7.0	0.1212
	240	-5.5	-8.4 to -2.0	-3.1	-4.7 to 0.0	0.0946
STAT1 Y701	0	118.1	115.2 to 121.6	110.4	104.7 to 113.5	**<0.0001**

Phospho epitope	Time (minutes)	Median	95% CI*	Median	95% CI*	P**
	15	-7.8	-9.1 to -6.3	-5.4	-6.6 to -4.0	**0.0040**
	30	-8.5	-10.1 to -6.4	-6.0	-8.2 to -3.8	**0.0070**
	60	8.8	1.4 to 11.9	15.7	5.6 to 24.4	0.0541
	120	143.0	127.8 to 169.7	135.9	114.0 to 154.6	0.5632
	180	117.9	105.2 to 131.4	109.8	99.2 to 130.8	0.4778
	240	92.5	83.1 to 101.6	97.9	86.6 to 109.3	0.3331
STAT1 S727	0	31.4	30.5 to 32.0	28.1	27.9 to 29.5	**<0.0001**
	15	-1.3	-1.5 to -0.8	-0.7	-1.3 to -0.5	0.0988
	30	-1.5	-2.1 to -1.2	-0.7	-1.1 to -0.3	**0.0189**
	60	-1.3	-2.0 to -0.4	-0.6	-1.4 to 0.0	0.1266
	120	4.8	3.6 to 7.3	5.9	4.5 to 7.9	0.4827
	180	7.5	6.2 to 8.9	8.5	7.3 to 10.7	0.2349
	240	5.9	4.8 to 6.8	7.6	5.7 to 9.1	**0.0414**
STAT3 Y705	0	118.2	112.6 to 128.3	129.7	114.2 to 133.3	0.4269
	15	-4.7	-8.9 to -3.4	-4.1	-7.3 to -2.8	0.5345
	30	-7.1	-10.3 to -5.3	-7.7	-10.8 to -4.8	0.9029
	60	15.8	8.1 to 22.2	17.0	13.3 to 25.4	0.1215
	120	100.4	81.7 to 141.0	124.8	111.0 to 143.3	0.0984
	180	81.0	64.5 to 103.6	98.7	82.1 to 111.7	0.1073
	240	61.6	39.1 to 69.2	68.6	57.5 to 77.0	0.2594
STAT3 S727	0	496.5	470.5 to 509.5	476.5	467.0 to 494.2	0.2868
	15	-6.6	-11.1 to -4.2	-2.7	-10.6 to 1.4	0.4731
	30	-13.9	-18.3 to -12.1	-12.8	-16.3 to -6.0	0.3233
	60	7.8	-0.6 to 27.1	14.5	2.6 to 28.3	0.6045
	120	162.4	132.9 to 259.4	236.2	205.5 to 267.4	0.0753
	180	187.8	139.3 to 213	202.3	172.7 to 246.1	0.0553
	240	125.3	82.7 to 138.4	140.1	120.6 to 152.1	0.0608

* 95% CI: 95% confidence interval of median, ** Unpaired Mann-Whitney test.

Table S3. Comparison of phosphorylation of ERK, Nf-κB, p38, STAT1 Y701, STAT1 S727, STAT3 Y705, STAT3 S727, STAT4 Y693, STAT5 Y694 (median MdFI and 95% confidence interval of median MdFI) in B cells from pSS patients (n=25*) and healthy donors (n=25**), by time following TLR7 and -9 stimulation of PBMC cultures. * 2 patients not included at 60 and 120 minutes, ** 1 healthy donor not included at 180 and 240 minutes.

Phospho epitope	Time (minutes)	pSS patients		Healthy donors		Comparison
		Median	95% CI*	Median	95% CI*	P**
NF-κB	0	112.7	109.2 to 117.4	115.9	107.1 to 119.0	0.5219
	15	66.7	46.9 to 98.2	55.1	46.5 to 63.9	0.1253
	30	86.2	70.3 to 129.5	77.2	69.3 to 93.6	0.3329
	60	110.7	90.9 to 135.0	103.7	93.7 to 127.5	0.8651
	120	98.7	88.5 to 107.9	97.6	92.1 to 111.1	0.9448
	180	96.2	80.9 to 102.6	98.3	90.5 to 106.6	0.3040
	240	86.1	78.3 to 93.3	94.6	82.4 to 101.3	0.0946
P38	0	56.7	55.3 to 58.3	60.5	55.9 to 63.4	0.0910
	15	7.7	4.6 to 11.6	4.3	3.0 to 7.3	0.0873
	30	11.5	7.2 to 14.2	6.6	5.8 to 10.2	0.1159
	60	18.1	11.6 to 20.7	12.0	9.2 to 16.8	0.1873
	120	15.1	14.0 to 20.6	13.8	10.3 to 19.1	0.2843
	180	13.0	12.0 to 14.9	11.0	8.8 to 15.6	0.4778
	240	8.6	6.7 to 13.2	9.1	5.7 to 12.6	0.8144
ERK1/2	0	71.2	68.0 to 75.1	73.0	70.5 to 75.1	0.4496
	15	6.5	4.6 to 9.1	7.5	6.5 to 10.1	0.5472
	30	8.5	6.1 to 11.6	9.0	5.7 to 12.9	0.5731
	60	9.2	6.3 to 11.6	9.2	6.1 to 11.9	0.9611
	120	13.2	9.7 to 16.0	13.6	10.6 to 16.2	0.7692
	180	12.0	10.2 to 15.2	11.8	8.8 to 13.4	0.6654
	240	14.3	10.6 to 19.7	13.0	9.6 to 15.5	0.2427
STAT4 Y693	0	8.6	8.2 to 9.1	9.6	8.3 to 10.8	0.1405
	15	-0.1	-0.7 to 0.4	-0.1	-0.8 to 0.5	0.8726
	30	-0.3	-0.6 to 0.6	0.0	-0.4 to 0.2	0.9791
	60	-0.4	-1.3 to 0.3	-0.2	-0.8 to 0.2	0.8164
	120	0.1	-0.2 to 0.8	0.0	-1.0 to 1.1	0.8173
	180	0.3	-0.6 to 1.7	0.0	-0.7 to 1.1	0.3531
	240	0.6	-0.1 to 0.8	0.2	-0.3 to 0.8	0.7687
STAT5 Y694	0	83.8	82.3 to 87.7	90.7	85.6 to 94.1	0.0539

	15	-19.9	-22.5 to -18.4	-21.0	-24.0 to -18.6	0.8126
	30	-19.6	-24.4 to -17.8	-22.0	-25.2 to -20.0	0.3329
	60	-15.8	-19.5 to -13.3	-18.9	-20.9 to -16.8	0.2089
	120	-6.7	-10.7 to 0.1	-6.0	-8.7 to 2.8	0.4575
	180	4.3	-1.7 to 9.8	10.1	-1.4 to 16.5	0.0729
	240	6.4	2.4 to 9.5	9.3	3.6 to 15.7	0.0729
STAT1 Y701	0	96.5	91.6 to 104.3	97.7	89.0 to 101.6	0.8877
	15	-4.6	-8.1 to -2.6	-4.1	-5.8 to -0.8	0.6402
	30	-8.4	-10.7 to -4.9	-4.9	-8.7 to -2.2	0.1353
	60	-1.8	-6.5 to 2.2	5.9	-5.6 to 9.8	0.1319
	120	63.7	54.3 to 82.7	58.2	52.9 to 86.8	0.6635
	180	150.6	129.0 to 169.3	149.3	114.2 to 171.9	0.5815
	240	148.0	135.4 to 172.0	151.9	132.1 to 162.7	0.8144
STAT1 S727	0	29.0	28.0 to 30.2	27.6	25.8 to 29.0	0.0646
	15	0.9	0.7 to 2.9	1.1	-0.1 to 1.9	0.2150
	30	5.7	4.1 to 7.4	3.2	1.6 to 3.7	**0.0001**
	60	12.9	8.3 to 17.7	7.2	5.4 to 8.5	**0.0021**
	120	25.2	23.0 to 30.4	18.3	15.0 to 21.9	**0.0003**
	180	45.9	37.9 to 49.6	40.8	34.5 to 45.9	0.2193
	240	39.4	31.3 to 47.2	37.9	30.9 to 46.2	0.8919
STAT3 Y705	0	81.24	80.2 to 84.7	83.92	79.7 to 91.0	0.2150
	15	1.6	-0.3 to 4.0	1.6	-2.3 to 2.7	0.3233
	30	0.3	-1.9 to 1.9	0.2	-5.8 to 1.0	0.5094
	60	1.6	-2.0 to 4.0	-0.4	-4.3 to 2.1	0.2015
	120	21.0	15.1 to 25.1	24.1	17.5 to 31.4	0.4575
	180	29.7	22.0 to 32.6	26.8	18.1 to 35.8	0.7687
	240	28.6	24.6 to 32.3	28.2	24.4 to 33.8	0.8298
STAT3 S727	0	271.3	267.0 to 292.6	295.1	270.2 to 330.4	0.0873
	15	41.2	22.3 to 49.4	20.0	7.7 to 26.1	**0.0035**
	30	83.9	63.3 to 109.9	59.4	43.5 to 71.8	**0.0080**
	60	138.8	112.0 to 155.0	96.5	77.3 to 127.0	**0.0069**
	120	178.4	155.2 to 196.9	145.2	121.4 to 156.7	**0.0074**
	180	184.0	164.9 to 208.3	168.4	145.0 to 194.5	0.2427
	240	152.8	126.5 to 174.6	156.5	132.7 to 169.3	0.7238

* 95% CI: 95% confidence interval of median, ** Unpaired Mann-Whitney test.

Table S4. Comparison of phosphorylation of ERK, Nf-κB, p38, STAT1 Y701, STAT1 S727, STAT3 Y705, STAT3 S727, STAT4 Y693, STAT5 Y694 (median MdFI and 95% confidence interval of median MdFI) in NK cells from pSS patients (n=25*) and healthy donors (n=25**), by time following TLR7 and -9 stimulation of PBMC cultures . * 2 patients not included at 60 and 120 minutes, ** 1 healthy donor not included at 180 and 240 minutes.

Phospho epitope	Time (minutes)	pSS patients		Healthy donors		Comparison
		Median	95% CI*	Median	95% CI*	p**
NF-κB	0	121.4	115.0 to 125.8	120.3	114.8 to 122.2	0.6130
	15	6.7	5.5 to 8.2	6.6	4.8 to 7.4	0.7247
	30	3.4	-3.4 to 6.9	4.2	2.9 to 6.4	0.2010
	60	6.3	2.0 to 10.1	11.9	9.4 to 13.1	**0.0261**
	120	19.2	14.0 to 35.6	44.6	33.6 to 49.0	**0.0003**
	180	12.1	7.0 to 19.9	26.0	15.7 to 31.7	**0.0056**
	240	-15.5	-36.4 to -3.0	6.4	-8.8 to 16.2	**0.0168**
P38	0	66.5	64.5 to 71.5	64.6	61.6 to 65.8	**0.0318**
	15	-1.0	-3.1 to 0.6	-2.4	-2.9 to -1.0	0.2452
	30	-1.1	-2.7 to 1.2	-1.2	-2.6 to -0.1	0.5219
	60	-0.9	-2.3 to 1.1	0.3	-1.3 to 2.9	0.5625
	120	13.6	11.6 to 15.9	28.2	17.8 to 32.0	**0.0003**
	180	15.6	11.6 to 19.5	19.8	15.8 to 24.3	0.0528
	240	7.9	5.8 to 10.6	11.6	8.2 to 15.7	0.0580
ERK1/2	0	73.1	69.4 to 75.7	71.7	67.3 to 74.0	0.2223
	15	2.3	-0.3 to 3.5	3.5	1.0 to 4.8	0.1570
	30	1.5	0.4 to 2.9	3.3	0.4 to 4.4	0.2531
	60	2.3	1.5 to 5.2	1.7	0.2 to 2.8	0.2015
	120	5.6	3.8 to 8.9	6.6	3.7 to 8.2	0.7085
	180	5.0	2.3 to 7.9	7.0	3.5 to 11.1	0.0946
	240	11.7	8.2 to 17.2	13.5	8.2 to 18.8	0.8767
STAT4 Y693	0	17.6	16.3 to 18.2	16.3	15.9 to 18.1	0.5862

	15	1.4	1.0 to 2.0	1.5	1.1 to 2.0	0.8726
	30	2.0	0.8 to 2.6	0.9	0.6 to 2.0	0.3940
	60	3.8	2.1 to 4.6	7.7	5.2 to 10.4	**0.0021**
	120	62.9	39.2 to 74.2	99.2	76.6 to 103.3	**0.0009**
	180	54.0	40.3 to 68.9	73.0	62.7 to 85.8	**0.0111**
	240	33.2	16.7 to 44.9	43.8	32.8 to 53.6	**0.0234**
STAT5 Y694	0	118.7	111.7 to 122.6	109.1	106.2 to 114.7	**0.0026**
	15	-22.7	-24.4 to -19.9	-20.1	-21.2 to -18.7	0.0565
	30	-22.5	-23.6 to -19.5	-18.7	-21.0 to -17.9	0.0737
	60	-19.6	-21.7 to -16.3	-17.4	-22.0 to -14.8	0.5904
	120	-14.2	-20.4 to -11.4	-14.1	-16.6 to -10.7	0.3126
	180	-11.2	-16.2 to -9.3	-13.4	-17.2 to -10.0	0.3230
	240	-10.7	-17.5 to -7.6	-11.1	-14.4 to -7.0	0.9075
STAT1 Y701	0	90.0	85.5 to 94.4	83.7	82.5 to 87.0	**0.0169**
	15	-3.2	-4.2 to 0.4	-1.0	-2.5 to 1.7	0.1458
	30	-2.7	-4.6 to -1.3	-0.3	-2.3 to 1.2	**0.0318**
	60	10.6	4.8 to 12.2	18.3	8.9 to 25.5	0.0753
	120	90.4	79.9 to 98.5	72.8	67.5 to 79.3	**0.0361**
	180	63.9	56.6 to 74.2	59.0	52.0 to 64.7	0.2592
	240	41.9	30.6 to 54.1	44.9	38.5 to 54.4	0.3744
STAT1 S727	0	30.3	29.6 to 31.6	28.5	27.1 to 29.5	**0.0070**
	15	-0.3	-1.3 to 0.6	-0.4	-0.8 to 0.7	0.3329
	30	-1.0	-1.9 to 0.0	-0.5	-1.5 to 0.1	0.1353
	60	-1.9	-2.4 to -1.3	-0.9	-1.7 to -0.2	**0.0380**
	120	4.5	2.8 to 5.8	4.4	2.7 to 5.5	0.9945
	180	4.4	2.3 to 6.0	4.2	3.3 to 5.6	0.6948
	240	1.3	-2.5 to 2.8	2.5	-0.8 to 3.4	0.1776
STAT3 Y705	0	97.8	92.6 to 100.3	94.9	91.6 to 97.8	0.1811
	15	-0.1	-3.6 to 2.3	0.4	-1.1 to 4.1	0.2781
	30	1.3	-1.5 to 5.5	0.9	-0.9 to 3.3	0.2868
	60	0.0	-2.6 to 2.6	1.0	-2.2 to 4.1	0.3754
	120	26.8	19.4 to 33.1	27.0	21.4 to 32.0	0.8651
	180	12.7	8.6 to 19.5	14.9	8.0 to 16.0	0.8302
	240	9.1	4.8 to 15.8	10.3	5.2 to 13.2	0.8452
STAT3 S727	0	257.4	241.3 to 298.4	275.3	260.1 to 295.1	0.1458
	15	-8.4	-13.7 to 0.2	-12.6	-15.0 to -7.9	0.2374
	30	-10.1	-13.7 to -2.4	-8.7	-11.2 to -6.8	0.8425
	60	-0.9	-6.4 to 1.0	-2.7	-6.9 to 5.9	0.8642
	120	60.8	39.7 to 75.8	90.4	66.4 to 108.5	**0.0026**
	180	40.6	22.8 to 57.7	57.3	38.8 to 91.2	**0.0275**
	240	-30.6	-129.2 to 7.4	14.2	-13.5 to 44.7	0.0580

* 95% CI: 95% confidence interval of median, ** Unpaired Mann-Whitney test.

Table S5. Comparison of plasma cytokine levels (median pg/ml and 95% confidence interval of median) showing significant differences between pSS patients (n=25) (except IL-15, n= 21; and IL-4, n= 22) and healthy donors (n=25) (except IL-6, n=24; TNF-α, n= 23; and IL-4, n= 16).

Cytokine	pSS patients		Healthy donors		Comparison
	Median	95% CI*	Median	95% CI*	p**
IL-1β	18.96	18.96 to 85.66	18.96	18.96 to 18.96	**0.0004**
IL-13	16	16 to 58.78	16	16 to 16	0.0207
IL-6	0.3254	0.3254 to 10.18	0.3254	0.3254 to 0.3254	**0.0043**
IL-12	200	200 to 288.2	140.4	110 to 140.4	<0.0001
MIP-1α	84.45	84.45 to 183.3	56.13	56.13 to 56.13	<0.0001
MIP-1β	66.05	46.95 to 156.6	46.95	46.95 to 46.95	0.0008
MCP-1	293.5	256.6 to 401.6	219.2	181.1 to 256.6	0.0029
IL-15	113.7	21.51 to 194.5	21.51	21.51 to 21.51	0.0001
IFN-α	139	88.51 to 139	88.51	88.51 to 88.51	<0.0001
TNF-α	7.758	7.758 to 45.95	7.758	7.758 to 7.758	0.0027
IL-2	6.368	6.368 to 20.93	6.368	2 to 6.368	<0.0001
IL-4	36.8	36.8 to 270.2	36.8	36.8 to 36.8	0.0049

* 95% CI: 95% confidence interval of median, ** Unpaired Mann-Whitney test.

Table S6. Comparison of plasma cytokine levels (median pg/ml and 95% confidence interval of median) showing significant differences between SSA+ pSS patients (n=12) and SSA- pSS patients (n=13), (except for IL-4, n=11 in each category).

Cytokine	SSA+ patients		SSA- patients		Comparison
	Median	95% CI*	Median	95% CI*	P**
IL-1β	108.1	18.96 to 182.6	18.96	18.96 to 34.35	**0.0199**
MCP-1	401	293.5 to 1337	256.6	219.2 to 293.5	0.0292
IFN-α	160	88.51 to 423	88.51	88.51 to 139	**0.0214**
IL-2	20.93	6.368 to 49.45	6.368	6.368 to 6.368	0.0049
IL-4	228.9	36.8 to 889	36.8	36.8 to 94.19	0.0339

* 95% CI: 95% confidence interval of median, ** Unpaired Mann-Whitney test.

Errata for
Towards stratification of patients with Sjögren's syndrome

Single cell analyses and immune profiling

Irene Sarkar

Thesis for the degree philosophiae doctor (PhD)

at the University of Bergen

Irene Sarkar 12/10/2020   12/10 2020 _[signature]_

(date and sign. of candidate)   (date and sign. of faculty)

Errata

Page 8 line 11: Change of word: "Patient heterogeneity is a major obstacle in disease management" – corrected to "Patient heterogeneity is a major obstacle to disease management"

Page 9 line 3 Misspelling: "abberations" – corrected to "aberrations"

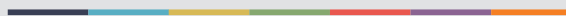Page 9 line 9 Misspelling: "immne" – corrected to "immune"

Page 41 To add to sentence: "Systemic disease can be controlled with non-steroidal anti-inflammatory drugs (NSAIDs) like hydroxychloroquine (HCQ) and corticosteroids like prednisone" – corrected to "Systemic disease can be controlled with non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) like hydroxychloroquine (HCQ) and corticosteroids like prednisone."

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