Flow cytometry based analyses as a tool in biomarker discovery for patient stratification in primary Sjögren's syndrome

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Scientific Environment

The following doctoral work was conducted in the period 2013 to 2017 at the Broegelmann Research Laboratory (http://www.uib.no/en/rg/broegelmann), Department of Clinical Science, University of Bergen, within the framework of the Bergen Research School of Inflammation (http://www.uib.no/en/rs/brsi). The work was carried out under the supervision of Silke Appel, Petra Vogelsang and Roland Jonsson.

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Some wise person once told me that the acknowledgements are the only section of the thesis that will be carefully read. To minimise unwanted effort I have embolden names so you can skip to the "important" bits.

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Abbreviations

AECC	American-European Classification Criteria	**NF-κB**	Nuclear factor kappa-light-chain enhancer of activated B cells
APCs	Antigen presenting cells	**NK**	Natural killer
BCR	B cell receptor	**NKT**	Natural killer T
CD	Cluster of differentiation	**OAS1**	2'-5' oligoadenylate synthetase 1
CV	Coefficient of variation	**PBMC**	Peripheral blood mononuclear cells
DCs	Dendritic cells	**PCA**	Principal component analysis
DNA	Deoxyribonucleic acid	**pDCs**	Plasmacytoid dendritic cells
EGM	Extraglandular manifestation	**PMT**	Photomultiplier tube(s)
ERK	Extracellular signal-regulated kinases	**PRRs**	Pattern recognition receptors
GAS	Gamma-activated sequences	**pSS**	Primary Sjögren's syndrome
GBP1	Guanylate Binding Protein 1	**RA**	Rheumatoid arthritis
HCQ	Hydroxychloroquine	**RIG**	Retinoic acid – inducible gene
IFI44	Interferon-induced protein 44	**RNA**	Ribonucleic acid
IFN	Interferon	**SLE**	Systemic lupus erythematosus
IFNAR	Interferon type I receptor	**SOCS**	Suppressor of cytokine signaling
Ig	Immunoglobulin	**SS**	Sjögren's syndrome
IKK	IkB kinase	**SSA**	Sjögren's syndrome antigen A
IL	Interleukin	**SSB**	Sjögren's syndrome antigen B
IRAK	Interleukin-1 receptor-associated kinase	**STAT**	Signal transducer and activator of transcription
IRF	Interferon regulatory factor	**TCR**	T cell receptor
ISRE	IFN-stimulated response elements	**TGF**	Tumor growth factor
JAK	Janus Kinase	**Th**	T helper
MAPK	Mitogen-activated protein kinase	**TLR**	Toll-like receptor
MFI	Median fluorescence intensity	**TNF**	Tumor necrosis factor
MHC	Major histocompatibility complex	**TRAF**	TNF receptor associated factors
MS	Multiple sclerosis	**Treg**	Regulatory T cells
MxA	Myxoma resistance proteins	**TRIF**	TIR-domain-containing adapter-inducing interferon-β

Summary

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by lymphocytic infiltrates of exocrine glands, notably the salivary and lacrimal glands combined with immune-mediated glandular destruction. The disease is chronic, disabling and there is no cure. Diagnosis of the disease is difficult, with the symptoms of the disease (dryness of the mouth and eyes, and fatigue) frequent in the population as a side effect of many drugs, other co-morbidities or aging. Many cases of pSS are hence misclassified or go unidentified. Like other autoimmune diseases pSS progression and phenotype are heterogeneous with many clinical presentations limited to local manifestations, while others develop extraglandular manifestations as well as life threatening conditions such as B cell lymphomas. New disease markers for pSS that are specific for diagnosis or useful to predict disease development have the potential to radically change how we treat, diagnose and define the disease.

The work contained in this thesis focused on the use of flow cytometry based assays in the search for disease markers for the identification and stratification of pSS patients.

In paper I we assessed a multiplex flow cytometry protocol used for the measurement of MAPK/ERK and JAK/STAT signaling networks in peripheral blood mononuclear cells for inter-assay precision for experimental variables (phospho-protein measured, cell type and stimulant). In addition, three different blood collection tubes were assessed for their effect on basal and induced intracellular signaling in different cell subsets. The method showed a high level of precision with median coefficients of variation under 10 %, while the use of heparin as an anti-coagulant was superior in retaining immune cell responsiveness compared to citrate. Citrate strongly affected NK cell responses to stimuli, while CPT based isolation methods were associated with higher basal phosphorylation.

In papers II and III the flow cytometry protocol presented in paper I was used to compare basal and IFNα or TLR7 and -9 stimulation induced phosphorylation states

in immune cells from pSS patients and healthy individuals. Both basal and induced phosphorylation differed significantly between pSS patients and healthy individuals, while induced phosphorylation also differed between by patient sungroups.

In paper IV, we compared immune cell quantities in peripheral blood of patients with pSS and healthy individuals, and associated changes with clinical manifestations of the disease. Primary Sjögren's syndrome patients displayed decreased absolute counts of diverse subtypes of lymphocytes and increases of monocytes and granulocytes compared to healthy individuals. Greater decreases of lymphocytes were associated with differing patient phenotype.

In conclusion analysis of both intracellular signaling pathways and cell quantification are promising techniques for the identification of biomarkers that could be used in diagnosis and stratification of pSS.

List of publications

The doctoral thesis is based on the following publications.

I. **Davies, R.,** Vogelsang, P., Jonsson, R., Appel, S. An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells. *J Immunol Methods* 436: 58-63 (2016)

II. **Davies, R.,** Hammenfors, D., Bergum, B., Vogelsang, P., Gavasso, S., Brun, J.G., Jonsson, R., Appel, S. Single cell based phosphorylation profiling identifies alterations in Toll-like receptor 7 and -9 signaling in patients with primary Sjögren's syndrome. *In manuscript*

III. **Davies, R.,** Hammenfors, D., Bergum, B., Vogelsang, P., Gavasso, S., Brun, J.G., Jonsson, R., Appel, S. Aberrant cell signaling in peripheral blood mononuclear cells upon interferon alpha stimulation in patients with primary Sjögren's syndrome associates with type I interferon signature. *In manuscript*

IV. **Davies, R.,** Hammenfors, D., Bergum, B., Jakobsen, K., Vogelsang, P., Brun, J.G., Bryceson, Y., Jonsson, R., Appel, S. Patients with primary Sjögren's syndrome have alterations in absolute quantities of various peripheral leukocyte populations. *Submitted*

Content

1. Introduction

1.1. The immune system

The immune system has evolved in order to maintain and protect the integrity of the organism. The system decides which cells, microorganisms and substances must be removed, as well as controlling the balance of others, for example commensal microbial flora [1] thus maintaining homeostasis and the integrity of the organism. The immune system is therefore integral to an organism's health, protecting against threats from infectious agents and abnormal self. This maintenance of homeostasis and biological integrity requires a delicate balance that protects the host from potential infectious agents, while minimizing potential collateral damage brought about by their control. A loss of balance in these processes may promote the emergence of serious infections or conversely immune system driven pathological inflammatory conditions.

Immune system driven pathological conditions can result from malfunctions within the innate arm of the immune system, with associated diseases termed auto-inflammatory [2]. Diseases involving the malfunction of the adaptive immune system are termed auto-immune, Sjögren's syndrome (SS) is included in this group [2]. The diverse range of environmental and biological threats and the high cost of inappropriate immune responses have provided a strong evolutionary driving force. This evolutionary drive has resulted in a highly complex and coordinated web of cellular interactions allowing for a fine tuned control, consisting of many stops, balances and feedbacks. In-turn this complexity allows for diverse points of origin that malfunction can occur in the system as well as propagate. Inconsequence malfunction within the immune system can lead to highly diverse sets of disease pathogenesis and manifestations.

Broadly speaking the vertebrate immune system can be divided into 2 distinct but interconnected functional divisions- the innate and the adaptive immune system. It is

generally accepted that the innate immune system provides a rapid, non-specific response to infection and the adaptive response is slow but highly specific [3].

1.2. Innate immunity

The innate immune response is initiated at the site of infection through pre-formed cells and immune factors, thus responding without delay. The innate immune system is composed of anatomical barriers (skin, mucous membranes etc.) and cellular components including neutrophils, basophils, mast cells, monocytes, macrophages, dendritic cells (DCs), natural killer T (NKT) and innate lymphoid cells including natural killer (NK) cells. Additionally, innate defence mechanisms include a number of soluble factors- complement proteins, natural antibodies, and cytokines that protect the host against various infectious agents [3].

Recognition of threats to the host by the innate immune system is driven through a number of different mechanisms that work in combination to trigger a particular immune response. Recognition of microbial threats is primarily through direct detection by a limited number of germ-line encoded pattern recognition receptors (PRRs) that identify evolutionary conserved invariant features of microbes, termed pathogen associated molecular patterns [4] or perhaps more accurately microbe associated molecular patterns.

Pattern recognition receptors divide into two groups: secreted PRRs and cell-associated PRRs (transmembrane and intracellular signal-transducing receptors) [5]. Secreted PRRs include antimicrobial peptides, collectins, lectins and pentraxins, and have a number of functions including direct microbial killing and enhancing phagocytosis [5]. Cell-associated PRRs are expressed constitutively on many types of innate immune cells and include a number of plasma bound and intracellular receptors, for example Toll-like receptors (TLR), C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors and retinoic acid- inducible gene (RIG)-like receptors [6].

Toll-like receptors can detect molecules termed damage associated molecular patterns [7, 8]. These molecules are normally hidden from PRRs through compartmentalization or sequestration and are released during cell lysis and tissue damage [8]. Phagocytes including monocytes, macrophages, neutrophils and DCs use this system to identify, engulf and destroy microbes, dead cells and tissue debris. After encountering pathogens, phagocytes produce and secrete proinflammatory cytokines that can induce DC maturation allowing them to prime immune responses, with maturation stimulus influencing the type of immune response [9] (see section 1.4.1.). Additionally, cells of the innate immune system can detect non-self through the monitoring of molecules normally expressed by healthy cells. This strategy is utilized by NK cells where identification of the major histocompatibility complex (MHC) class I prevents activation of an immune response, while cells with no or low expression of MHC will be killed [3, 10].

1.3. Adaptive immunity

The main cellular components of the adaptive immune system are B and T lymphocytes. B cells express a membrane bound immunoglobulin (Ig) known as the B cell receptor (BCR) which can bind soluble antigen in its native form. Following activation B cells differentiate into memory and effector B cells known as plasma cells [11]. Plasma cells are capable of secreting antigen specific Ig known as antibodies which play a diverse range of functions for example blocking infectivity (blocking attachment, entry into host cells, inhibiting pathogen lifecycle), killing pathogens, activation of complement, antibody-dependent cellular cytotoxicity and increasing phagocytosis [12].

T cells express a T cell receptor (TCR) which recognises antigen presented on MHC. T cells can be divided into two subsets based on expression of the co-receptors cluster of differentiation (CD) 4 and CD8. Activation of naïve T cells causes proliferation and differentiation into memory T cells and effector T cells, with CD8+ naïve T cells differentiating into cytotoxic T cells, and CD4+ naïve T cells into helper T (Th) cells or regulatory T cells (Treg) [11]. These subsets of T cells play different roles in the

immune response with CD4+ Th cells helping direct the immune response through the secretion of cytokines (see section 1.4.3.), while CD8+ cytotoxic T cells kill cancerous and virus infected cells.

Unlike receptors of the innate system, BCR and TCR are not encoded in the germ line but are generated de novo in each organism through the recombination of genes. This allows for the generation of an almost unlimited spectrum of antigen specific receptors [11, 13, 14]. The high diversity of possible receptors allows for a more specific and stronger response and is essential in adapting for rapidly mutating threats [11]. Because of the high diversity of possible receptor specificity, prior to infection low levels of immune cells displaying each receptor for its cognate antigen (peptide or protein capable of inducing an immune response) are present. A strong immune response therefore requires not only lymphoid activation and maturation but significant clonal expansion [11, 15]. This creates a time lag during which period the body relies on the non-specific innate immune response for protection. Secondary encounters of the antigen are however rapid with the adaptive immune response producing a specific immunological memory of the infection [11, 15]. This rapid secondary response depends on the generation of memory B and T cells. These cells develop from naïve progenitor cells throughout the course of infection and remain circulating in the blood and lymph long after the infection's clearance [11].

1.4. Coordination of the immune response

1.4.1. Innate control of the adaptive response

The coordination of an immune response is finely tuned and takes place at many levels. Induction of adaptive immunity is dependent not only on direct antigen recognition by the antigen receptors but also signals delivered by the innate immune system. This system was suggested over two decades ago by Charles A. Janeway Jr, when he postulated that the recognition of pathogen associated molecular patterns by the innate system delivers essential signals to the adaptive immune system [4]. The evolution of this system allows for an extra layer of self and non-self discrimination

and distinction between harmful and benign microbes [4]. Innate control of the adaptive arm of the immune response has also been recognized as being a critical step in determining the nature of the adaptive response, with the nature of the pathogen determining the PRRs it activates, which in turn dictates the immune response elicited against it [5].

Antigen presenting cells (APCs), in particular DCs, continually monitor lymphoid and non-lymphoid sites. The detection of viral or microbial cell components through PRRs, induces endocytosis or phagocytosis of the microbe. This leads to activation of the immature DCs and loss of endocytic capacity and induction of migratory properties. The activated DCs then migrate to draining lymph nodes and mature allowing them to present antigen to T cells [16].

Innate instruction of the adaptive response occurs initially at this point through the interaction between APCs and T cells [17]. Antigen recognition by TCR requires antigen presentation by the APCs in context of a MHC molecule. MHC molecules consist of four classes − MHC class I and II which encode polymorphic cell surface antigens, as well as class III and IV [18]. MHC class I molecules are present on most nucleated cells and are responsible for the presentation of intracellular antigen, or extracellular antigen through cross presentation by DCs [19]. MHC class II in contrast is limited to professional APCs − DCs, macrophages and B cells in particular, and presents extracellular antigen following endocytosis and digestion in lysosomes [20]. MHC class III and IV molecules include a number of proteins not involved in antigen presentation including complement proteins, cytokines, and heat-shock proteins [18, 21].

MHC class I bound antigen is recognised by CD8+ cytotoxic T cells, while CD4+ Th cells recognise antigen presented by MHC class II molecules. Recognition by the TCR of the MHC − cognate antigen complex is required for activation of naïve T cells in addition to two other signals, expression of co-stimulatory molecules B7 (CD80/CD86) on APCs and recognition by CD28 on the T cell, and stimulation of the T cell with cytokines secreted by the APCs [22]. Activated CD4+ Th cells can in

turn provide a second signal to naïve B cells bound to its cognate antigen that require an additional signal for activation in what is termed T cell-dependent activation. This produces higher affinity and functionally more versatile antibodies then T cell-independent B cell activation where the additional signal is provided through TLRs or crosslinking of BCR [11].

1.4.2. Intercellular communication in the immune response

An essential component of the immune response is the coordination of activities between cellular players. To accomplish these coordinated responses, cells send, receive and integrate a multitude of signals in extensive and highly complex networks, informing other cells of changes in their environment. These signals arise from direct cell to cell interactions or through the detection of soluble cell signaling molecules. Mechanisms of action of signaling molecules can be categorised as autocrine (acts on the cytokine secreting cell itself), paracrine (acts on cells in close proximity), juxtacrine (requiring cell to cell contact) and endocrine (acts on cells in distant regions of the body) (Figure 1). Additionally, the molecule may display a number of functional properties including pleiotropism, redundancy, additive, synergism and antagonism [23].

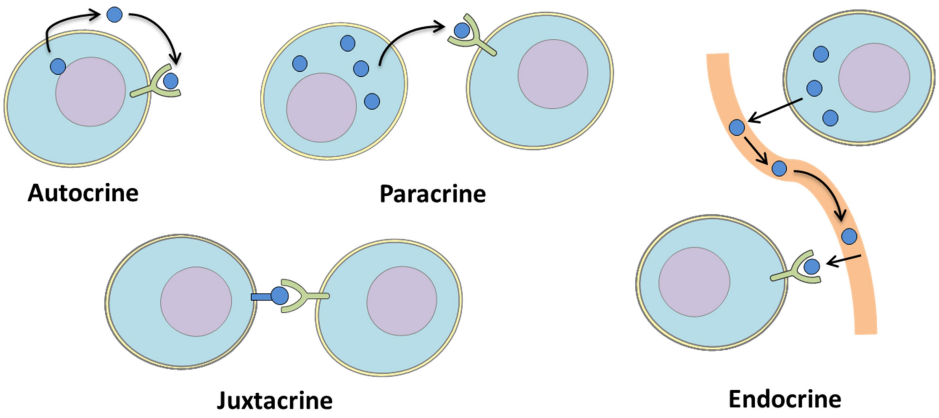

Figure 1. *Mechanisms of action of intercellular signaling molecules. Figure was produced using Servier Medical Art.*

The immune system incorporates a number of specialized molecules to communicate between cells. However, the principle cell signaling molecule of the immune system is the cytokine. Cytokines are a diverse group of molecules whose primary function is the regulation and coordination of immune responses. For example, they help B cells to produce antibodies, undergo class switching and affinity maturation; as well as recruiting, activating, and maintaining CD8 T cells, macrophages, neutrophils as well as other effector cells [24]. During the initial immune response the combination of cytokines produced by cells involved in the innate immune response creates a cytokine profile. The cytokine profile along with other signals including the type and amount of antigen and co-stimulatory molecules directs the differentiation of different T-cell subsets, in particular the Th cells [25]. The differentiation of these cells determines their cytokine secretion profile and plays a crucial role in determining the ultimate direction of the adaptive immune response.

1.4.3. The helper T cell paradigm

Th cells are divided into three major subsets- Th1, Th2 and Th17, based on their effector functions and cytokines secreted (Figure 2). The major role of Th 1 cells is the defence of the host from intracellular pathogens through promoting macrophages and cytotoxic T cell immune responses. Th1 cell differentiation from naive T cell is driven by interferon (IFN) γ and interleukin (IL)-12 and the transcription factors T-bet and signal transducers and activator of transcription (STAT) 4. Th1 cells in turn support further Th1 differentiation through producing IFNγ and IL-12 while suppressing the Th2 driven response through inhibitory action of IFNγ [24].

Th2 cells drive immune responses requiring humoral components to eliminate the pathogen, in particular through IgE production, eosinophil recruitment and clearance of extracellular parasites. IL-4 along with IL-2, IL-7, TSLP (thymic stromal lymphopoietin) with the transcription factors GATA3 and STAT5 drive Th2 differentiation. Th2 cells produce IL-4 in a positive feedback loop with IL-4 also suppressing Th1 driven responses. Th17 cells play crucial roles during immune

responses against extracellular bacteria and fungi; they are IL-23 responsive and produce many cytokines not produced by Th1 or Th2 cells including IL-17A, IL-17F and IL-22. Th17 differentiation is driven by tumor growth factor (TGF)-β, IL-6, IL-21 and IL-23 with the transcription factors RORγt/STAT3 [24].

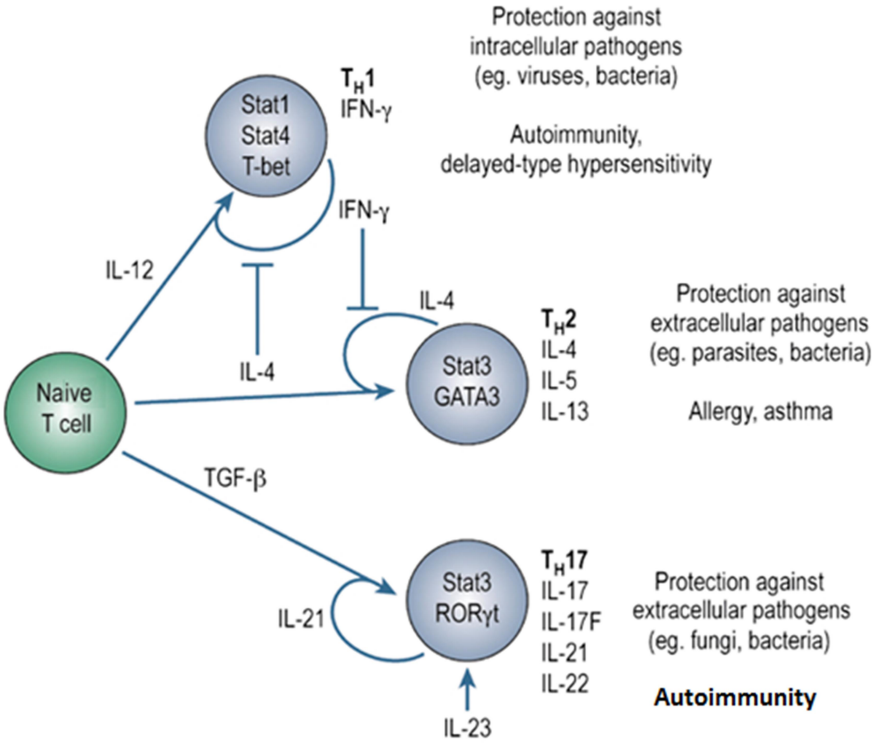

Figure 2. *Overview of the basic CD4 Th cell subsets and the transcription factors and cytokines involved in their induction and their effector profile. Figure adapted from Deenick and Tangye (2007) [26].*

1.4.4. Type I interferon in the immune response

The IFN family is comprised of type I (including IFNα and IFNβ among others), type II (IFNγ) and type III (IFNλ) IFN and were named based on their ability to interfere with viral replication [27]. Type I IFN in humans consist of more than 13 structurally similar cytokines which all signal through the same receptor known as the IFN type I receptor (IFNAR), which in turn influences IFN-stimulated genes generally through the actions of IFN regulatory factors (IRF) [28]. All nucleated cells can be induced to

produce type I IFN [29], however plasmacytoid DCs (pDCs) which account for 0.2 to 0.8 % of peripheral blood mononuclear cells (PBMC) constitutively express IRF7 and can produce 100 to 1000 times more type I IFN than other blood cells upon viral infection [30].

Type I IFN is produced in response to bacteria, viruses or microbial nucleic acids through activation of PRRs including TLRs, RIG-like receptors and nucleotide-binding oligomerization domain-like receptors. On the cell surface TLR4 induces type I IFN following recognition of lipopolysaccharide from bacteria, and signals through the adaptor molecule TIR-domain-containing adapter-inducing IFN-β (TRIF), which activates TANK binding kinase 1, which in turn leads to the activation of IRF3 [28].

Four TLRs detect viral nucleic acids, with TLR3 recognizing double stranded ribonucleic acid (RNA), TLR7 and 8 recognizes single stranded RNA and TLR9 recognizing non-methylated viralCpG-containing deoxyribonucleic acid (DNA). Unlike TLR4, the TLR3, -7, -8 and -9 are expressed on endosomal membranes and their activation therefore requires the endocytosis of the virus and digestion of the viral envelope and capsid protein by host cell enzymes [31]. Alternatively, these TLR in pDCs can recognize some viruses following autophagy of infected cells [32]. Like TLR4, production of type I IFN through TLR3 uses the TRIF, TANK binding kinase 1 and IRF3 [28]. In contrast to TLR3 and TLR4, production of type I IFN through TLR7, -8 and -9 is through the adaptor myeloid differentiation primary response gene 88 (MyD88) in complex with TNF receptor associated factor (TRAF) 6 and interleukin-1 receptor-associated kinase (IRAK) 1 and 4, which activates IRF-3, -5 and -7 [33]. An overview of TLR and their respective ligands, activated pathways and induced products is given in Figure 3.

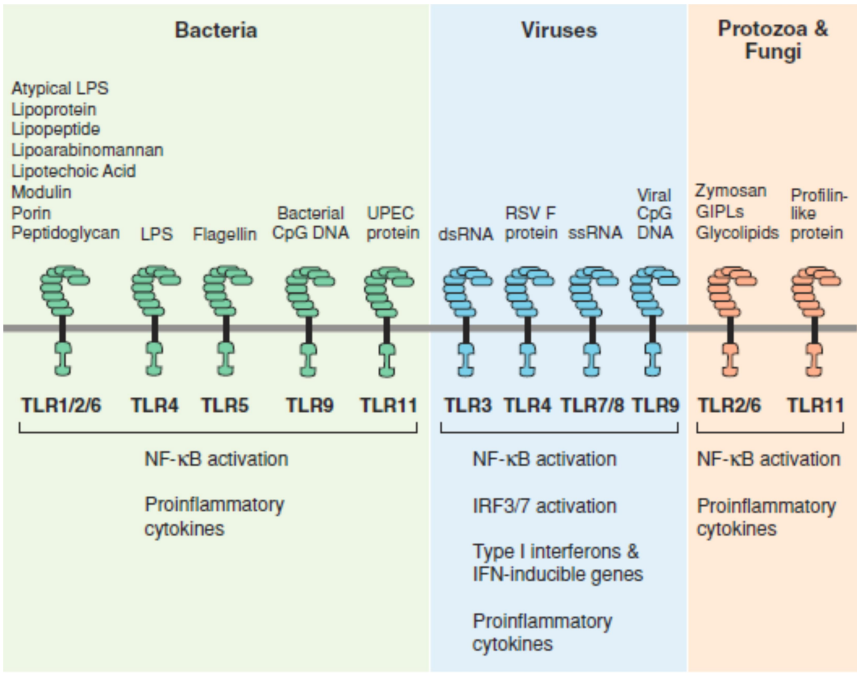

Figure 3. *TLR ligand specificities. TLRs recognize diverse PAMPs from bacteria, viruses, protozoa, and fungi. Following TLR binding to its respective ligand, NF-κB and IRF3/7 can be activated dependent on the ligand and TLR. Activation of TLR induced pathways can lead to the production of type I IFN and proinflammatory cytokines. Figure and text adapted from West et al. 2006 [34].*

The majority of cells also express cytosolic RNA helicases receptors of the RIG-like receptors superfamily including RNA helicases RIG-1, MDA5 (Melanoma Differentiation-Associated protein 5) and LGP2 (Laboratory of Genetics and Physiology 2). These receptors sense RNA which induces downstream signaling by interacting with the adaptor protein MAVS (mitochondrial antiviral-signaling protein). MAVS allows for the activation of TANK-binding kinase 1- IkB kinase (IKK)ε, which is responsible for the activation of IRF3 and -7, and IKKα and IKKβ, responsible for NF-kB activation resulting in type I IFN production [35]. Expression of IRF5 and -7 is also upregulated by type I IFN [36, 37], thereby cells without constitutive expression of IRF5 or -7 require a signal through IRF3 to activate type I IFN expression and "prime" the cell to produce type I IFN [30].

Secreted type I IFN can then activate cells in a paracrine or autocrine manner through binding the IFNAR. Binding of the receptor induces activation of Tyk2 and Janus Kinase (JAK) 1 which recruits and activates STAT1 and STAT2. STAT1 and STAT2 heterodimers can form a complex with IRF9, called the IFN-stimulated gene factor 3 which enters the nucleus and binds to IFN-stimulated response elements (ISRE) activating type I IFN induced genes (see Figure 4) [38]. Although the exact biological function of many gene products of type I IFN induced genes are unknown, many are known to be involved in antiviral responses including myxoma resistance protein (MxA) [39, 40], whose expression is stimulated exclusively by IFN-α/β or IFN-λ [41]. A large number of products are also associated with angiogenesis, apoptosis and cell proliferation [39, 42-44]. Type I IFN induced gene products are also involved in initiating type I IFN production (TLR7 and IRF5) [45], as well as down regulating of type I IFN response through induction of negative regulators including the suppressor of cytokine signaling (SOCS) family [46, 47].

1.5. Intracellular signaling pathways

Intercellular signaling allows cells to receive and send messages, however, to respond a cell must convey the signal through the cell to appropriate response elements. To accomplish this process cells utilize a number of mechanisms which transmit information through the cell through conformational changes in proteins. These proteins are incorporated in long chains to form pathways as well as interconnected networks; with the transfer of information through the cell involving the linking of different changes of state. This large scale linking allows incorporation of many signals allowing countless finely tuned responses to the multitude of signals the cell may receive. Many of these signaling events are transmitted through ligand binding which results in changes in the proteins activity. Signal transduction utilizes a number of chemical reactions to induces change in protein conformation and activity, many of these reactions are however inherently slow [48].

Cell signaling systems therefore use numerous enzymes as catalysts including protein kinases to catalyze phosphorylation reaction and adenylyl cyclase to catalyze the

formation of cyclic adenosine monophosphate from adenosine triphosphate. This allows changes to occur in timeframes necessary for cellular responses. Additionally, cells may use enzymes such as phosphatases which can destroy these protein modifications giving another layer of control [48]. A number of major pathways are used in intracellular signal transduction; those relevant to this thesis (JAK/STAT, Mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are described in the subsequent section. Because the responses and functions of each intracellular signaling molecule are diverse the following review is limited to the context of this thesis.

1.5.1. JAK/STAT

The JAK / STAT signaling pathway regulates the cellular response to a number of cytokines and growth factors. Signal transduction by the pathway utilizes tyrosine kinases called JAKs and transcription factors called STATs to transduce a signal received by an extracellular receptor to the nucleus. The pathway operates through binding of a ligand to the extracellular domain of a JAK associated membrane bound receptor results in receptor dimerization and JAK phosphorylation. This in turn results in the JAKs phosphorylating the cytoplasmic domain of the cytokine receptor, creating a binding site for STAT. The bound STATs tyrosine residues are phosphorylated by JAKs, resulting in disassociation of STAT. The phosphorylation also promotes STAT dimerization which is essential for nuclear translocation and retention as well as DNA binding [49-51].

The mammalian STAT family consists of 7 different STATs (STAT 1-6, -5a and -5b), and 4 JAKs (JAK 1-3 and Tyk2). STAT and JAKs are functionally heterogeneous, with different ligands resulting in differing combinations and responses and signaling specificity. Additional to phosphorylation at tyrosine residues, STAT1 and -3 can undergo serine phosphorylation independent of JAK through serine kinases including extracellular signal-regulated kinases (ERK) and protein kinase C for STAT3, and p38 and protein kinase C for STAT1 [52]. Serine in

addition to tyrosine phosphorylation can modulate the transcriptional activity of STAT contributing to gene responses adding an additional layer of control [52, 53].

Type I IFN can activate STAT1 and STAT3 in most cell types, while activation is cell type dependent for STAT4, STAT5 and STAT6 [38]. Type I IFN signaling through STAT1 is generally accepted to be proinflammatory, antiproliferative and proapoptotic and activates a number of STAT1-dependent inflammatory genes including chemokine (C-X-C motif) ligand 9 and 10, and B-cell activating factor. In contrast, type I IFN signaling through STAT3, STAT4 and STAT5 often promotes cell survival, proliferation and differentiation [54]. STAT3 has been observed to be capable of negatively regulating IFN responses and has been proposed to inhibit TLR signaling either through inducing anti-inflammatory molecules such as IL-10 or direct suppression of NF-κB [38, 55].

Experiments with mice lacking STAT1 indicate that the anti-apoptotic responses in T cells to type I IFN are primarily mediated by STAT3 and to a lesser extent STAT5A/B [56]. STAT4 is involved in the anti-viral effect and production of IFNγ. Switching from type I IFNs activation of STAT1 to STAT4 due to STAT1 inhibition by TCR derived signals in CD8+ T cells during lymphocytic choriomeningitis virus infection enables optimal antigen-specific CD8+ T cell expansion and production of IFNγ promoting immunity to lymphocytic choriomeningitis virus [54, 57]. Mouse models of lymphocytic choriomeningitis virus-induced hepatitis and IFNα therapy of individuals with hepatitis C also show that that induction of cytotoxicity and production of IFNγ in NK cells is dependent on differential STAT1/4 phosphorylation [58-60].

These studies show that NK cells display a high basal expression of STAT4 but reduced STAT1 compared to other cell subsets. This pre-disposes the cell to STAT4 activation by type I IFNs and IFNγ expression [58]. Total STAT1 levels are induced during viral infections as a result of type I IFN exposure, this change acts to promote the activation of STAT1 and increased cell cytotoxicity but limits both the activation of STAT4 and IFNγ expression [58-60].

Differential STAT activation therefore in part determines the outcome of type I IFN signaling by shifting the balance between suppressive, anti-proliferative, anti-viral and proinflammatory gene expression [38] with different STAT complexes formed in response to type I IFN controlling the distinct gene expression programmes. For example, the IFN-stimulated gene factor 3 complex, composed of STAT1, STAT2 and IFN-regulatory factor 9 binds to ISRE sequences activating anti-viral genes. In contrast, STAT1 homodimers bind to gamma-activated sequences (GAS) and induce proinflammatory genes [38] (Figure 4).

The JAK/STAT pathway is negatively regulated on a number of levels, such as suppression of cell surface IFNAR expression [38], type I IFN induction of negative regulators including SOCS family members, ubiquitin carboxy-terminal hydrolase 18 and microRNA [38, 61]. Cross regulation between STATs has also been observed with negative regulation of STAT1 by STAT3 occurring through competition for common receptor docking sites [62].

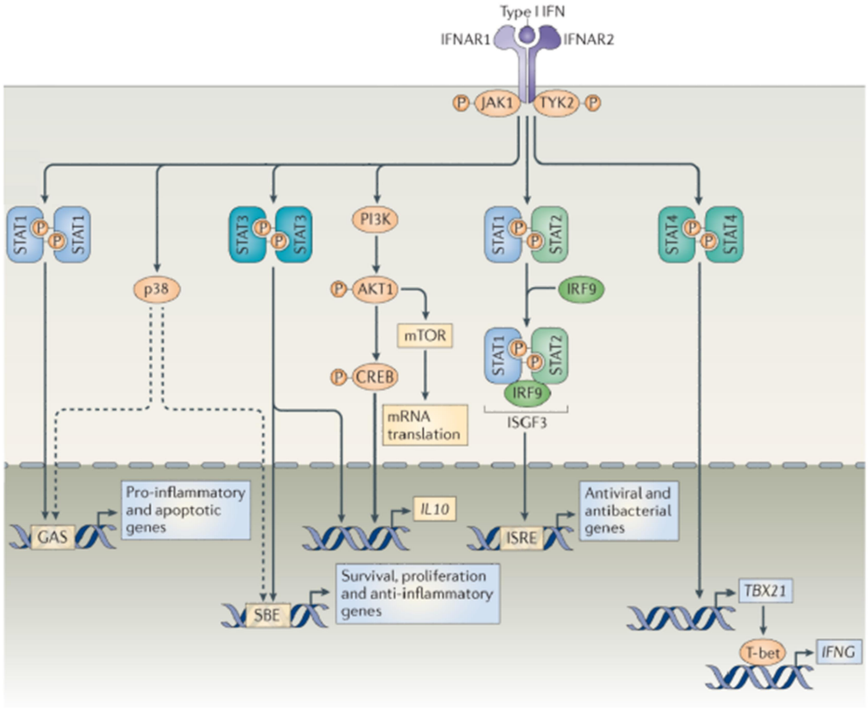

Figure 4. *Signaling pathway activated by type I IFN. Type I IFN can activate a number of pathways utilizing STAT and MAPKs initiating different responses. STAT1 homodimers formed in response to type I IFN bind to IFN activated GAS enhancer elements in promoters of IFN stimulated genes. This results in the transcription of genes encoding proinflammatory cytokines and apoptotic factors. STAT3 homodimers can also be formed in response to type I IFNs; this can result in the transcription of both pro and anti-inflammatory cytokines including IL-10. STAT1-STAT2 heterodimers formed following activation by type I IFNs bind to IFN regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (complex), which binds ISRE activating anti-viral and antibacterial genes. Independent of STAT, type I IFN can also signal through the phosphoinositide 3-kinase (PI3K)–AKT pathway to produce IL-10, mammalian target of rapamycin (mTOR) which regulates mRNA and p38 which is an upstream regulator of several genes regulated by ISRE and GAS elements. CREB, cyclic adenosine monophosphate-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 [63].*

1.5.2. The MAPK cascade

The MAP kinase cascade is among the most prevalent cascades in eukaryotes and regulates a number of fundamental cellular processes including proliferation, transformation, apoptosis and differentiation [64-66]. The pathway utilizes three kinases that act in series. The most upstream referred to as MAPKKK, which phosphorylates and activates MAPKK, in turn activating MAPK. MAPK acts as the effector of the pathway, acting on hundreds of different substrates including transcription factors, transcription suppressors, and chromatin remodeling proteins. In mammals there are three main MAPK families − ERKs, JNKs (Jun amino-terminal kinases) and p38/SAPKs (stress-activated protein kinases). Activation of MAPK requires dual phosphorylation that results in exposure of the kinase active site and allows for substrate binding [65, 67].

The pathway can respond to an extensive number of different stimuli producing highly specific and fine turned outcomes. Negative regulators affect the strength and duration of the transduced signals, in particular MAPK activity can be inhibited like the STAT family through endogenous phosphatases which can dephosphorylate both tyrosine and serine/threonine residues within a single substrate [67].

The ERK1/2 pathway enhances the production of a number of proinflammatory cytokines including tumor necrosis factor (TNF) [68], IL-6 [69], and anti-inflammatory cytokines including IL-10 [70], while p38 activation has been implicated in the expression of proinflammatory cytokines including IL-6, IL1β and TNFα [71].

Both ERK and p38 can be activated in the production and response to type I IFN. For example, p38 activation in response to type I IFN is required for transcription of genes regulated by ISRE and GAS elements in a STAT independent manner (see figure 4) [63]. Several studies have also indicated that p38 is required for anti-viral and growth inhibitory effects of type I IFN, while ERK participates in the response to viral infections [63]. Both ERK1/2 and p38 can also be activated by various TLR ligands, activation of AP-1 (activator protein-1) in TLR signaling for example is

mostly regulated by MAP kinases including p38 and ERK [72]. AP-1 activation through TLR7 or -9 leads to the transcription of genes encoding proinflammatory cytokines and chemokines such as TNFα, IL-6, IL-8, and IL-1β [34] (Figure 5). These responses are however context dependent, for example signaling through ERK in conjunction with STAT3 in B cells following stimulation with TLR7/8 and TLR9 agonists can induce production of the anti-inflammatory cytokine IL-10 with production further enhanced by IFNα in TLR7/8 induced responses [73].

1.5.3. NF-κB associated pathways

The NF-κB protein complex regulates a range of genes controlling the transcription of cytokines and antimicrobial effectors as well as genes that regulate cellular differentiation, survival and proliferation. In mammals, the NF-κB family is composed of two subfamilies – the NF-κB proteins and Rel proteins, and 5 family members − p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelA (p65), RelB, and c-Rel [74].

The pathway is known to signal through either the canonical (classical) or non-canonical (alternative) pathway. In the classical pathway, receptor activation (TCR, BCR etc.) leads to activation of IKK complex (IKKα, IKKβ and NEMO). The activated IKK complex in turn phosphorylates IkB which is bound to the inactive NF-κB. The phosphorylation of IkB results in its polyubiquitination and degradation by the 26S proteasome. This releases the NF-κB dimers which translocate to the nucleus, binding activator regions increasing specific gene transcription. In contrast, the non-canonical (alternative) pathway relies on the activation of IKKα. IKKα phosphorylates p100 resulting in its ubiquitination and proteasomal processing to p52. This creates NF-κB p52/RelB complexes that translocate to the nucleus upregulating gene transcription [74].

Signaling through NF-κB plays an important role in the immune system, regulating many genes required in the development of the immune system as well as those responsible for both adaptive and innate immune responses. The pathway is utilized by a number of receptors involved in the innate response, in particular TLR, where

NF-κB is one of the main transcription factors affected by TLR signaling. Both TLR7 and TLR9 signal through the adaptor MyD88 to NF-κB or IRF7, with the most frequently activated form of NF-κB in TLR signaling being a heterodimer composed of RelA and p50 [75]. In general, activation of NF- κB, like AP-1 leads to proinflammatory cytokines and responses [75, 76], while activation of IRF7 leads to the production of type I IFN [77] (Figure 5).

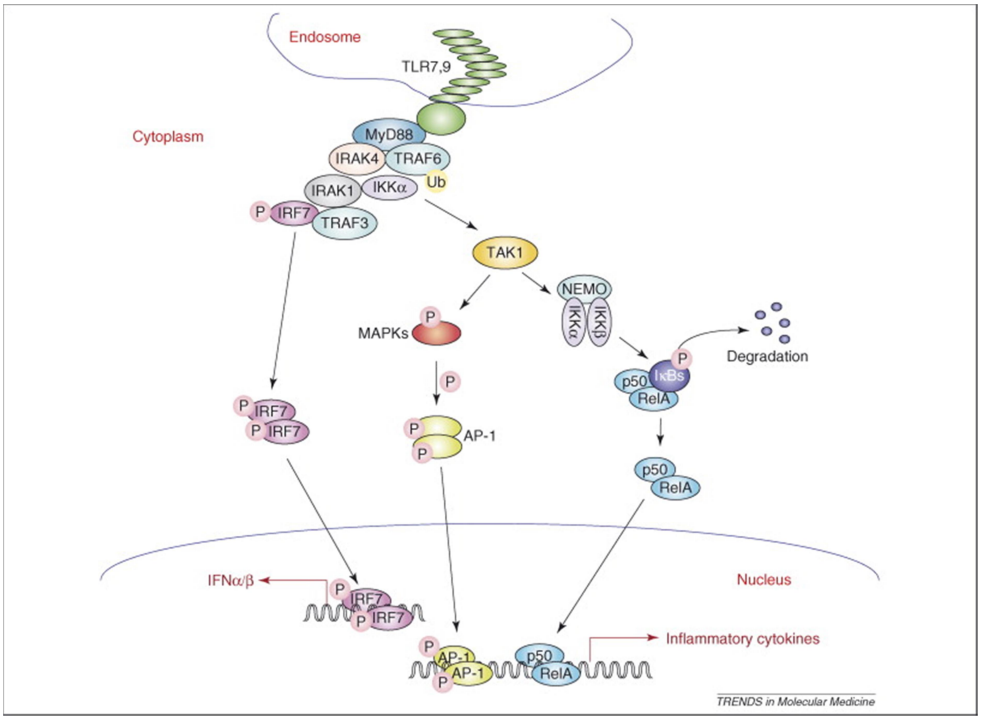

Figure 5. *TLR7 and TLR9 mediated signaling. TLR7 and TLR9 reside in endosomal compartments of cells. Following binding of TLR to ssRNA (TLR7) or unmethylated CpG dinucleotides (TLR9), a MyD88-dependent pathway signals through activation of TAK1-mediated NF-κB and MAPK pathways which regulates transcription of genes encoding inflammatory cytokines. Alternatively IRF7 forms a signaling complex with MyD88, IRAK4, TRAF6, IRAK1 and IKKα. IRF7 is phosphorylated by IRAK1 and IKKα, dimerizes and regulates the expression of type I IFNs, including IFN-α and IFN-β. Figure adapted from Kawai and Akira 2007 [75].*

Although not being the main contributor to type I IFN production, NF-κB activation can influence its production and response. IRF3 along with NF-κB and the JAK-

STAT signaling cascade is essential in positive feedback regulation of type I IFN genes and induction of IRF7 expression in early stages of virus infection when endogenous level of IRF7 in the cell are low [78]. Activation of NF-κB has also been observed to compensate for genetic defects in type I IFN pathway of IRF7 deficient mice allowing survival to otherwise lethal poxvirus infections [79].

1.6. Autoimmunity and autoimmune disease

Physiological autoimmunity is defined as an immune response against self components. Autoimmunity is present in a healthy immune system and is assumed to assist in the normal homeostatic mechanisms of the organism, for instance by natural autoantibodies that can target self nuclear and cytoplasmic debris enhancing their phagocytosis [80]. The mechanisms involved in a switch from physiological autoimmunity to pathological autoimmunity are poorly understood, but is characterized by a breakdown of self-tolerance leading to an adaptive immune response to self-antigens and damage to cells and tissues.

1.6.1. Tolerance

Tolerance can broadly be defined as the non-reactivity of the immune system to an antigen after repeated exposure. A number of regulatory mechanisms have evolved that occur in immature lymphocytes at generative lymphoid organs (central tolerance) or in mature lymphocytes in peripheral sites (peripheral tolerance).

Because of the random recombination of genes used to generate different specificities of receptors in immature B and T cells, receptors specific for self-components can be produced. To prevent self-targeting, both immature T and B cells undergo negative selection (clonal deletion) during their maturation [81, 82]. Central tolerance for B cells takes place in the bone marrow where immature B cells that recognise self molecules undergo receptor editing resulting in expression of a new Ig light chain, if the receptor editing fails the B cell is deleted [81, 83]. Central tolerance for T cells takes place in the thymus. T cells undergo positive selection where only T cells that

recognise self-MHC molecules are permitted to survive, while negative selection removes T cells that bind self peptides above an affinity threshold [82, 84].

It is known that central tolerance does not prevent all self-reactive cells from entering the periphery. A number of mechanisms therefore exist in the periphery that protect from these self-reactive cells including anergy, suppression and deletion. Anergy occurs in mature B and T cells when they are activated without proper stimulation signals. This occurs in mature T cells when the T cell encounters its cognate antigen presented by an APC without the co-stimulatory signals (CD28/B7) through non expression of B7, or engagement of B7 by CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) or PD-1 (programmed cell death protein 1) [85, 86]. B cells recognising self-antigen without T cell co-stimulation become unresponsive or die from apoptosis [87] and T cells chronically stimulated by antigen can be deleted through Fas- or Bim-mediated apoptosis pathways [86].

Immature CD4+ T cells within the thymus that recognise self-antigens strongly may develop into natural Tregs, and Tregs known as inducible Tregs can be induced through the treatment of naïve peripheral CD4 T cells with TCR stimulation and with TGFβ plus IL-2 [24]. Both Treg subsets circulate in the periphery and are thought to be involved in self-tolerance and immune modulation exerting their effects through the suppression of proliferation and IFN-γ production of effector T cells for example [24, 88].

1.6.2. Autoimmune diseases − etiology and pathogenesis with emphasis on pSS

Autoimmune diseases comprise a range of organ-specific diseases including multiple sclerosis and systemic disorders for instance rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and SS. During their lifetime, 5 to 10% of all individuals will go on to develop some kind of autoimmune disease [89]. Common among all autoimmune diseases is a breakdown of the systems that maintain tolerance to self-components, resulting in immune responses to these components and extensive

pathology. The development and initiation of an autoimmune disease is thought to result from a combination of genetic elements and environmental triggers, with pathogenesis occurring long before clinical presentation [90] as depicted in the proposed eitopathogenic events in SS (Figure 6). The full picture is however far from complete.

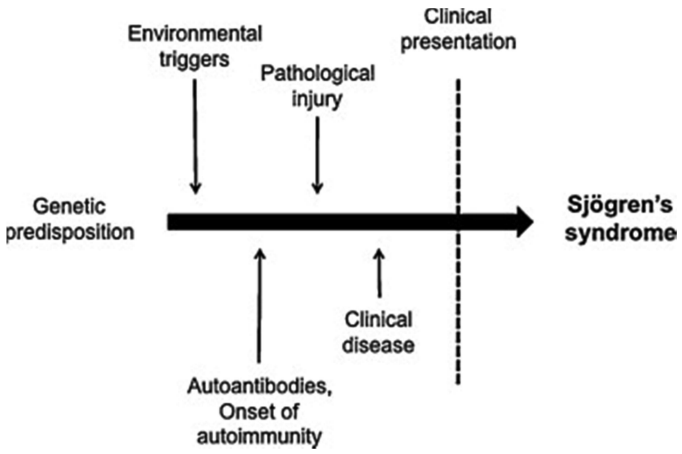

Figure 6. *Proposed etiopathogenic events prior to diagnosis of Sjögren's syndrome. Figure from Jonsson et al. 2011 [90].*

The central question regarding the etiology of autoimmune diseases is how self-tolerance fails and how self-reactive lymphocytes are activated. Initiation is thought to result from the exposure of predisposed individuals to an environmental trigger. Following activation of self-reactive lymphocytes, numerous interacting factors propagate the inflammatory response and subsequent tissue damage. These factors reinforce each other to perpetuate a response to a persistent self-antigen. For example, inflammation induced tissue damage may result in the release and modification of other self-antigens and activation of lymphocytes, in a phenomenon known as epitope spreading [91]. As a result, autoimmune diseases tend to be chronic, progressive and self-perpetuating. Aberrations common in autoimmune diseases that may play a role in their propagation include shifts of immune cell subset frequency [92], cell receptor expression [93], chemical messengers [94] and autoantibody production [95, 96].

Genetics has been identified as an important factor in the development of disease. First degree relatives of autoimmune disease patients display generally a five time greater risk than the general population of developing an autoimmune disease [97]. Genome wide association studies in human autoimmune disorders have identified many alleles which pose a risk. The strongest associations have been found within the MHC locus, in particular HLA (Human leukocyte antigen) class II molecule HLA-DR3 which is linked to SS, SLE and autoimmune myositis. Several non-MHC susceptibility genes have also been identified. In SS, IRF5, STAT4, BLK and IL12A have significant associations [97], of which IRF5 and STAT4 have an additive effect [98]. Such findings support the concept of a quantitative threshold of immune signaling, where the small effect of many alleles could combine to enhance susceptibility to autoimmunity [97].

Genetic differences do not however offer the full picture, autoimmune diseases occur in both monozygotic twins in no more than 20-30% of the cases thus indicating environmental factors also play a critical part [97]. Environmental factors thought to play a role in autoimmunity commonly act on pathways in which gene polymorphisms associate with disease [97]. For example, infectious agents like viruses are thought to trigger autoimmunity by interaction with PRRs such as TLRs and several genetic variants associated with SS feature in downstream signaling from TLRs or their regulation, including IFR5 [99, 100], IL-10 [101], IκBα (nuclear factor of kappa light polypeptide gene enhance in B-cell inhibitor, alpha) [102] and TNIP1 (TNFAIP3 interacting protein 1) [100].

A common feature of autoimmune diseases is their higher prevalence in females. The strongest sex ratio biases are observed in SS, SLE, autoimmune thyroid disease and scleroderma where the ratio of women to men is 7:1 to 10:1 [103]. Sex hormones or sex-linked genes therefore likely influence the susceptibility of developing an autoimmune disease. Significant evidence exists for a hormonal role in primary Sjögren's syndrome (pSS), for example in pSS patients the common age of onset is around menopause and mouse studies show that estrogen is protective to the development of pSS, and ovariectomy leads to SS like disease [104]. An X

chromosome additive effect is also observed with females with triple X syndrome (47, XXX) showing a higher prevalence of both pSS and SLE then normal 46, XX females [105] indicating possible influence of sex-linked genes.

1.6.3. The interferon signature in autoimmunity

It has been reported that patients with various autoimmune diseases including SLE, RA and SS display an increased expression of type I IFN regulated genes in both PBMC and tissue known as the type I IFN signature, [106, 107]. In SLE about half of adults display an IFN signature [108], which correlates with disease severity and activity [106, 108]. Further, the signature has been observed to be stable over time, despite flares of the disease [109, 110]. In RA incidences of an IFN signature in patients has been reported in a approximately 25- 50% of assessed patients [111, 112]. Over half of pSS patients with pSS exhibit a type I IFN signature [113]. Patients with pSS exhibiting a type I IFN signature display higher ESSDAI (European League Against Rheumatism SS disease activity index) scores; higher levels of anti Ro and La autoantibodies; higher serum IgG; lower C3 and lower absolute counts of lymphocytes and neutrophils [113].

At least three possible mechanisms are speculated to be behind the type I IFN signature in autoimmune diseases, with contributions of each likely differing among diseases and between patients with similar disease [106]. The first possible mechanism is type I IFN production through type I IFN producing cells, in particular pDCs which are activated in the majority of diseases displaying a type I IFN signature [106]. A number of type I IFN inducers, in particular immune complexes are present in many autoimmune diseases including pSS. Immune complexes of autoantibodies and auto antigen that contain self nucleic acids can activate TLR7 and -9 which in turn induce the production of type I IFN [106].

The second mechanism involves genetic factors. High proportions of identified risk genes identified for development of autoimmune diseases encode products involved in type I IFN system including those involved in the production of or response to type I IFNs. Gene polymorphisms of IRF5 have been associated with SLE and pSS

patients [114, 115]. Further, IRF5 expression is elevated in SLE patients [116], is activated in these individuals [117] and is associated with serum IFNα activity [118]. In addition, STAT4 is also identified as a risk gene in SLE, RA and pSS and confers increased sensitivity to IFNα in SLE patients [119-121]. Polymorphisms in TYK2 (tyrosine kinase 2) which is required for signaling through the IFNAR are also associated with SLE [122] and confer an increased sensitivity to IFN.

The third mechanism involves a lack of regulation of pDCs or expression of IRGs. Normally, following the clearing of an infection, the type I IFN system is switched off and the immune system returns to homeostasis [45]. There is evidence in autoimmune disease indicating a loss of this regulation. For example, monocytes from healthy individuals reduce the IFNα production of pDCs stimulated with RNA containing immune complexes in PBMC cultures, while monocytes from SLE patients are less inhibitory [123]. In pSS patients, anti-Ro52 antibodies may also interfere with type I IFN negative feedback [124]. Ro52 which is also known as tripartite motif-containing protein 21 is an IFN-inducible E3 ubiquitin-protein ligase that promotes ubiquitination and proteasomal degradation of IRF3 and IRF7 [125, 126]. Anti-Ro52 antibodies from SS patients have been observed to inhibit the E3 ligase activity of Ro52 [127] potentially removing its negative regulation of IRF3 and IRF7.

Increases in type I IFN activity in autoimmune diseases could manifest in a number of ways. Type I IFN can exert their effects on immune cells either directly or indirectly through the induction of chemokines, cytokines or by stimulation of cell types which participate in the activation of other immune cells. For example, type I IFN can act as an immune adjuvant and increase the expression of MHC class I molecules [128], enhance NK cell cytotoxicity and their ability to produce IFNγ [129], stimulate production of IgG subtypes and induce long-lived antibody production and immunological memory [130], and CD4 T cell differentiation into IFN-γ- secreting Th1 T cells [131].

In SS, a transient or persistent viral infection of epithelial cells leading to a genetically determined amplified induction of type I IFN production in particular IFNα by locally recruited pDCs, has been postulated as a likely scenario in its development (an overview of this model is depicted in Figure 7). This further leads to activation of glandular epithelial cells through increased expression of MHC and costimulatory molecules, and apoptosis or necrosis of the epithelial cell releasing potential autoantigens including RNA binding SS antigen A (SSA) and SS antigen B (SSB) [132]. Effects of IFN as mentioned previously could lead to the production of autoantibodies. Once produced, autoantibodies will form immune complexes with their respective autoantigen.

The immune complexes can in turn activate type I IFN pathways through TLR dependent and independent triggering of IFN producing cells, for example by pDCs after internalization via the Fc receptor for IgG, FcγRIIa. Produced IFN can then sustain a positive feedback and promote autoimmunity through many of the mechanisms mentioned previously and lead to impaired function in affected salivary and lacrimal glands [132]. Where there is a large amount of inflammatory mediators or migration of autoimmune cells to other organs, extraglandular manifestations (EGM) may occur [132]. Autoantibodies may also participate in the development of EGM through the formation of immune complexes which can lead to organ damage or directly target organs, for example anti-Ro52 antibodies can bind fetal cardiomyocytes potentially causing congenital heart block [132].

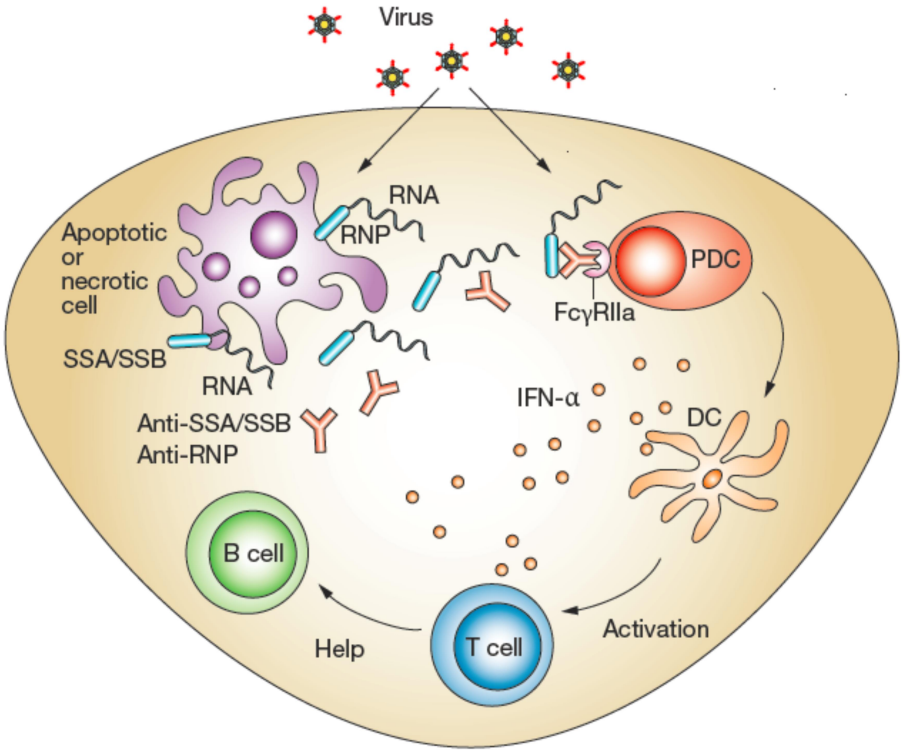

Figure 7. *Disease model of SS in minor salivary glands. Viral infection of epithelial cells leads to production of IFNα by pDCs leading to increased necrosis or apoptosis of the epithelial cells. Necrosis or apoptosis leads to exposure of SSA/SSB ribonucleoproteins. B cells produce autoantibodies against the ribonucleoproteins and form immune complexes which are internalized by pDCs though association with the Fc receptor for IgG, FcγRIIa, resulting in production of IFNα. IFNα can then stimulate DC maturation, activation of T cell and production of autoantibodies by B cells which further facilitate an autoimmune loop. DC, dendritic cell; FcγRIIa, Fc receptor for IgG FcγRIIa; IFNα, interferon alpha, PDC, plasmacytoid dendritic cell; RNP, ribonucleoprotein. Figure and text adapted from Nordmark et al. 2006 [132].*

1.6.4. Challenges in the diagnosis and treatment of autoimmune diseases

As autoimmune rheumatic diseases share many common features and clinical presentations including arthralgia and arthritis, myalgia, sicca symptoms, and

pulmonary, renal, and neurological involvement, they impose significant diagnostic challenges [133]. Correct diagnosis is crucial because of distinctions in clinical course, treatment options (which may have significant adverse effects) and prognosis of the diseases [133]. Up to 50% of patients with apparent autoimmune rheumatic diseases cannot be diagnosed in the first 12 months. Some of these unclassified patients will progress to a defined autoimmune rheumatic disease within 5 years, while a small proportion resolve completely, and most remain symptomatic but undefined [133]. In some cases, features from two autoimmune rheumatic diseases can be present, with management largely guided by the dominant clinical phenotype [133]. Delays in diagnosis after a patient becomes symptomatic are further exacerbated with significant evidence demonstrating that many autoimmune diseases have a pre-clinical period of development, for instance specific autoantibodies are present up to 18-20 years before symptom onset in pSS [134, 135].

Significant strides have been made in the recognition of cells and molecules that are important in the pathophysiology of autoimmune diseases. This has led to the identification of a number of potential targets and the development of a number of compounds that modulate or inhibit survival of B cells, inhibit T cell function, and inhibit cytokines and complement [136]. Identification of the role of B cell in autoimmune pathophysiology has led to treatments including rituximab and belimumab, monoclonal antibodies that deplete B cells [136]. However, significant difficulties have been encountered from the transition from bench to bedside of promising drug candidates. This failure may stem from the complex interplay of the cells and secreted products of the immune system. Additionally, the high degree of heterogeneity within a disease and the inclusion of unsuitable patients in clinical trials may impact whether a compound meets its endpoint in trials [136].

1.7. Sjögren’s syndrome

Sjögren’s syndrome is a systemic autoimmune disease characterized by lymphocytic infiltrates of the exocrine glands notably salivary and lacrimal glands combined with immune-mediated glandular destruction [90]. Estimates of prevalence vary due to

differences in diagnostic criteria but pSS has been estimated to affect about 0.1 to 4% of the global population [137], with more stringent estimates being 0.05% [137], 0.16% [138] and 0.09% [139] using the revised American–European Consensus Group (AECC) criteria [140].

The hallmarks of the disease are a dryness of the mouth (xerostomia) and the eyes (keratoconjunctivitis sicca) that result from immune-mediated glandular destruction [90]. This dryness and other clinical manifestations lead to a significant decrease of life quality of those affected by the disease. Sjögren's syndrome can occur alone, termed pSS or associated with other autoimmune disorders, most commonly SLE, RA or scleroderma, termed secondary SS [141]. The disease predominantly affects women at a 9:1 ratio to men, with a peak incidence between the 4^{th} and 6^{th} decade of life [90].

1.7.1. Clinical features

Patients with pSS can display a diverse number of both local and systemic clinical features. As mentioned previously, SS exhibits local manifestations through oral and ocular dryness resulting from disease mediated destruction and dysfunction of salivary and lachrymal glands [90]. The resulting oral dryness can lead to increased oral infections (primarily candidiasis), mucosal friability and dental caries [90]. Ocular dryness can result in damage of the corneal and conjunctival epithelia, as well as discomfort, and functional disability through visual impairment. Ocular manifestations may also lead to complications including corneal ulceration and eyelid infections [142].

Systemic manifestations in pSS are highly diverse, and 30 to 70% of patients develop systemic involvement before or after diagnosis of pSS [142]. Primary Sjögren's syndrome patients frequently display increased fatigue and musculoskeletal manifestations including arthralgias and myalgias. Skin involvement, in particular xerosis, is frequently encountered in addition to Raynaud's phenomenon, purpura and annular erythema [142]. Pulmonary involvement in pSS consists of various forms of airways disease (bronchiectasis, obstructive airway disease) and interstitial lung

disease [143]. Patients with pSS may present involvement of the entire gastrointestinal tract. Epigastric pain, dyspepsia and nausea can occur, pSS patients may also exhibit jejunitis, sigmoiditis, and inflammatory bowel disease [144]. Association with chronic liver diseases is well documented, with increased incidences of hepatomegaly, pruritus, palmar erythema and jaundice among patients [144].

1.7.2. Diagnosis and treatment

Diagnosis of SS as used by the papers incorporated in this thesis is based on the AECC for SS (Table 1). It is noted however that the American College of Rheumatology and the Sjögren's International Collaborative Clinical Alliance (SICCA) have recently published their updated criteria for use in individuals with signs and / or symptoms suggestive of SS [145]. The AECC criteria comprises of a questionnaire on subjective symptoms, and objective tests for oral and ocular dryness. To be classified with pSS, patients must display lymphocytic infiltration in minor salivary glands, with a focus score ≥ 1 (50 lymphocytes per 4 mm^2), or autoantibodies (anti-Ro/SSA and/or anti-La/SSB) as well as oral and ocular features [140]. In practice the diagnosis of SS might be difficult, as SS presents symptoms characteristic of multiple disease phenotypes, medication side effects and general aging.

Table 1. American-European Classification Criteria (AECC) for Sjögren's syndrome

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 anaesthesia (≤ 5mm in 5 min)
   Rose bengal score or lissamine green score ≥ 4 (according to Bijsterveld's scoring system)
4. Histopathology: a focus score ≥ 1 (50 lymphocytes per 4 mm^2) in minor salivary glands
5. Oral signs: a positive result for at least one of the following three tests:
   Unstimulated whole salivary flow (≤ 1.5 ml in 15 min)
   Parotid sialography showing presence of diffuse destruction without major duct obstruction
   Salivary scintigraphy showing delayed uptake, reduced concentration or delayed excretion of tracer
6. Serology: presence of autoantibodies (anti-Ro/SSA and/or anti-La/SSB) in serum

Note: Diagnosis of pSS requires four out of six criteria, including item 4 or item 6. Diagnosis of secondary SS requires a well-defined connective tissue disease and any one from items 1-2 and any two from items 3-5. Table from Vitali *et al*., 2002 [140].

Currently there is no cure or treatment of the disease, with management based on symptom relief and prevention of complications. Initial therapy includes sialogogues and eyedrops [146]. Systemic manifestations may be treated with non-steroidal anti-inflammatory drugs including hydroxychloroquine (HCQ), and in case of visceral involvement (vasculitis, neuropathy, nephritis etc.), corticosteroids [146]. However, there are no evidence based guidelines for the management of pSS, with therapeutic treatment based on personal experience, expert opinion, and reported studies [146]. The lack of effective treatments and guidelines is linked to the poor understanding of the disease etiology and pathogenesis. Study of disease etiology is difficult in part because of a long delay between disease onset and presentation of clinical symptoms. Additionally, because of the heterogeneity of the disease, development of new therapies may require targeting subgroups of patients to achieve levels of efficacy required to pass clinical trials.

1.7.3. Health care costs

Primary Sjögren's syndrome imposes a significant impact on the health care system, as well as a significant economic burden. An analysis of healthcare usage by pSS patients in the United Kingdom by Callaghan *et al*. 2005 [147] showed that health care costs for pSS patients were doubled compared to a control group (age matched, female Caucasians without inflammatory rheumatic conditions). These costs were a consequence of increased visits to healthcare providers (dentists, ophthalmologists, general practitioners, and rheumatologists), hospital stays, diagnostic costs and drug therapy. The indirect cost of pSS through loss of economic productivity (through labour and other activities including housework and childcare) is not well documented compared to healthy members of the population. However, Bowman *et al.* 2010 [148] estimated costs to be comparable to RA patients (69 to 83%). The authors concluded that indirect costs were due primarily as a result of lower probabilities of holding a job and reduced work schedules due to illness.

1.8. Biomarkers

Biomarkers traditionally are anatomical, physiological, biochemical, molecular parameters or imaging features, that can be used in diagnostics of a disease, monitoring or predicting the effect of treatments, as well as measuring disease progression or predicting future severity [149]. Most often the term biomarker has largely been limited to molecular or biochemical markers [150]. Biomarkers can be classified as a) antecedent biomarkers − assess the risk of developing the disease, b) screening biomarkers − identify individuals with subclinical disease, c) diagnostic biomarkers − aid in diagnostic of overt disease, d) staging biomarkers − estimate disease severity and e) prognostic biomarkers that provide information on the course of the disease, predict response to therapy, or monitor efficacy of a therapeutic strategy [150]. Traditional biomarkers are used widely in the clinic, e.g. the measurement of blood pressure to assess cardiovascular health, or blood glucose levels for diabetes, as well as in research. Probably one of the most well-known examples is the Philadelphia chromosome, where a shortened chromosome 22

resulting from a translocation between chromosomes 9 and 22 is associated with chronic myelogenous leukemia. The translocation results in the creation of the BCR-ABL oncogene and a gene product with increased tyrosine kinase activity that induces the onset of leukemia [151]. Researchers were able to use this knowledge to predict which patients would benefit from tyrosine-kinase inhibitors, resulting in the development of the drug imatinib (Gleevec) [151].

1.8.1. Biomarkers in rheumatology

Biomarkers currently used in the practice of rheumatology include genetic markers, gene expression products, autoantibodies, cytokines and growth factors, tissue abnormalities and quantities and relative numbers of cell subtypes [149]. Overall development and implementation of new biomarkers in the practice of rheumatology in the last decade has been poor compared to their use in research and treatments of cancers. Very few markers are currently available for indicating disease progression, severity, management and diagnosis, and there is no dispute that new biomarkers in rheumatology are needed. For example, a significant proportion of individuals receiving anti-TNF therapy in the treatment of both RA [152] and psoriasis [153] have an inadequate response.

The use of appropriate biomarkers might enable identification of non-responders before therapy is initiated, decreasing costs and preventing unwanted complications from a therapy that was not going to be effective. Further, biomarkers may allow for shorter diagnosis time and therapeutic intervention. The identification of pre-clinical autoimmune disease may enable therapeutic intervention to prevent disease progression [154]. An outline of different types of biomarkers that could be used during the course of SS is given in Figure 8.

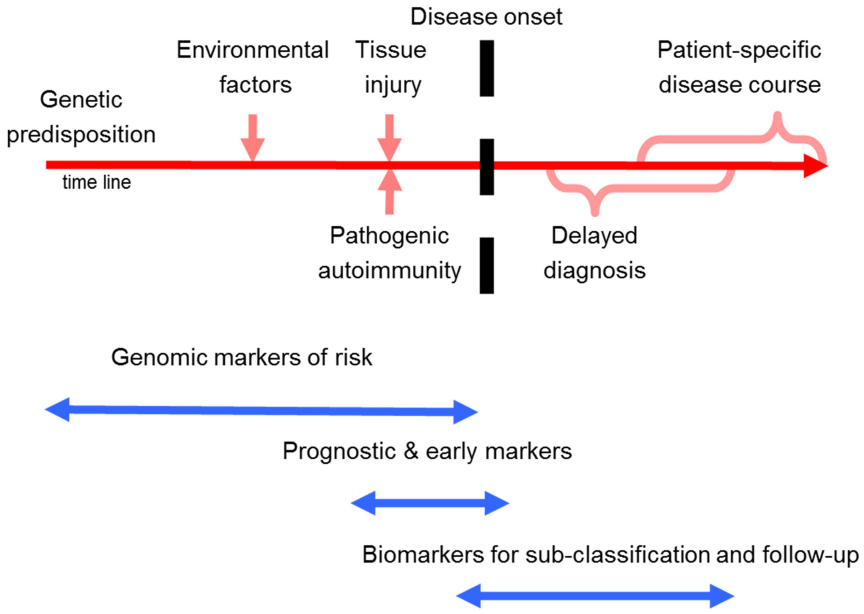

Figure 8. *Potential for different types of biomarkers during the proposed etiopathogenic events prior to diagnosis of Sjögren's syndrome. Figure adopted from Nicolas Delaleu.*

A few factors exist that explain the disparity between the success in the use of biomarkers in the treatment of cancers and rheumatic diseases. First, most rheumatic diseases can be considered syndromes in that they are classified based on a set of identifying features; they do not however manifest, progress or respond the same way in different patients because of differences in genetic, hormonal, environmental and other factors. This substantial heterogeneity poses significant difficulties not just in regards to treatment and identification of disease, but also in the development of biomarkers. Second, unlike cancers, the mechanisms that underpin rheumatic disease development and progression are also not well understood; therefore development of biomarkers relating to these underlying mechanisms is impeded. Finally, because of the complexity of the immune system and the high amount of crosstalk between cells it also seems unlikely that single biomarkers would be adequate to relate clinical symptoms to disease mechanisms or predict outcomes of interest.

Despite the challenges involved with the development of biomarkers in rheumatic diseases, progress is being made. Autoantibodies can offer prognostic and diagnosis information due to specificity for particular clinical phenotypes and are routinely used in clinical evaluation [133]. RA can be divided based on positivity for anti-citrullinated protein antibodies, with positivity representing a more severe form of the disease [155]. Multiple sclerosis can be classified according to whether the disease is driven by Th1 or Th17 cells, with IFN-β treatment ineffective against Th17- cell driven disease [156]. Increases in the type I IFN signature in RA patients treated with a TNF-α inhibitor indicate poor clinical outcome [157], and a type I IFN signature in RA is also predictive of nonresponse to B-cell depleting therapy [158, 159]. A type I IFN signature in SLE correlates with more severe disease and cerebritis, nephritis, and hematological involvement [108]. T cell surface levels of CD44 isoforms − CD44v3 and CD44v6 in SLE have been found to correlate with disease activity scores, positivity of anti-dsDNA, and the presence of lupus nephritis, indicating their use as possible biomarkers for disease activity [160]. In the future it is hoped that biomarkers could aid in the management of rheumatic diseases by establishing a molecular taxonomy of the diseases as well as facilitating the stratification of current clinical classification into subtypes that may guide clinical decision making processes.

1.8.2. Biomarker discovery

The goal of biomarker discovery is the identification of measurements that predict clinical data or outcomes. Research into potential biomarkers may include the analysis and measurement of proteins [161], gene expression [162], metabolites [163] and cellular processes [164] among others. The process to the implementation of a new candidate biomarker requires numerous steps. First suitable candidates are identified; this is accomplished by two strategies- deductive reasoning and an unbiased approach. The use of deductive reasoning to identify candidates based on preexisting understanding of the pathophysiology of disease, while an unbiased approach can be employed where molecular and proteomic techniques are used to identify candidate biomarkers on differential expression between normal and diseased

states [150]. In case of the “unbiased” strategy, the identification of the candidate is followed by quantification, where an alternative method is used to confirm the differential expression. The candidate marker then requires confirmation whether the candidate has adequate potential for success to justify further investigation. This requires analysis to be extended to large number of samples to include a broad range of patients and controls, in an effort to access the specificity of the biomarker. The final step requires the validation of the marker in a clinical setting, this involves the systematic study of other clinical covariates and of pathophysiologically related condition associations between the marker and disease state [150].

When successfully validated, a number of properties determine whether a biomarker is useful in a clinical setting. First and foremost the strength and consistency of the biomarker’s association with the outcome, or disease [150]. The importance of these factors can vary depending on a biomarker’s intended use. Biomarkers used to screen large healthy populations for disease need to be specific, while cost effective. Markers used to monitor disease or treatment response in a few individuals can be more expensive as well as less sensitive and specific as the patient can be repeatedly tested [150]. Second, whether it improves or provides new information compared to existing tests [150]. Third, the assay costs, accessibility and difficulty of interpretation. And lastly whether the biomarker helps the clinician manage patients or provide benefit to the patients welfare (such as psychological benefits) [150].

2. Aims

The main objectives of the presented work were to identify changes in immune cells from peripheral blood in pSS patients that could be used as biomarkers for patient identification and stratification. Thus, the following aims were outlined:

1. Analyze MAPK/ERK and JAK/STAT signaling networks in human peripheral blood cells by measuring intracellular phosphorylation states by flow cytometry. Compare phosphorylation states in unstimulated and stimulated immune cells from patients with pSS and healthy individuals. Evaluate signaling profiles against the clinical manifestations of the disease (papers I, II and III). We hypothesized that aberrant intracellular signaling could play a significant role in the pathogenesis of pSS. Hence study of intracellular signaling could allow for new insights of their role in autoimmune diseases and enable development of new diagnostic and stratification tools, improve current therapies and identify new therapeutic targets.

2. Quantify immune cells in peripheral blood of patients with pSS and compare against healthy individuals and associate clinical manifestations of the disease with immune cell subset counts (paper IV). Immune cell makeup of cellular infiltrates of exocrine glands have been documented to change with degree of infiltrate severity in pSS [165, 166] and associate with different clinical feature [167]. Shifts in immune cell proportions and concentrations have been noted in the peripheral blood of pSS patients compared to healthy individuals [168-171]. We hypothesized that shifts in immune cell concentrations in peripheral blood could be informative of disease progression and co-morbidities. Hence the study could allow for development of diagnostic and stratification tools, possibly improving current therapies

3. Methodological aspects

The following section describes the main methods and techniques used during the course of completion of the thesis.

3.1. Flow cytometry as a tool in biomarker discovery

Flow cytometry is a laser-based analysis technology used in the characterisation of particles (most commonly cells) in a fluid. The instrument is capable of performing multiple quantitative measurements on a particle rapidly and simultaneously. Prior to measurement, particles in suspension are hydrodynamically focused so that they separate from each other within a fluid stream. The stream passes through one or more lasers, with the resulting florescent and scattered light detected by photomultiplier tube(s) (PMT) (Figure 9), where it is amplified converted to a voltage pulse and then a digital value. Although measurements are performed on a large number of particles, the strength of the method lies in the fact that each measurement is performed on a single particle, allowing identification and grouping of individual particles for example as cellular subpopulations. The method draws on the use of fluorescent reagents, most commonly fluorchrome coupled monoclonal antibodies, to further identify the characteristics of individual particles. The fluorchrome can be excited by a specific wavelength of light provided by a laser, and emits on a lower specific wavelength. The emitted light of specific wavelength is discriminated by the use of appropriate optical filters before detection of the emitted light, greatly increasing the number of parameters that can be detected. Because of the ability of flow cytometry to measure multiple parameters at a single cell level it is increasingly being recognized as a valuable tool in biomarker research.

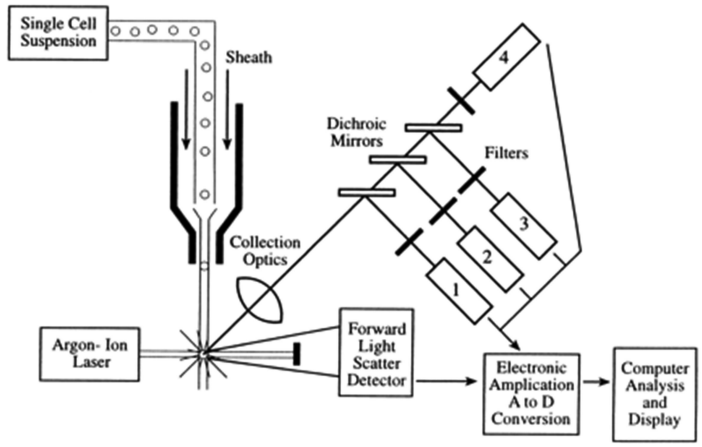

Figure 9. *Schematic of a flow cytometer. A single cell suspension is hydrodynamically focused to intersect an argon-ion laser. Signals are collected by a forward angle light scatter detector, a side-scatter detector, and multiple fluorescence emission detectors. The signals are amplified and converted to digital form for analysis. Text adapted and figure from Brown and Wittwer 2000 [172].*

For example, flow cytometry assays have been utilized for biomarker detection through measurement of receptor occupancy for generating pharmacodynamics biomarker data, informative in identifying optimal drug doses [173], activation status in diagnosing pulmonary active tuberculosis [174], fusion proteins in cell lysates of leukemia patients [175] and phosphorylation of intracellular signaling proteins to identify neutralizing antibodies in multiple sclerosis patients treated with IFN-β [176].

3.2. Phosphoflow cytometry

Phospho-specific flow cytometry, also known as phosphoflow, combines the identification of individual cells and subtypes through CD markers, with the analysis of activated intracellular signal transduction pathways [177]. Phosphoflow allows for the observation of these individual cells and distinct cellular subsets, revealing dysfunctions that may otherwise be masked in complex cell mixtures [177]. This approach has been in continuous development for application in diagnostics since its

first proof-of-principle in risk stratification of acute myeloid leukaemia [178] and holds great promise in identifying the role of signaling events in autoimmune disease. Identification of dysfunctions in intracellular signaling in patients with autoimmune diseases could help direct research into targeted treatments and diagnostic indicators, and when coupled with clinical outcomes provide biomarkers for therapeutic efficacy.

The identification of the activation of signaling is through the detection of phosphorylation on proteins involved in signal transduction or transcription. Compared to the detection of surface markers, phosphoflow cytometry faces different challenges. First, the targets are intracellular and are inaccessible to antibody, and second, after stimulation, phosphorylation is a transitional event and therefore the phosphorylated protein is not stable [177]. To overcome these problems, cells are generally fixed with formaldehyde or paraformaldehyde to cross-link the phospho-proteins and stabilize them for detection. The fixed cells are then permeabilized to allow for the entry of phospho-specific antibodies [177]. Use of the fixation and permeabilization agent is chosen based on the balance between stability of essential surface markers to permeabilization treatment while optimizing the detection of the phosphorylation. Hence all antibodies used for surface marker detection should be validated on cells following permeabilization and fixation. The basic steps of the method are depicted in Figure 10.

Methodology between paper II and III varies slightly. Prior to fixation, cells were resuspended by pipetting in paper III. This step was added due to excessive cell losses during prior testing of the assay. The cell loss was presumably because of sedimentation of cells during long incubation, followed by poor resuspension because of the low volume of a fixative. This likely lead to clumping of cells and cell loss. In paper II an alternative adjustment to the methodology was made, the fixative was pre-diluted before addition to cells, increasing the volume added and improving resuspension and minimising cell clumping.

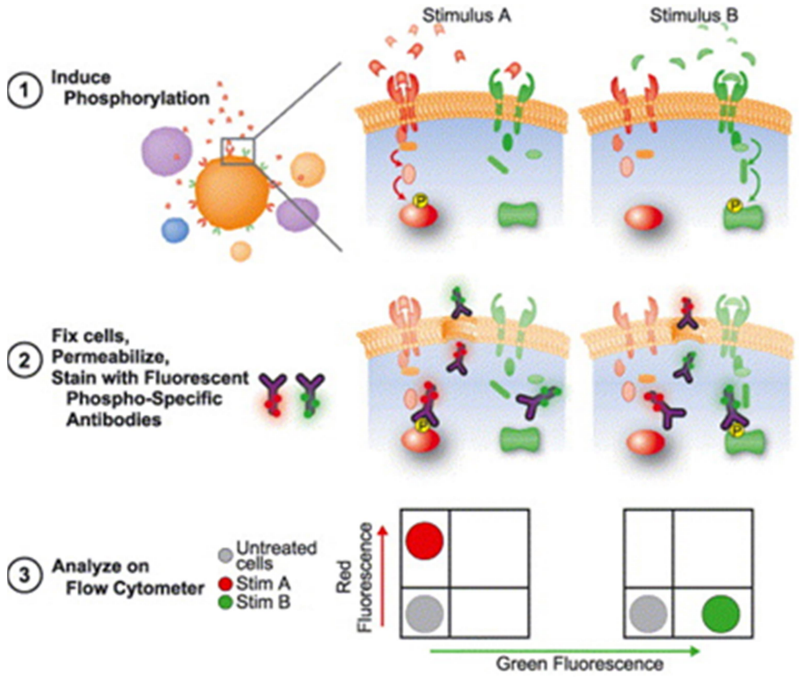

Figure 10. *Basic steps in phosphoflow cytometry. (1) A heterogeneous sample of cells is treated with two different stimuli to induce distinct signaling cascades and phosphorylation of two target proteins. (2) The cells are then fixed, permeabilized, and stained with fluorophore-conjugated phospho-specific antibodies to the phosphorylated forms of the proteins and surface markers to identify cell type. (3) The cells are then analyzed on a flow cytometer. Figure and text adapted from Krutzik et al. 2004 [177].*

Phosphoflow cytometry is capable of detecting abnormal signaling in peripheral blood samples from patients. These changes have been successfully used in the prediction of disease outcomes and treatment responses. For example, individual differences in activation-induced signaling of phospho-proteins have produced patient classifications that are predictive of response to therapy in acute myeloid leukemia [178], informed on IFN-β activity in the treatment of multiple sclerosis [176] and on clinical efficacy of DC-vaccinations in glioblastoma patients [179].

3.3. Quantitative analysis by flow cytometry

Flow cytometry can be used to provide accurate measures of relative cellular abundance of various cell subtypes in heterogeneous samples such as peripheral blood. However, technical limitations preclude most flow cytometers from accurate absolute quantifications. This is because only a fraction of the specimen is sampled by the forward scatter-activation system, while defined volumes require the assumption of uniform cell suspension [180]. Analysis of cellular populations based on relative abundance results in a significant loss of information and can give false impressions in regards to the changes within leukocyte populations. For example, the use of a drug that depletes CD20+ B cells would result in an increase in T cell percentages because of the B cell depletion, although the absolute number of T cells remains the same. Historically, the need for absolute counts was highlighted in acquired immune deficiency syndrome diagnosis, where CD4+ T cell counts ≤200 µl of peripheral blood has been included as an defining event, as these measurements are useful predictors for the onset of opportunistic diseases such as *Pneumocystis carinii* pneumonia [181].

3.4. Antibody selection and panel design

A list of all antibodies and their targets used in the papers encompassed in this thesis is displayed in Table 2, further details can be found in the respective papers.

Table 2. Antibodies used in flow cytometry experiments

Fluorochrome	Target	Cell	Clone	Supplier	Paper
Alexa fluor® 488	CD20	B cells	H1	BD Biosciences	I, II, III
PE	CD56	NK, NKT cells	N901	Beckmann Coulter	I, II, III
BV786	CD3	T, NKT cells	SK7	BD Biosciences	I, II, III
PerCp-Cy™5.5	ERK1/2 (pT202/pY204)	N/A	20A	BD Biosciences	I, II, III
PerCp-Cy™5.5	STAT1 (pY701)	N/A	4a	BD Biosciences	I, II, III
PerCp-Cy™5.5	STAT3 (pY705)	N/A	4/P-STAT3	BD Biosciences	I, II, III
PE-Cy™7	NF-κB p65 (pS529)	N/A	K10-895.12.50	BD Biosciences	I, II, III
PE-Cy™7	p38 MAPK (pT202/pY204)	N/A	36/p38	BD Biosciences	I, II, III
PE-Cy™7	STAT5 (pY694)	N/A	47 / STAT5(pY694)	BD Biosciences	I, II, III
Alexa fluor® 647	STAT4 (pY693)	N/A	38/p-STAT4	BD Biosciences	I, II, III
Alexa fluor® 647	STAT1 (pS727)	N/A	K51-856	BD Biosciences	I, II, III
Alexa fluor® 647	STAT3 (pS727)	N/A	49/p-STAT3	BD Biosciences	I, II, III
Qdot® 605	CD45	Leukocytes	HI30	Molecular probes	IV
APC-Cy™7	CD16	Granulocytes, monocytes, NK cells	3G8	BD Biosciences	IV
FITC	CD14	Monocytes	MEM-18	Immunotools	IV
Horizon V500	CD3	T cells	UCHT1	BD Biosciences	IV
Pacific blue™	CD4	Helper T cells	RPA-T4	BD Biosciences	IV
PerCP-Cy™5.5	CD8	Cytotoxic T cells	RPA-T8	Biolegends	IV
PE-Cy™7	CD56	NK, NKT cells	NCAM16.2	BD Biosciences	IV
Alexa Fluor® 647	CD20	B cells	2H7	Biolegends	IV
PE	CD38	Activation marker	HB7	eBiosciences	IV

The antibodies used for the identification of cells through identification of combinations of certain cell surface molecules were chosen based on their ability to differentiate the main cell subsets in peripheral blood (paper IV) or PBMC (paper I – III). Antibodies used in the analysis of intracellular cell signaling pathways were chosen due to ability to be induced by TLR7 and -9 stimulation or IFNα and prominent roles in inflammatory or anti-inflammatory immune responses.

All antibodies used were titrated for their specific experimental conditions to find optimal staining for phenotypic and phospho-antigens. For papers I – III, antibodies to phospho-antigens were titrated on unstimulated and stimulated samples to find the concentration required to maximise fold change. Additionally, final concentrations were chosen where small changes in antibody concentrations gave little differences in fold change. Phenotypic markers were titrated to give good separation of negative and positive populations, while minimising high levels of staining which might spillover into channels used to measure phospho-antigens.

For paper IV phenotypic and activation markers were titrated to maximise the difference between the negative and positive population where no shift of the negative due to non-specific binding occurs. This is important for the successful use of fluorescent minus one controls. Additionally, barcoding dyes (papers I – III**)** were titrated with concentration of dyes in the 3 x 3 matrix selected based on their ability to resolve each sample in the matrix, as well as minimising spillover into critical channels as assessed by a barcode only control.

Antibodies used in phosphoflow **(**papers I − III**)** were tested with their respective fixation and permeabilization agents as epitopes are sensitive to fixation and permeabilization agents which can result in a loss of recognition and changes in staining intensity of antibodies.

Choice of fluorophores used in the antibody panel were based on wavelength of available lasers and the filters on the flow cytometer with bright fluorophores used for rare antigens, and dim for common antigen (CD45, CD3); however some concession had to made to to availability. For example, we had to use the use PerCp-

Cy™5.5 for ERK1/2, STAT1 (pY701) and STAT3 (pY705), which is relatively dim. Choices were also based on minimising spillover into important and sensitive channels reducing the need for compensation and subsequent introduction of error.

3.5. Fluorescent cell barcoding

Papers I − III utilized fluorescent cell barcoding of PBMCs as outlined in Krutzik *et al.* 2011 [182]. Following fixation and permeabilization with methanol, PBMCs were stained according to a 3 x 3 barcoding grid using 3 levels of pacific orange and pacific blue succinimidyl ester dyes prior to combining the 9 samples together. The dyes are reactive to amine functional groups present primarily on protein lysine side chains and at the N-terminus, while non-reacted dyes are subsequently washed off [182]. This allows for simultaneous antibody staining and data acquisition, with the different samples being distinguishable during software analysis based on their fluorescence intensity of the barcoding dyes. The procedure eliminates variation between samples measured together thus increasing assay robustness while also reducing antibody consumption and sample acquisition time. Because of the reduction in antibody consumption and increased throughput the method is suited to tasks requiring the measurement of many samples [182].

3.6. Flow cytometric controls

Critical to successful application of flow cytometry is the use of appropriate controls. Controls are essential in flow cytometry as they provide context from which one may interpret test samples. Flow cytometry experiments commonly contain at least three types of controls: setup, gating controls and biological comparisons controls [183]. Setup controls are used in the setup or checking the setup of the instrument. Gating controls are used to help distinguish specific from non-specific binding. This allows for determination of positivity and negativity and accurate setting of gates. Biological controls are those that provide biologically relevant comparison conditions, for example healthy donor samples. A comprehensive overview of the subject can be found in a paper written by Maecker and Trotter 2006 [183].

Various flow cytometry controls were utilized for each assay. All flow cytometry based assays included in the thesis (papers I − IV) utilized BD cytometer setup and tracking beads for determining minimum baseline PMT voltages for assays, and monitoring cytometer setup and performance (laser alignment, laser time delay, sensitivity etc.). In addition, initial experimental setups utilized single antibody stained and unstained cell samples were used for optimization of PMT voltages. Additionally, PMT voltages were set to minimise spillover into channels for measurement of phospho-epitopes to reduced measurement errors caused by compensation. This was of particular importance in barcoded samples (paper I – III) where a barcoded sample that has not been stained with antibody (Barcode only control) was also used to check that the median fluorescence intensity (MFI) on other parameters of interest did not fluctuate in response to barcode intensities.

All experiments (papers I − IV) included single fluorescent stained compensation controls (beads or cells) for the measurement and removal of fluorescent spillover before subsequent analysis. Fluorescent minus one controls where samples that include all antibody conjugates present in test samples minus one conjugate were used in the setting of cell population gates in paper IV when there was no clear division between positive and negative population. In stimulation assays (papers I − III), unstimulated samples were used to distinguish positive from negative events. In additional the assays utilized cryopreserved PBMC from a single donor, frozen in multiple aliquots, thawed and processed with each experimental run unstimulated and stimulated as a positive control to monitor inter-assay variation and for inter-assay normalization.

For quantitative analysis (paper IV) internal microsphere counting standards were measured co-currently with cells during sample acquisition.

3.7. Collection of peripheral blood and cryopreservation of PBMC

In all papers peripheral blood from pSS patients or healthy individuals was utilized. Patients were recruited from the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway (papers II − IV). All patients fulfilled the pSS AECC [140] and displayed no additional autoimmune diseases or lymphoma. Healthy individuals were recruited from the blood bank at the Haukeland University Hospital in Bergen, Norway (papers I − IV). All individuals provided written informed consent. The study was approved by the regional ethical committee (#2009/686). Samples from pSS patients and healthy individuals were collected in parallel to reduce the influences of seasonal effects.

Because blood samples from patients were often sporadic, cryopreservation (papers I – III) was used to minimise time and possible time associated variation between assays, as well as maximize the number of samples analysed per assay. To maximise cell recovery, cell health and cellular response following cryopreservation and thawing, serum support mediums such as fetal calf serum are commonly used during freezing. To avoid unspecific stimulation that could result from use of fetal calf serum and possible batch to batch effects, we used a non-Animal Origin, Chemically Defined Freeze Medium- ProFreeze^{TM}-CDM, in addition to the serum free cell media X vivo-20^{TM} and dimethyl sulfoxide. PBMC samples were frozen with a CoolCell® freezing chamber at -70 °C overnight before being moved to a -150 °C for long term storage. The CoolCell® freezing chamber ensured a consistent and reproducible -1 °C/minute cell freezing rate [184]. This controlled temperature reduction and the use of a cryoprotectant such as dimethyl sulfoxide minimises damage to the cells by increasing permeability of the plasma membrane, as well as disrupting the formation of larger ice crystals [184, 185]. Thawing was accomplished rapidly (37°C) to avoid ice recrystallization and osmotic stresses, and based on reported higher cell viability and recovery than passive thawing [184].

3.8. Stimulation of patient and healthy donor PBMC

In papers I – III PBMC samples were stimulated *in vitro*. Stimulants were titrated, concentration of stimulants that produced robust and strong responses were used in subsequent assays. Sterile conditions were adhered to as to prevent contamination with bacteria or other compounds which could activate pathways in cells. Stimulation was performed following a 2 hour resting period to allow for a reduction in basal levels of cell signaling in PBMC following thawing. Stimulation was performed in a chemically defined, serum-free hematopoietic cell medium- X vivo-20^{TM}, as opposed to media supplemented with fetal calf serum which is commonly used. The reasoning behind this was to avoid batch to batch variations of fetal calf serum, as well as to avoid potential stimulation of immune cells by components of fetal calf serum.

In papers II and III the stimulants IFNα2b, TLR7 ligand CL097, TLR9 ligand ODN 2006 and TLR9 ligand ODN2395 were used. ODN 2006 and ODN 2395 are type B and C CpG oligonucleotide, respectively. CpG ODNs are synthetic oligonucleotides that contain unmethylated CpG dinucleotides in a particular context, and are present in greater frequency in bacterial and viral DNA compared to human DNA [186]. Type C triggers IRF7 mediated intracellular signaling pathways from early endosomes leading to strong IFNα induction, while type B and C stimulates NF-κB mediated signaling from late endosomes resulting in strong B cell activation [187]. CL097 is a derivative of the imidazoquinoline compound R848 and mimics viral components (ssRNA). TLR7 and -9 ligands were used in assays as viral infection is a suspected trigger behind pSS. In addition, pSS is associated with a type I IFN signature, with TLR7 and -9 activation capable of inducing pathways involved in the production of type I IFNs. IFNα2b was used because as one of the major type I IFNs it is capable of inducing type I IFN gene expression through activation of the JAK / STAT pathway.

3.9. Gene expression

In papers II and III gene expression of MxA, 2'-5' oligoadenylate synthetase 1 (OAS1), interferon-induced protein 44 (IFI44), Guanylate Binding Protein 1 (GBP1) and 18S rRNA was measured, to investigate relationship between IFN responsive gene expression and cell signaling. 18S rRNA was used as reference gene, and relative expression levels were calculated as $2^{-\Delta Ct}$. 18S rRNA was used as a reference gene because of its invariant expression level throughout tissues and cells. Additionally, 18S rRNA is seen to be more reliable than other commonly used reference genes [188].

The type I IFN-inducible genes MxA, IFI44 and OAS1, and IFNγ responsive gene GBP1 were measured because of the association of a type I IFN signature with increased disease activity in pSS [113]. MxA has been shown to be a reliable biomarker for identifying systemic type I IFN bioactivity in pSS patients [189], and is inducable by type I and III IFN [190]. IFI44 and OAS1 are strongly induced by IFN [191, 192], with IFI44 only activated by type I IFN and not type II [192]. GBP1 is most abundantly induced by IFNγ than IFNα [193].

The IFN score was calculated according to Feng et al. [194] 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 x SD of healthy controls was utilized.

Associations with increased type I IFN-inducible gene expression and patients with a positive type I IFN score with potentiated intracellular signaling pathways may indicate a mechanistic links between the features.

3.10. Data analysis

Initial visualization and analysis of flow cytometry data was either through cytobank [195] (papers I − III) or Flowjo (Tree Star) (papers II − IV). Identification of immune cell populations was based on light scatter properties or relative expression of CD markers, with culture conditions also identified in papers I – IV based on their staining intensity of amine-reactive dyes. In paper IV**,** leukocyte subtypes counts per microliter of blood were calculated based on event count within gate relative to a bead standard in the respective sample and total number of beads in the sample, with the absolute counts obtained by dividing the number of positive cell events by the number of bead events, and then multiplying by the BD Trucount^{TM} bead concentration. In papers I − III MFI was recorded in channels used to measure phosphor antibodies.

3.10.1. Principal component analysis

Multivariate data can be difficult to visualize and interpret because of the sheer number of data points, therefore principal component analysis (PCA) was used to identify important variables and visualize the datasets (papers II − IV). PCA is a mathematical algorithm that reduces dimensionality of the data while retaining most of the variation in the data set [196]. It does this by creating new uncorrelated variables that successively maximize variance and are linear functions of those in the original dataset. The new variables are termed principal components [196]. The largest variability is explained by the first principal component and each successive principal component explains less variability then the prior one [197]. Samples can then be visualized in a score plot allowing for the identification of patterns within the data [197].

Variables are visualized in loading plots and are interpreted in conjunction with the score plot to identify the influence each variable has on the spread of samples in the score plot. Variables are treated as vectors and the position of variables on the loading plot are informative of their relationship to one another [196]. 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 through either the software R version 3.3.1. (http://www.r-project.org/) (paper IV) or Unscrambler® (CAMO) (papers II − III).

3.9.2. Hierarchical clustering

Unsupervised hierarchical clustering was used for identification of subgroups within pSS patients in paper IV. Hierarchical clustering seeks to build a hierarchy of clusters where objects (pSS patients in this thesis) that are more similar cluster closer together. The basic process of hierarchical clustering involves- 1. Assigning each object to its own cluster, where distances between the clusters equal the similarities between the objects they contain. 2. Finding the most similar pair of clusters and merge them into a single cluster. 3. Computing distances (similarities) between the new cluster and each of the old clusters. 4. Repeating 2 and 3 until all objects are contained in a single cluster. Ward's method was used for hierarchical clustering in this thesis. Ward's method merges 2 clusters based on the size of an error sum-of-squares criterion, where each merger seeks to minimize the increase in the total within-cluster error sum of squares [198]. Hierarchical clustering was performed with the software R version 3.3.1. (http://www.r-project.org/) and R package FactoMineR [199].

3.10.3. Statistics

As papers II – IV were exploratory no post-hoc tests were conducted, results are reported in full in supplementary information. Because many measurements showed skewed distributions, non parametric unpaired Mann-Whitney tests were used for univariate comparisons of continuous measures − cell quantities and clinical data (paper IV) and MFI specific for cell type and time points (papers II and III), and correlations were performed using Spearman's rank test. Pearson's chi-squared test (χ^2) was used for comparisons of categorical data (paper IV). Repeated measures one-way analysis of variance, with the Greenhouse correction and Holm-Sidak's multiple

comparisons test was used when comparing fold change (arcsinh) in channels measuring phosphorylated proteins in PBMC for three different isolation methods (paper I). Fold change was calculated through Microsoft Excel using the formula (ASINH (MFI stimulated/cofactor)) – (ASINH (MFI unstimulated/cofactor)), with an assigned cofactor of 150. Coefficient of variation (CV) values for process triplicates reported in paper I were calculated in Microsoft excel from MFI values of target phospho-proteins normalized against their respective unstimulated samples. All statistical group comparisons were done through Prism 6 (GraphPad Software, Inc., USA).

4. Summary of the main results

Paper I

An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells.

A multiplex flow cytometry protocol was assessed for inter-assay precision for experimental variables (phospho-protein measured, cell type and stimulant). Coefficient of variations calculated from process triplicates of normalized MFI of phospho-proteins displayed median CVs under 10% when grouped according to cell type, stimulation agent and phospho-protein measured, while the CV for each triplicate did not exceed 20% indicating good reproducibility of the protocol. The protocol was used in papers II and III. Additionally, three blood collection methodologies were assessed − lithium-heparin tubes followed by density gradient centrifugation, and CPT sodium citrate or sodium heparin tubes. Heparin tubes were observed to give stronger activation following stimulation with recombinant human IFNα2b and PMA compared to citrate, with citrate strongly affected NK cell responses to stimuli, CPT based isolation methods were associated with higher basal phosphorylation [200].

The lithium-heparin peripheral blood collection protocol was therefore used for subsequent experiments (papers II and III).

Paper II

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

The multiplex flow cytometry protocol optimized in paper I was used to make quantitative basal measurements of the phospho-proteins ERK1/2, NF-κB p65, STAT1 (Y701), STAT1 (S727), STAT3 (Y705), STAT3 (S727), STAT4 (Y693), p38 and STAT5 (Y694) in T cells, B cells and NK cells from female pSS patients and age

matched female healthy individuals, and measurements over a 4 hour time period following TLR7 and -9 stimulation. Basal and TLR7 and -9 stimulation induced measurements showed significant differences in the phosphorylation profiles between samples from pSS patients and healthy individuals.

PCA showed that basal phosphorylation profiles could be used to differentiate pSS patients from healthy donor samples through stronger phosphorylation in NK and T cells relative to B cells. PCA using induced MFI at 15 minutes ($MFI^{15min} - MFI^{basal}$) after TLR7 and -9 stimulation showed a spatial shift of EGM- patients away from healthy individual samples, while spatial groupings of the majority of EGM+, SSA- and medicated patients overlapped with healthy individual samples. The loading plots indicated the shift was primarily through stronger induction of phosphorylation in B cells from EGM- for NF-κB, P38 and STAT3 S727 following TLR 7 and -9 stimulation.

74% of the patients had a positive IFN signature. These patients differed from the IFN signature negative patients regarding their phosphorylation profiles, in particular with increased induction of phosphorylation of P38, NF-κB and STAT3 S727 in B cells following TLR7 and -9 stimulation. TLR7 and -9 stimulation induced phosphorylation of P38, NF-κB and STAT3 S727 in B cells correlated with the gene expression of 3 type I IFN inducible genes.

Paper III

Aberrant cell signaling in peripheral blood mononuclear cells upon interferon alpha stimulation in patients with primary Sjögren's syndrome associates with type I interferon signature

The multiplex flow cytometry protocol optimized in paper I was used to make quantitative basal measurements of the phospho-proteins ERK1/2, NF-κB p65, STAT1 (Y701), STAT1 (S727), STAT3 (Y705), STAT3 (S727), STAT4 (Y693), p38 and STAT5 (Y694) in T cells, B cells and NK cells from female pSS patients and age

matched female healthy individuals, and measurements over a 4 hour time period following stimulation with IFNα2b.

Cells derived from pSS patients displayed small but significant increases in the basal phosphorylation level of numerous signaling proteins compared to cells from healthy individuals. The phosphorylation profiles following stimulation with IFNα2b differed significantly between pSS patients and healthy individuals, especially regarding STAT1 Y701.

PCA was used to further investigate the induced MFI at 15 minutes (MFI^{15min} – MFI^{basal}) after stimulation with IFNα2b and search for groupings which may be associated with SSA autoantibody and EGM expression. Spatial grouping pSS patient samples following PCA exhibited a skewed distribution away from healthy individual samples with movement dominated by induced phosphorylation of STAT1 Y701 in T cells, NK and B cells. Medicated and SSA autoantibody negative patients grouped closer to healthy individuals samples than non-medicated and SSA autoantibody positive patients.

A type I IFN signature was found in 64% of patients. Increased STAT1 phosphorylation in B cells was limited to type I IFN signature positive patients compared to healthy individuals and IFN negative patients. NK cells from type I IFN positive patients displayed reduced phosphorylation of STAT1 S727, STAT4 Y693 and P38, and increased phosphorylation of STAT1 Y701 compared to healthy individuals and type I IFN negative patients, even if not always reaching statistical significance. IFNα induced phosphorylation of STAT1 Y701 in B cells correlated with type I inducible gene expression.

Paper IV

Patients with primary Sjögren's syndrome have alterations in absolute quantities of various peripheral leukocyte populations

Flow cytometry was used for quantitative counts of leukocyte populations in peripheral blood of patients with pSS and age and sex matched healthy individuals. Comparisons of quantities of each immune cell subtype in peripheral blood were made between pSS patients and healthy individuals, SSA+ or SSB+ patients and SSA-SSB- patients, and EGM+ and EGM- patients. Reductions in the concentration of lymphocyte subsets and increases in monocytes and granulocytes in the peripheral blood of pSS patients compared to healthy individuals were observed. EGM+ and SSA+ or SSB+ patients exhibited greater reductions in lymphocyte subsets then EGM- and SSA-SSB- patients. Hierarchical clustering identified a subset of patients with higher concentrations of NKT cells, CD56hi NK cells, CD20+CD38- B cells and CD8+CD38- T cells that was associated with weaker clinical symptoms than the other clusters as indicated by their lower prevalence of organ-specific EGM and SSA and/or SSB autoantibodies, possibly marking a milder disease phenotype.

5. Discussion

The developed phosphoflow protocol presented in paper I was reliable as given by reported CVs between assays. The largest variations in phosphorylation measurements were observed for monocytes, followed by CD3+CD56+ cells and CD20+ B cells. A number of factors could be attributed to this observation including the number of cells measured; CD3+ T cells which were the most abundant cell subset analyzed displayed the lowest level of variation for example. Additionally, variation in the health of cells might have influenced the result, different cell subsets may respond to freezing / thawing differently or react differently in culture.

The inclusion of a fixable live-dead dye could further exclude dead cells by removing background due to the non-specific binding of antibodies to dead cells [201]. Prior screening of PBMC samples however indicated viability of greater than 95 % and negligible levels of apoptotic cells using Muse® Annexin V and Dead Cell Assay Kit. However, as this assay does not differentiate between cell subsets there could potentially be higher levels of apoptosis in rarer subsets. We cannot therefore exclude the effect of apoptotic cells in changes in signaling profiles in rarer subsets. This could be the case for signaling differences of NK cells observed in papers II and III. Increased pecentages of apoptotic NK cells are a feature of pSS patients [170]. Therefore differences in signaling observed in NK cells in pSS may be a consequence of this.

Greater variation was also observed in cells stimulated with LPS and PMA. The increased variation following LPS stimulation could result from the fact that the major responders are monocytes and B cells, therefore variation could be more associated with the cell type rather than the stimulant. Alternatively, variation may be a consequence of pathway kinetics following induction by the stimulant. Because of technical limitation, in particular multiple targets, 15 minutes was used in stimulations. This timescale is not optimal for all stimulants or phospho targets and may influence observed variation. For example, a rapidly decaying phosphorylation following PMA stimulation may increase error associated with small differences in

fixation times relative to other targets displaying less rapid phosphorylation / de-phosphorylation.

Variation was larger in measurements of STAT4 Y693, STAT1 S727, P38 and STAT5 Y694 than the other antibodies used in the assay. The greater variation in the phospho-epitope staining is likely a consequence of the strength of the signal, with STAT4 Y693, STAT1 S727 and P38 giving the lowest basal MFI measurements, and STAT5 Y694 showing the 5th lowest basal measurements of the 9 targets.

The use of lithium-heparin tubes for blood collection and density gradient centrifugation with lymphoprep™ for PBMC isolation gave consistently lower basal measurements than the isolation methods, utilizing CPT™ with sodium citrate or sodium heparin. The cause is unclear, but may result from lower viability, increasing levels of non-specific staining or difference in the induction of the pathways caused by the different isolation methods.

The use of lithium-heparin as an anti-coagulant was superior in retaining PBMC responsiveness compared to sodium citrate and sodium heparin over multiple signaling pathways and cell types.

Citrate in particular strongly reduced levels of phosphorylation in multiple signaling pathways. Interestingly, this effect was stronger in NK cells, in particular the decreased response of STAT4 Y693. One would expect that the differences in signaling could be due to the different mechanisms of action of both sodium citrate and heparin as anti-coagulants. Citrate acts as an anti-coagulant presumably by sequestering Ca^{++} ions [202], in contrast, heparin binds to and enhances the inhibitory activity of the plasma protein anti-thrombin against several serine proteases of the coagulation system [203]. A previous study comparing lithium-heparin, sodium heparin and sodium citrate anti-coagulants on cytomegalovirus-specific responses, analyzed by IFNγ assays on PBMC and whole blood, yielded higher CMV-specific responses in whole blood using lithium-heparin [204] also indicating an edge for it as an anti-coagulant of choice.

Measurements of induced and basal signalling in papers II and III demonstrated cell specific differences in phosphorylation of signaling molecules in PBMC between groups (pSS and healthy individuals, and presence of autoantibody or EGM). As mentioned previously, the strength of phosphoflow is the ability to resolve the phosphorylation status of proteins in individual cells. However, this benefit is in part dependent on the analytical methodology used downstream from these measurements.

During data analysis we used MFI in identified populations. Differences observed could therefore be a result of shifts of sub populations within the T, B and NK cell subsets rather than potentiation of a signaling pathways. Subpopulations could display different responses to the stimuli or basal phosphorylation levels thus affecting the MFI for the observed cell subset. Phosphorylation curves do not however indicate any sub-responses (e.g. bimodal curves), though shifts could be slight and still have a significant effect on median MFI of the population. Population shifts were observed in paper IV and by other authors [168, 169].

Basal phosphorylation was measured in both papers II and III, and comparisons between pSS patients and healthy individuals corresponded well. Greater spread of measurements and lower differences between pSS and healthy individuals were however observed in paper III. The differences are likely resultant from a change in the methodology between the two papers as mentioned previously.

We detected increased basal levels of STAT5 Y694 phosphorylation in T and NK cells. Increased basal levels of STAT5 phosphorylation have previously been shown in T cells from pSS patients compared to healthy donors [205]. However, in contrast to our study, the authors also found significant differences in B cells for basal phosphorylation of STAT5 Y694 and no differences for phosphorylation of STAT1 Y701 in T cells [205]. Moreover, Ramos et al. found significant differences in basal phosphorylation levels of STAT3 Y705 in T cells [206] which is in contrast to our findings. These differences are likely the result of the use of cryopreserved PBMCs and long culture period (6 hours) in our study, as both other studies used freshly isolated cells.

Increased induction of NF-κB, P38 and STAT3 S727 were observed in B cells following stimulation with TLR7 and -9 following exclusion of patient prescribed medications. Their induction was also stronger in patients without EGM. The cause or the functional relevance of the aberrant phosphorylation is unknown. B cells for their part do not differ in TLR7 and -9 expression between pSS patients and healthy individuals, which may affect their response to their respective ligands [207]. However, increased secretion of a number of cytokines and chemokines have been observed following stimulation of B cells with TLR7 and -9 ligands including IFNα following stimulation with TLR7 ligands [208]. Additionally, NF-κB can bind to promoter regions of many genes including proinflammatory cytokines such as TNFα and IL1β [79] which can be secreted by B cells [209]. TNFα and IL1β are present at higher concentration in peripheral blood of pSS patients along with increased numbers of TNFα and IL1β secreting cells [210, 211].

A number of polymorphisms associated with pSS and autoantibody presence in pSS could potentially effect signaling through NF-κB, P38 and STAT3 S727. TANK (TNF receptor associated factors family member-associated NF-κB activator) for example encodes a protein that is a negative regulator of proinflammatory cytokine production induced by TLR signaling, and is thought to suppresses TLR signaling by controlling the ubiquitination of TRAF [212]. Changes to its function could potentially affect all three of the fore mentioned pathways following TLR stimulation. Mice deficient in TANK spontaneously developed lupus-like autoimmune nephritis [212] indicating a role in autoimmunity, while pSS patients with germinal centre like structures shows a positive association with polymorphisms in the gene [213]. A20 (Antiapoptotic signaling protein) encoded by TNFAIP3 (tumour necrosis factor-alpha-induced protein 3) is also a negative regulator of TLR stimulated activation of NF-κB and polymorphisms in TNFAIP3 are associated with SS [214]. Additionally, an association with two polymorphism in TNIP1 (inhibitor of nuclear factor kappa-B kinase subunit epsilon) that encodes ABIN1 (A20-binding inhibitor of NF-κB) a repressors of NF-κB and of IKBKE (Inhibitor of nuclear factor kappa-B kinase subunit epsilon), which is an NF-κB activator, and antibody-positive

pSS has been observed in a Scandinavian cohort [215]. Many of these associations are however weak and often poorly reproducible indicating a possible redundancy in the system with many polymorphisms likely to have similar consequences on a pathway.

Increased STAT1 Y701 activation in response to IFNα confirms previous work of Pertovaara *et al*. 2016 [216] where B cells in peripheral blood from pSS patients exhibited significantly increased STAT1 Y701 phosphorylation following stimulation with IFNα, while T cells displayed a non-significant but increased response to IFNα stimulation [216]. Phosphorylation of STAT1 in B cells and monocytes from pSS patients has also previously been observed be potentiated following stimulation with IFNγ and IL6, and CD4+ T cells following stimulation with IL6 [216]. Therefore, as noted by Pertovaara, *et al*. 2016 [216] it seems unlikely that differences are through receptor expression and more likely through commonalities in their signaling pathways.

Increased responses through STAT1 following IFNα stimulation in pSS patients is in contrast to SLE patients, where decreased responses have been observed [217]. Interestingly, increased serum levels of IFNα have been found in SLE patients most notably during disease flares [218, 219], while serum IFNα concentrations from pSS have differed significantly with groups finding no change [220], decreases [221] and increases [222, 223] compared to healthy individuals. Approximately half the patients in both diseases display a type I IFN signature [108, 113]; the fore mentioned differing response to IFNα may indicate different mechanisms behind increased type I IFN gene expression. A primarily TLR driven increase in IFNα production leading to up regulation of IFN regulated genes may dominate in SLE leading to increased type I IFNs, whereas pSS patients show an increased responsiveness to type I IFN. The existence of two differing mechanisms driving increased IFN regulated gene expression in pSS could explain the inconclusive efficacy of HCQ in pSS patients [224-226] in contrast to SLE [227]. Because the therapeutic effect of HCQ is attributed to its suppression of endosomal TLR activation [228], it would likely be

more effective in treating diseases with a primarily TLR driven pathogenesis or co-morbidities.

In contrast to STAT1, IFNα induced repression of phosphorylation of numerous other signaling proteins were observed. STAT3 S727 for example, although not significant in our study, indicated slightly reduced phosphorylation following IFNα stimulation in T and B cells, which was significant in T cells following removal of medicated patients, while phosphorylation of STAT5 in B and T cells was reduced. This finding was similar to reports for SLE, where Huang *et al*. 2011 observed reduced responses of STAT3 and STAT5 to IFNα in T and B cells [217]. Additionally, a similar pattern of responses (increased STAT1 induction and reduced induction of other) 120 minutes following TLR7 and -9 stimulation, further indicating a predisposition to STAT1 activation over other pathways.

Papers II and III documented strong deregulation in NK cell signaling pathways. As mentioned previously, NK cells display high basal expression of STAT4 and reduced STAT1 compared to other cell subsets [58]. This pre-disposes the cells for STAT4 activation by type I IFNs and IFNγ production [58]. Total STAT1 levels are then induced during viral infections as a result of IFN exposure and this change acts to promote the activation of STAT1 and promotes cell cytotoxicity but limits both the activation of STAT4 and IFNγ expression [58-60, 129].

Our study showed increased basal signaling by NK cells through STAT1 Y701 and STAT1 S727. Following exposure of NK cells to IFNα the response through STAT1 Y701 was greatly increased, while responses through STAT4 Y693 were decreased. A similar response was also observed after 120 minutes following stimulation with TLR7 and -9 ligands, possibly through self stimulation with cell produced cytokines in culture. Such a profile would likely polarize NK cells in pSS towards a low IFNγ producing phenotype and increased cell cytotoxicity [58-60, 129]. However, no increase in NK cell killing ability has been observed on a per cell basis compared to healthy individuals in pSS patients [170]. Interestingly NK cells from pSS patients are hypo-responsive to IFNα induced cell cytotoxicity [229].

Whether this aberrant profile is an association with polymorphisms in STAT4 commonly associated with pSS [121] is unknown, however pSS is associated with a polymorphism in NKp30, an NK-specific activating receptor that regulates cross talk between NK and DCs and type II IFN secretion [230], indicating a possible role of the IFNγ / cell cytotoxicity axis in NK cells in pSS. Further, low relative phosphorylation of STAT4 to STAT1 in response to IFNα in NK cells resembles profiles of individuals with hepatitis C infections receiving IFNα therapy [59]. Our finding of higher STAT1 to STAT4 activation is perhaps not surprising with systemic autoimmunity being noted to mimic many features of viral immunity [231].

STAT3 was also reduced in NK cells 120 minutes following TLR7 and -9 stimulation. Activation of STAT3 is involved in the prevention of apoptosis and enhances cell survival and proliferation [232]. Reduced STAT3 activation in NK cells may therefore be associated with the decreased numbers of NK cells, decreased cytotoxicity and increased percentages of apoptotic NK cells documented in pSS patients [170].

PCA utilizing the IFNα induced sample dataset indicated a stronger induction of STAT1 Y701 by IFNα in B cells of SSA autoantibody positive patients. The connection between type I IFN and activation of autoreactive B cells is well established through observation of patients undergoing IFNα therapy developing *de novo* autoantibodies or increased titers of pre-existing autoantibodies [233, 234]. Similarly B cells displayed stronger induction of NF-κB and STAT3 S727 following TLR7 and -9 stimulation in SSA autoantibody positive patient. These differences could represent shifts in B cell subpopulations, for example pSS patient have shifts in B cell subpopulations based on their expression of CD38 and IgD [235]. Different subpopulations could display different levels of signaling components, such changes have been documented during B cell development for NF-κB complexes [236]. Differences in subset responses to TLR7 and -9 stimulation as well as IFNα therefore could explain the displayed potentiated signaling in pSS patients.

PCA following TLR7 and -9 stimulation also suggests that it is possible to subdivide pSS patients based on presence of EGM. EGM- patients displayed enhanced TLR responses through NF-κB, p38 and STAT3 S727 in B cells compared to EGM+ patients. This finding was regardless of the use of glucocorticoid prednisone, which inhibits NF-κB activation [237], and HCQ which inhibits TLR7 and -9 signaling [238]. The reason for the lack of EGM in patients with increased signaling following TLR7 and -9 stimulation is unclear, of interest is the observation that the same patients with high TLR signaling all showed lower responses to IFNα (data not included in study) suggesting a negative regulatory effect or differing pathogenesis. SOCS1 mRNA expression levels have been found to be up-regulated in PBMC from pSS patients [216] and can be induced by TLR ligands including CpG-DNA [239]. Additionally, SOCS1 mRNA expression in these patients did not correlate with the IFNα, IFNγ and IL-6-stimulated pSTAT1 levels in B cells and monocytes or with the IL-6-stimulated pSTAT1 levels in CD4+ T cells which could potentially induce the expression of SOCS1 mRNA [240]. As SOCS1 can suppress signaling from a number of cytokine, IFNα included [241]. It is possible the lower responses of these patients to IFNα could result from a TLR driven increased expression of SOCS1.

Patients prescribed medication displayed increased responses through STAT3 S727 in T cells following IFNα stimulation, although little effect of the medication on STAT1 Y701 activation was observed. This indicates medication could at least in part affect the relative responses of the JAK/STAT pathways perhaps indirectly through inhibiting TLR induced expression of products regulating or promoting IFNα responses, whether this translate into therapeutic benefits is uncertain.

The expression of type I IFN genes (OAS1, IFI44 and MxA) in PBMC from pSS patients showed a positive correlation with induction of STAT3 S727, NF-κB and P38 phosphorylation in B cells in response to TLR7 and -9 stimulation.

Associations with increased induction by TLR7 and -9 and increased type I IFN gene expression could exist for a number of reasons. For example, promoters for early type I IFNs (IFNα4 and IFNβ) have NF-κB response elements that are essential for

constitutive and early expression of IFNβ following viral infection [242], therefore increased responsiveness of NF-κB could potentially drive increase early type I IFN expression. Additionally, NF-κB is activated together with IRF3/IRF7 following direct viral invasion of the cytoplasm through cytoplasmic RNA helicases [6]. Together they induce antiviral genes, including those that encode IFNα and IFNβ [6], potentiated NF-κB signaling could thereby affect this induction. Further, as many of the same components also function in NF-κB, STAT3 S727 and P38 TLR driven responses as in other type I IFN inducible pathways, it is plausible higher levels of type I IFN gene expression is indirectly associated with the increased responses of STAT3 S727, NF-κB and P38.

Alternatively increased expression of type I IFN induced genes could potentiate TLR7 and -9 driven pathways through changing the relative positive and negative regulators of the fore mentioned signaling pathways.

Induction of STAT1 Y701 in B cells following stimulation with IFNα showed a positive correlation with the expression of Type I IFN genes, while the majority of other pathways showed negative correlations. This indicates that potentiation of this pathway could play a role in type I IFN induction or induction of type I IFN could play a role in the potentiation of these pathways.

Absolute counts of T lymphocytes were observed to be decreased in pSS patients compared to healthy individuals primarily through a non-specific loss of CD4+ T cells and to a lesser degree CD8+ through the loss of inactivated cells. Additionally, pSS patients displayed reductions in B cells through the reduction of inactivated cells as well as reductions in NK cells. These findings are in line with previous reports showing reductions in lymphocyte subsets in peripheral blood of pSS patients compared to healthy individuals [168-171].

The losses in CD38- B cells and CD38- CD4+ and CD8+T cells, as well as NK cells tended to be stronger in SSA+ or SSB+ patients than SSA-SSB- patients as well as EGM+ patients compared to EGM- patients. Previous haemocytometer based counts of leukocytes in peripheral blood by Sudzius *et al.* 2015 [168] have shown that both

SSA-SSB- and SSA+ or SSB+ pSS patients, display decreases in absolute counts of T cells, CD4+ T cells and CD8+ T cells compared to healthy individuals. The decreases were more pronounced in SSA+ and/or SSB+ patients. In addition, autoantibody positive patients had decreases in NK and NKT cells compared to healthy individuals.

Their findings correspond well to our observations with larger decreases in our study observed in SSA+ patients, although quantities of T cells in SSA- patients and healthy individuals were not significantly different, nor were quantities of CD8+ T cells for SSA-SSB-, and SSA+ or SSB+ compared to healthy individuals. In addition absolute counts using a mass cytometer by Mingueneau *et al.* 2016 [169] similarly found reductions in CD4+ T cells for both SSA+ and SSA- pSS patients, and NK cells in SSA+ pSS patients compared to healthy donor samples but no differences in B nor CD8+ T cells in either group.

In our previous papers (II and III) increased activation of STAT1 Y701 was observed, both basal and following induction with IFNα. Signaling through STAT1 is a requirement for the antiproliferative effects of IFNα [243]. The increased activation of STAT1 may in part explain the low counts of lymphocytes in pSS observed by us and others [168, 169]. IFNα has previously been speculated to in part explain lymphopenia observed in SLE patients [218], while lymphopenia has been hypothesised as a requirement in the induction of autoimmunity [244, 245] , with the two-hit model of autoimmunity stating that autoimmunity requires two insults to hit together − lymphopenia and the absence of responsiveness of T cells to TGF-β [246, 247]. Further, B-cell activating factor is expressed at higher concentrations in serum of pSS patients [248, 249] and can rescue high-affinity self-reactive B cells from peripheral deletion. This has been observed to be inhibited at high levels of intercellular competition [250]. An increased response to IFNα therefore could play a role in pSS pathogenesis through its antiproliferative effects. Although we do not have direct evidence of a connection between lymphopenia and increased IFNα responses, both lower lymphocyte counts and stronger IFNα response through

STAT1 Y701 were similarly positively associated with incidences of SSA autoantibodies and prevalence of EGM in patients.

Peripheral blood cell counts likely reflect systemic inflammation, organ involvement in the disease, systemic disease activity, as well as medical therapy which is discussed later. As mentioned previously lymphopenia is likely involved in autoimmune etiology and pathogenesis. Thereby non-specific decreases in lymphocyte counts may provide an indicator of autoimmunity with reduced counts also in RA [251], insulin-dependent diabetes mellitus [252] and SLE [253] among others, and inflammation in general, and not a specific disease. The use of cell count in diagnosis however is not without precedent in rheumatology where presence of lymphopenia or leukopenia is used in SLE diagnosis in conjunction with other measures [254]. Although lymphopenia may give an indication of worsening disease, it is unlikely to be reflective of an individual disease or disease subtype unless used in conjunction with other criteria or if changes reflect organ involvement or other disease differentiating features.

A number of factors limit the significance of the findings presented, in particular the use of medication by patients with in the cohort, sample size and clinical information available regarding the cohort.

Numerous patients included in these studies were receiving prednisolone or HCQ. HCQ inhibits TLR7 and -9 signaling [228, 255] and this was evident in reduction of TLR stimulation driven signaling in patients using the drug. Similarly the activation of NF-κB is inhibited by commonly used medications including sodium salicylate (aspirin) [256, 257] and ibuprofen [237].

Differences in cell concentrations between pSS patients and healthy individuals remained significant with the removal of patients receiving medication. However, this does not exclude the possibility that medications used by patients could similarly affect cell concentrations. Prednisolone has been observed to cause leucocytosis in patients with leucocytosis associated diseases, through increases in granulocytes, monocytes and lymphopenia [258]. The observations were temporal with increased

counts of monocytes and granulocytes for the 15 to 21 days of the study, while lymphocyte counts were decreased after 8 days and remained decreased for the reminder of the study. Another report following administration of prednisolone to individuals with fever of unknown origin showed rapid decreases in lymphocyte and monocyte counts, followed by a rebound in their number over a 24 hour period [259].

Little evidence in published literature indicates an association with HCQ and lymphopenia nor increased granulocytes or monocyte counts with the exception of HCQ inducing apoptosis in a dose and time dependent manner (up to 48 hours) in *in-vitro* cultures of lymphocytes from SLE patients and healthy individuals in a single study [260].

The reported effects of medications commonly prescribed to pSS patients indicate that interpretation of results could in part be confounded by their use, in particular prednisolone. Only a small number of patients included in this study were prescribed prednisolone (13 / 86), while more patients were prescribed HCQ (27 / 86). No information regarding time of administration or dose of medication relative to blood sampling time was avaliable. Further, medication is typically used to treat complications in pSS therefore discerning between the effects of the presence of EGM and the increased use of medication is difficult.

Sample sizes used in each study allowed for enough power to differ between healthy individuals and pSS patients, as well as basic subdivisions of the pSS cohort (EGM, autoantibodies and medication). Further dissections and comparisons of groups for example by EGM patients using medication resulted in very small group sizes increasing the possibility of chance associations, therefore these were not included.

The pSS cohort used in the study lacked information which may be beneficial in future studies. For example, we previously mentioned medication can have an effect on both quantities of immune cells, as well as intracellular signaling. Although the impact of medication was examined in our study, we have no knowledge on the dose or the time of administration of medication relative to sampling period. Additionally, ESSDAI (EULAR SS Disease Activity Index) which gives a single measurement of

disease activity [261] was not available in many patients included in the study so it was excluded from analysis. This feature could be used to assess whether degree of signaling or lymphopenia for example are associated with worsening of disease activity and should be included in future analysis. Further, time since diagnosis or presentation of clinical symptoms could enable the assessment of how measured variables may change over the course of the disease.

Subjects used as controls in the studies were obtained from the blood bank at the Haukeland University Hospital in Bergen. It is unlikely that profiles of these individuals would represent individuals commonly encountered in a clinical situation. Therefore their use in assessing markers for identification of pSS is questionable. More appropriate controls would likely include individuals displaying sicca symptoms.

6. Summary and conclusion

Measurements of basal and induced intracellular pathways in mononuclear cells from peripheral blood from pSS and healthy individuals are informative of sample identity (healthy individual or pSS patients) and the expression of autoantibodies or systemic manifestations in pSS patients. Increased phosphorylation of induced pathways can be further linked with a type I IFN signature in patients. Our results suggest that the type I IFN signature in pSS could result through increased responsiveness of TLR7 and -9 possibly through increased expression of type I IFNs, as well as enhanced activation of STAT1 by IFNα in these patients.

Cell concentrations of leukocytes in peripheral blood were also associated with sample identity (healthy individual or pSS patients), systemic involvement and autoantibodies and allowed for identification of a cohort without organ associated EGM.

Both the phosphorylation of signaling molecules and concentrations of leukocytes in peripheral blood are likely affected by anti-inflammatory and immune modulating drugs commonly used in the treatment of the disease, thereby limiting their potential use in disease stratification.

Because of the heterogeneity of the disease cohort, and non-independence of many factors, clear conclusion regarding how measured markers are influenced by disease manifestations is problematic.

Both measures however show promise for stratification and identification of pSS. Future research utilizing larger sample sizes and more defined cohorts is required, in particular further dissection of patient groups by EGM expression and medication use are therefore required in future work attempting to stratify the disease. Biomarkers for identification of pSS patients require more appropriate controls, for example individuals displaying sicca symptoms, in addition to potential biomarkers usefulness being assessed in relation to current classification criteria.

7. Future perspectives

A significant positive correlation was observed between the expression of type I IFN genes in PBMC and phosphorylation of STAT1 Y701 in response to IFNα in B cells. Of interest is whether the increased gene expression associates at a single cell level in a cell subset specific manner and whether this corresponds to the cell subsets displaying increased responsiveness; or throughout all immune cells. Analysis of type I IFN induced genes was conducted in PBMC without prior cell stimulation, measurements following IFNα stimulation in different cell subsets should also be conducted. Single cell analysis of gene expression [262] is therefore of interest. Similarly positive correlation between expression of type I IFN genes in PBMC and phosphorylation of NF-κB, P38 and STAT3 S727 following stimulation of TLR7 and -9 were observed. Whether this relates to a cell specific increased induction of type I IFN should be investigated, or other molecules involved in type I IFN pathways.

The enhanced TLR7 and -9 responses through NF-κB, P38 and STAT3 S727 in B cells in patients without EGM compared to those with EGM should be further investigated. In particular, the possibility of a greater use of medication not prescribed by the patient's rheumatologist by EGM+ patients such as sodium salicylate (aspirin) [256, 257] and ibuprofen [237] should be excluded, a more defined cohort is therefore required to confirm this observation. If confirmed, further investigating of these groups should include whether they display differences in single nucleotide polymorphisms associated with the pathways or whether the groups display different concentrations of components involved in the fore mentioned signaling cascades. Identifying pathologically different subgroups would help in identifying potential therapeutic targets in SS, for example patients with B cells showing increased induction of cell signaling following TLR7 and -9 stimulation may respond better to HCQ.

The cause of the increased pathway induction should be examined. As previously mentioned a number of polymorphisms have been associated with pSS which play a role in signal transduction. Subdividing patients by polymorphisms and associating

with signaling profiles may allow for identification of genetic profiles which lead to similar signaling profiles thus facilitating patient stratification. Additionally, it is likely that feedbacks taking part in the cell signaling cascades will change the relative concentrations of signaling components and their positive and negative regulators. Relative concentration at a single cell level should be investigated.

Finally, TLR7 ligand CL097 and TLR9 ligands ODN 2006 and ODN2395 were used in combination in the TLR signaling assay due to limited availability of PBMC for each patient. It will be informative to know whether the differences encountered in phosphorylation following induction by TLR stimulation is specific to a particular TLR.

A number of modifications could be made with regards to future studies attempting to identify potential biomarkers in pSS. Selection of targets in papers II and III was largely through a mixture of an unbiased approach and the use of a deductive reasoning. In contrast, targets used in paper IV reflect a general phenotyping panel to identify common cell types in peripheral blood. The identification of cells or combinations of cells associated with the disease or its progression through identification of cells lost to these processes or increased activation / migration may be more effective for patient stratification. For example Maehara *et al*. [263] described that the expression of Th2 and certain Tfh-related molecules were associated with lymphocytic accumulation and ectopic GC formation. Therefore follicular Th cells in pSS may be a suitable target for peripheral blood analysis if these are lost in the blood during these processes. Additionally, cells which have previously been observed to differ between similar diseases may provide information to help differentiate between similar autoimmune diseases. For example Szabo *et al*. [264] detected increased proportions of double-negative B cells and plasmablasts in SLE, while they were decreased in pSS. And as mentioned previously changes in B cell subpopulations based on their expression of CD38 and IgD have been documented in pSS patients [235] which appear to be specific to pSS when compared to RA and SLE [265].

The strong associations of autoimmunity with type I IFN suggest pDCs should be included in future analysis; the phosphorylation of IRF3, IRF5 and IRF7 which activate type I IFN genes following activation of TLR7 and -9 is of special interest. Signaling through RIG-1 is involved in the production of type I IFN in the majority of cell types and therefore measurement of this pathway should also be considered. Monitoring of IRF9 and STAT2 is also of extreme interest due to their involvement in type I IFN responsive antiviral pathways [266].

Because of limitations imposed on the number of targets that can be measured simultaneously by fluorescence spectral overlap in flow cytometry we limited our analysis to 8 different fluorochromes in 3 different panels in papers II and III in an attempt to minimize the effects on channels used for measuring phosphorylated proteins, while paper IV utilized 9 different fluorochromes in the analysis. Due to the exploratory nature of our analysis analyzing more targets simultaneously would be beneficial. Mass cytometry can allow for a more in depth analysis [267] as antibodies are labelled with isotopes instead of fluorochromes. Therefore fluorescent overlaps of emissions do not need to be removed during analysis as per conventional flow cytometry. This allows for the measurement of greater numbers of markers simultaneously allowing for greater refinement of interesting populations. Mass cytometry would allow for the analysis of over 40 markers simultaneously, allowing the user to ask questions about relative expression of each phosphorylated proteins to one in another in each cell. Greater resolution of cell subtypes will also provide further information, for example if these changes are due to shifts in cell subtype population of potentiated signaling.

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Technical note

An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells

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ABSTRACT

Phosphoflow cytometry is increasingly being used as a tool for the discovery of biomarkers used in the treatment and monitoring of disease and therapy. The ability to measure numerous phospho-protein targets simultaneously at a single cell level accurately and rapidly provides significant advantages over other methods. We here discuss important considerations required to successfully implement these methods. Three different blood collection tubes (lithium-heparin tubes, CPT with sodium citrate and CPT with sodium heparin) were evaluated, with PBMC isolated through lithium-heparin tubes/lymphoprep displaying reduced basal and increased stimulation induced phosphorylation compared to the other two methods. Further, we provide a protocol outlining an 8 color assay developed for the study of intracellular signaling in peripheral blood mononuclear cells. The assay allows for the quantitative measurement of the phospho-proteins ERK1/2, NF-κB p65, Stat1 (Y701), Stat1 (S727), Stat3 (Y705), Stat3 (S727), Stat4 (Y693), p38 and Stat5 (Y694), as well as the identification of T cells, B cells, natural killer cells and monocytes. The assay additionally incorporates fluorescent cell barcoding, reducing assay costs and increasing throughput while increasing data robustness. Inter-assay precision was assessed over a month long period for all experimental variables (phospho-protein measured, cell type and stimulant). Coefficient of variations (CVs) calculated from process triplicates of normalized median fluorescence intensity (MFI) of the phospho-proteins displayed median CVs under 10% when grouped according to cell type, stimulation agent and phospho-protein measured, while the CV for each triplicate did not exceed 20%.

1. Introduction

By using highly specific fluorochrome conjugated antibodies, flow cytometry allows for quantitative multi-parameter analysis of single cells within complex cell populations. The additional use of fluorochrome conjugated antibodies to the phosphorylated forms of molecules phosphorylated during signaling cascades allows for the analysis of signaling networks within complex cell populations such as peripheral blood mononuclear cells (PBMCs). This provides quantitative data of phosphorylation events at a single cell level; without the need for prior separation of the cells of interest (Krutzik and Nolan, 2003). The pathway of interest can be further investigated through its activation either in vivo or in vitro. This technique termed phospho-flow cytometry has shown significant promise in stratification of disease (Irish et al., 2004, Brown et al., 2015), monitoring disease progression (Cesano et al., 2013) and identification of biomarkers for monitoring therapeutic intervention (Gavasso et al., 2014). Additionally the method can be used in conjunction with fluorescent cell barcoding to enable a higher throughput while minimizing reagent consumption and maximizing data robustness through the multiplexing of samples prior to staining; making this method ideal for profiling of multiple samples (Krutzik et al., 2011).

We here provide a protocol outlining an 8 color assay developed for the study of intracellular signaling in PBMCs. In the procedure, PBMCs are stimulated, fixed, permeabilized and stained with 3 different antibody panels to identify various cell types - T cells, B cells and natural killer (NK) cells, and phospho-proteins-NF-κB p65 (pS529), ERK 1/2 (pT202/pY204), P38 (pT180/pY182), Stat4 (pY693), Stat1 (pY701), Stat1 (pS727), Stat3 (pY705), Stat5 (pY694) and Stat3 (pS727). Additionally, we utilize 2 amine reactive dyes, Pacific Orange™ (PO) and Pacific Blue™ (PB) to barcode 9 different samples, allowing for a higher

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throughput while minimizing antibody consumption. Further, we discuss important issues related to the testing and development of in-house assays such as the choice of the blood collection tube.

2. Materials and methods

A schematic representation of the work flow and gating strategy is shown in Fig. 1.

2.1. Antibodies

The following phospho-specific monoclonal antibodies were used in 3 different panels during the flow cytometry protocol: 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™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™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™), Alexa Fluor® 488 conjugated anti-CD20 (clone H1 (FB1), BD Biosciences) and PE conjugated anti-CD56 (clone N901, Beckmann Coulter, CA, USA).

2.2. Blood collection

Blood was collected at the blood bank at the Haukeland University Hospital in Bergen, Norway. All blood donors provided written informed consent. Peripheral blood was collected in Lithium-heparin tubes (BD diagnostics) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with lymphoprep™ (Axis-Shield, Oslo, Norway) as per manufactures instructions. For the experiments analyzing the effect of the isolation method, BD Vacutainer® CPT™ with Sodium Citrate or Sodium Heparin (BD diagnostics) were utilized. PBMCs were washed twice with phosphate buffered saline (PBS; Lonza, Basel, Switzerland) before being resuspended in ice cold 50% X-vivo 20™ and 42.5% Profreeze™-CDM non-animal origin (NAO) chemically defined freeze medium (both from Lonza) and 7.5% DMSO (Hybrid max, Sigma D2650) at 3 to 5×10^6 cells/ml. PBMCs were then frozen in a CoolCell® (Biocision; San Rafael, CA, USA) freezing chamber at −70 °C overnight before being moved to a −150 °C freezer for approximately 6 months.

2.3. Cell culture and stimulation

Before stimulation, cryopreserved PBMCs were rapidly thawed using a water bath set to 37 °C and washed once in prewarmed X-vivo 20™ by centrifugation at 300g for 5 min. The cells were then resuspended in X-vivo 20™ and rested at 37 °C at 5% CO_2 for 30 min before the cell

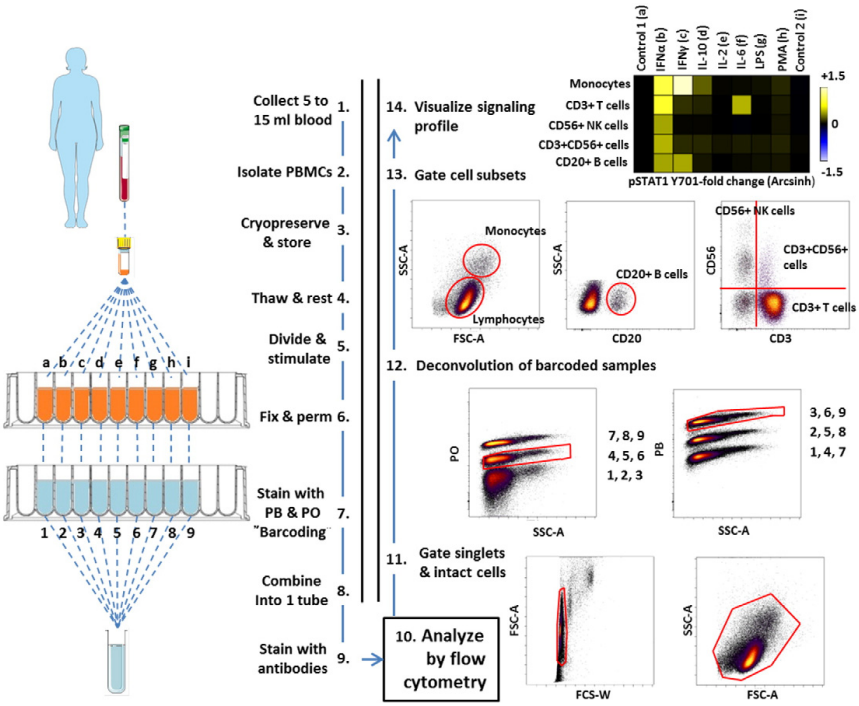

Fig. 1. Schematic representation of the work flow and gating strategy used in the analysis of intracellular signaling pathways in PBMCs. Blood was collected in Lithium-heparin tubes (BD diagnostics), and PBMCs were isolated by density gradient centrifugation before cryopreservation. At a later date PBMCs samples were thawed and rested in serum free media and then divided into 9 wells and stimulated as described in materials and methods. After stimulation, samples were fixed, permeabilized and stained with different concentrations of pacific blue (PB) and pacific orange (PO) dyes. Samples were washed to remove excess dye and combined into one tube and stained with fluorochrome conjugated antibodies as given in Materials and methods. Samples were analyzed on a flow cytometer, 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 PB and PO stains. Cell subtypes were identified based on their FSC-A and SSC-A scatter properties as either monocytes or lymphocytes. Lymphocytes were then subtyped as B cells (CD20+), T cells (CD3+CD56−), NK cells (CD3−CD56+) or CD3+CD56+ NKT cells based on surface antigen expression. Cells within each subtype were analyzed based on the change of MFI in each stimulation condition relative to the unstimulated reference sample. Data was analyzed, scatter graphs and heat maps were produced in cytobank (Kotecha et al., 2010).

concentration was adjusted to 1.25×10^6 cells/ml in X-vivo 20™ and 400 μl was dispensed into 9 wells of a Megablock® 96 well plate (Starstedt, Nümbrecht, Germany). The cells were rested at 37 °C with 5% CO_2 for 11/2 h to decrease basal phosphorylation levels. Following, the PBMCs were either left unstimulated (2 samples) or were stimulated for 15 min with cytokines (100 ng/ml), LPS (10 μg/ml; Sigma-Aldrich) or PMA (100 ng/ml; Sigma-Aldrich) at 37 °C with 5% CO_2. Recombinant human (rh) interleukin-10 CHO (IL-10), interleukin-2 (IL-2), interleukin-6 (IL-6), interferon-alpha 2 beta (IFNα2β), IFN-gamma (IFNγ) were from ImmunoTools (Friesoythe, Germany). Lyophilized cytokines and LPS were reconstituted in X-vivo 20™, while PMA was reconstituted in DMSO. All compounds were diluted in 100 μl X-vivo 20 ™ and added to 400 μl of cells resulting in a final cell concentration of 1×10^6 cells/ml during stimulation. All assays were performed in serum free media (X vivo-20™) to allow for a constant stimulation environment for the PBMCs and avoid unspecific stimulation.

2.4. Fluorescent cell barcoding and phospho-epitope staining for flow cytometry

PBMCs were fixed by adding 16% PFA (Electron Microscopy Sciences (Hatfield, PA, USA) warmed to 37 °C directly into the culture medium 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 1000 g for 5 min. The PBMCs 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 PBMCs 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 PBMCs were then washed once and the 9 different dye concentration/combination samples were combined into one sample. The sample was washed and incubated with 1 μl Fc receptor block (Miltenyi Biotec, Bergisch Gladbach, Germany) per 1×10^6 cells for 10 min on ice. Following, the sample was subdivided into 3 volumes 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.

2.5. 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. Gating was conducted as shown in Fig. 1: First, single cells were gated based on forward scatter area (FSC-A) and forward scatter width (FSC-W) properties, 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 PB and PO stains against SSC-A. Cell subtypes were identified based on their FSC-A and SSC-A scatter properties as either monocytes or lymphocytes. Lymphocytes were further subtyped as B cells (CD20+), T cells (CD3+CD56−), NK cells (CD3−CD56+) or CD3+CD56+ NKT cells based on surface antigen expression. The fold change (arcsinh) was calculated from median fluorescence intensity values (MFI) of unstimulated controls to MFI of each stimulated samples for each identified PBMC subtype. Fold change (arcsinh) was calculated through Cytobank or alternatively through Microsoft Excel using the formula (ASINH (MFI stimulated/cofactor)) - (ASINH (MFI unstimulated/cofactor)), with an assigned cofactor of 150. To assess the precision of the multiplexed assay complete process triplicates were run using cryopreserved PBMCs from 3 different donors over a 1 month period. MFI values of target phospho-proteins were normalized against their respective unstimulated samples and coefficient of variation (CV) values were calculated based on the normalized triplicate values. Additionally we cryopreserved PBMCs from a single donor with unstimulated and stimulated samples run in each assay for a positive control, inter-assay normalization, assessing assay to assay variability and calculating relative basal phosphorylation level in the unstimulated sample.

3. Results

3.1. The PBMC isolation method affects their signaling profile in a cell and pathway dependent manner

We first compared the effect of different PBMC isolation methods. The signaling profile of PBMCs separated using CPT sodium citrate or sodium heparin tubes as well as collection with lithium-heparin tubes followed by density gradient separation of PBMCs are shown in Fig. 2. The figure shows fold changes (arcsinh) for monocytes, T cells (CD3+), NK cells (CD3−CD56+), CD3+CD56+ NKT cells and B cells CD20+) measured in channels for pERK, pNF-κB, pP38, pStat1 S727, pStat1 Y701, pStat3 S727 pStat3 Y705, pStat4 Y693 and pStat5 Y694. Measurements were made for all 3 donor PBMCs and fold changes were calculated by comparing unstimulated samples to their respective stimulated samples for each donor and basal measurements were calculated against an unstimulated single donor control. The signaling profile was affected in a cell and pathway dependent manner. Differences among the groups were assessed through repeated measures one-way ANOVA, with the Greenhouse-Geisser correction and Holm-Sidak's multiple comparisons test, $p \ddot{} 0.05$ was considered significant. Basal measurements of cells derived from different PBMC isolation methods differed significantly. Lithium-heparin tubes followed by density gradient separation of PBMCs showed significantly lower basal phosphorylation of Erk1/2, P38, Stat1 Y701, Stat3 S727 and Stat5 Y694 in multiple cell types than those isolated through CPT sodium citrate or sodium heparin tubes. PBMC samples showed significant differences in fold changes following stimulation between PBMC isolation methods. Fold changes of PBMCs isolated using tubes with heparin additives were comparable, but differed significantly from the citrate tube. In particular a decreased phosphorylation fold change for NF-κB, Stat1 Y701, Stat3 Y705 and Stat5 Y694 following stimulation when compared to their respective unstimulated controls was exhibited. These differences were shown in multiple cell types but were most notable in NK cells, where for example pStat4 had mean fold change of 1.29 when peripheral blood was collected in lithium-heparin tubes compared to 0.337 with CPT tubes with sodium citrate. These decreases were often not corresponding with increases in basal signaling (e.g. Stat4 Y693 and Stat3 S727 in NK cells) and therefore do not explain the observed repression

3.2. Phosphoflow distinguishes cell and treatment specific signaling profiles

Examples of results from following the described method is shown as a heat map in Fig. 3A. The figure displays a scale indicating fold change (arcsinh) relative to their respective unstimulated sample. Different cell subsets in PBMCs are activated to different degrees dependent on the agent used in cell stimulation. Interferon α was shown to activate multiple phospho-proteins, this was strongly shown in pStat4 and pStat1 (Y701) and to lesser degrees pStat3 (Y705), pStat3 (S727) and pStat5, with the level of activation dependent on cell type. While

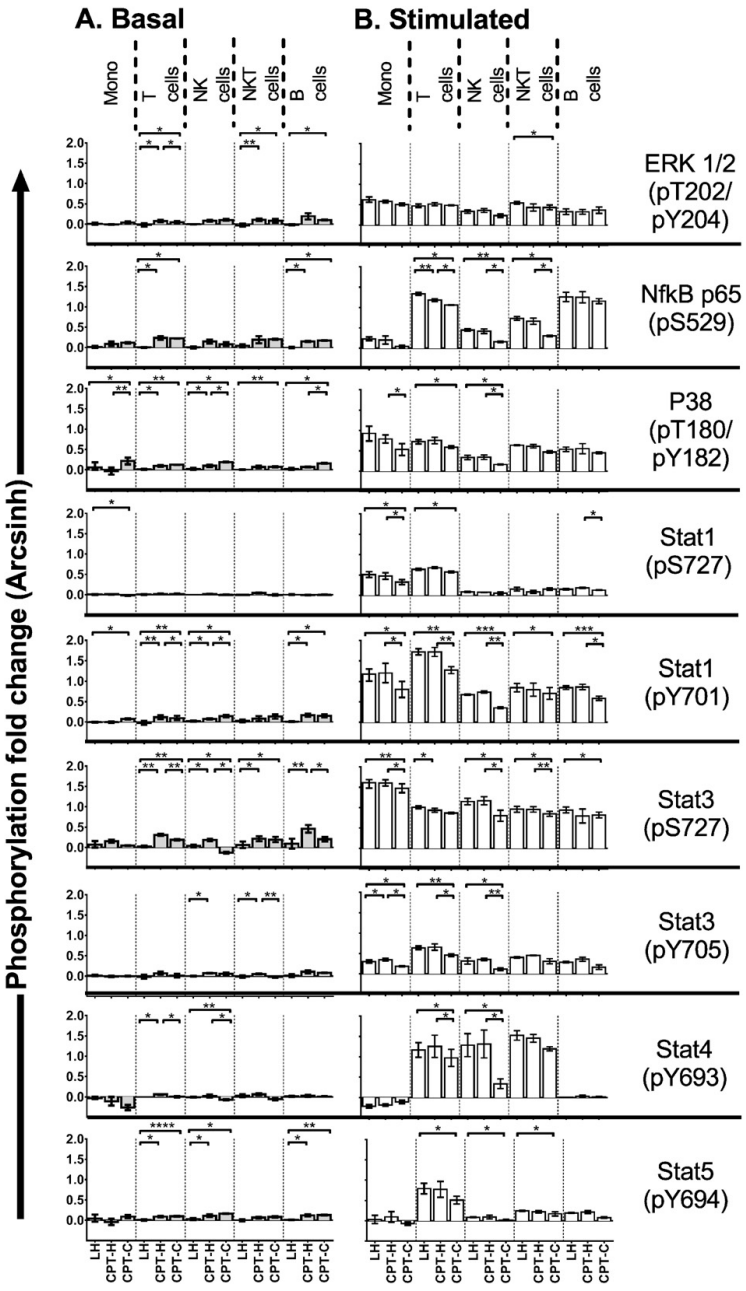

Fig. 2. Effect of PBMC isolation method on cell signaling. The Isolation method is displayed at the base of the figure with lithium-heparin (LH) tubes, CPT with sodium-citrate (CPT-C) or sodium-heparin (CPT-H) shown. Cell type is shown on top and phospho-protein measured on the right of the figure. The axis for phosphorylation fold change (arcsinh) following stimulation and basal fold differences (arcsinh) are shown on the left hand side of the figure. Basal fold differences are shown on the left hand side of the figure (A) and are represented by grey bars while fold changes following stimulation are shown on the right (B). The bars show the mean and standard deviation. The PBMC isolation method was shown to have an effect on degree of signaling in PBMCs after stimulation with PMA and interferon α for 15 min and basal levels. These effects varied with cell type and kinase measured. In most cases peripheral blood mononuclear cells isolated using CPT (CPT-H and CPT-C) showed higher degrees of basal phosphorylation then PBMCs isolated by lithium-heparin tubes and density gradient centrifugation. Heparin based isolation methods resulted in stronger post stimulation fold changes. A repeated measure one-way ANOVA, with the Greenhouse-Geisser correction and Holm-Sidak's multiple comparisons test was used to analyze differences between groups, significant p values are denoted as * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

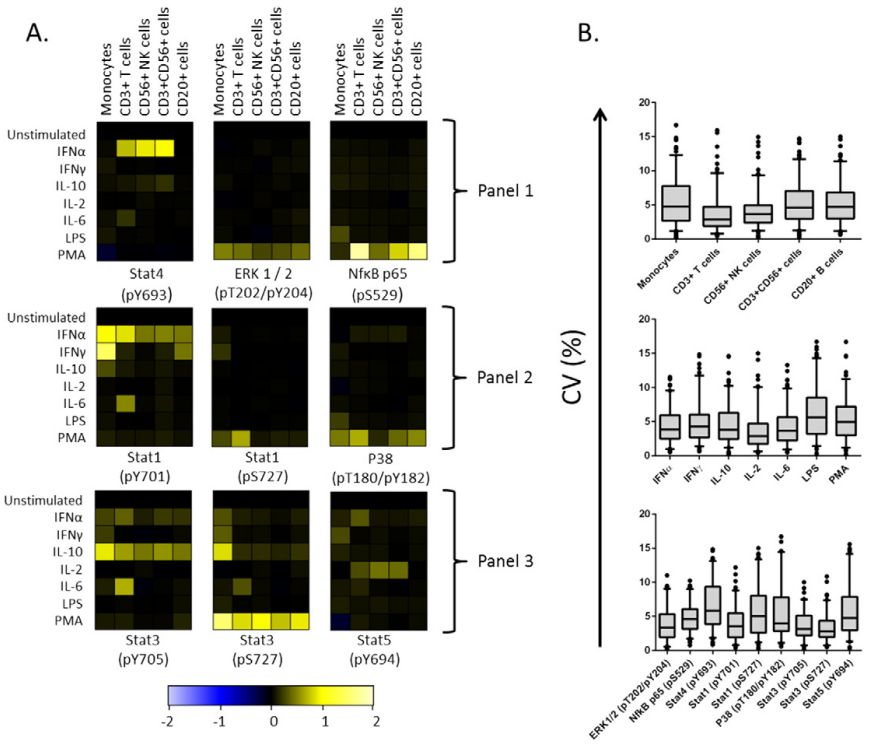

Fig. 3. Phosphoflow distinguishes cell and treatment specific signaling profiles. A. Heat map representation of the stimulation profile of PBMCs. Data was generated as outlined in the protocol with antibody panels used in the analysis shown to the right of the maps. All cells were gated according to the schematic shown in Fig. 1. The columns represent the cell subsets, T cells, B cells, NK cells, CD3 + CD56 + cells and monocytes. Each row represents a sample stimulated with a cytokine or chemical, with the phospho-specific antibody detected shown at the base of each map. The color of each block represents the fold change (arcsinh) in the channel corresponding to the analyzed phosphorylated protein relative to the un-stimulated sample, and the degree of change shown in the scale at the figure base. Data was analyzed and heat maps produced in cytobank (Kotecha et al., 2010). B. Precision of flow cytometry based phospho-protein measurements of PBMCs from 3 different donors. Analysis of PBMCs shows excellent precision of normalized MFI values of phospho-proteins. CV values for the multiplexed assays measured in complete process triplicates, grouped according to cell type analyzed (upper panel. $n = 189$), stimulation agent (medium panel. $n = 135$) and phospho-protein measured (lower panel. $n = 105$). Upper panel: The cell specific group median assay CVs were 4.76% (monocytes), 2.88% (CD3 + T cells), 3.64% (CD56 + NK cells), 4.61% (CD3 + CD56 + cells) and 4.73% (CD20 + B cells), with 95% of the results having CVs within 10% (CD3 + T cells and CD56 + NK cells) and 13% (monocytes, CD3 + CD56 + cells and CD20 + B cells). Medium panel: CV values grouped by stimulation agent showed medians of 3.84% (IFNα), 4.27% (IFNγ), 3.8% (IL-10), 2.88% (IL-2), 3.64% (IL-6), 5.62% (LPS) and 4.96% (PMA), with 95% of the results having CVs within 10% (IFNα and IL-6), 12% (IFNγ, IL-10, IL-2 and PMA), and 15% (LPS). Lower panel: CV values grouped by phospho-protein measured had median assay CVs of 3.36% (pERK 1/2), 4.62% (pNfκB), 5.81% (pStat4), 3.56% (pStat1(Y701)), 5.05% (pStat1(S727)), 3.98% (pP38), 3.16% (pStat3(Y705)), 2.80% (pStat3(S727)) and 4.79% (pStat5), with 95% of the results having CVs within 10% (pERK 1/2, pNfκB, pStat1(Y701), pStat3(Y701), pStat3(S727)) and 15% (pStat4, pStat1(S727), pP38, pStat5). Box plots show the medium value plotted as a line with each box displaying the distribution of the inner quartiles and vertical lines showing 95% of the data.

IFNγ activation profile displayed greater cell subset specificity, strong activation was limited to pStat1 (Y701), pStat1 (S727), pStat3 (Y705), pStat3 (S727) and pStat5 in monocytes; as well as pStat1 (Y701) in B cells. Interleukin-10 showed a greater degree of specificity in phospho-protein activation, strong induction was shown in pStat3 (Y705) in all cell types with relatively lower levels of activation of pStat3 (S727). Interleukin-2 activation was largely limited to activation of pStat5 in T cells, NK cells and CD3 + CD56 + NKT cells. IL-6 activation was more pronounced in T cells in particular pStat1 (Y701), pStat3 (Y705) and pStat3 (S727); while LPS displayed a higher levels of activation in monocytes of NF-κB and P38. PMA showed high phospho-protein activation in diverse cell types, with strong activation of ERK 1/2, NF-κB, P38 and Stat3 (S727).

3.3. Multiplex assay shows good reproducibility over phosphor-proteins, cell types and stimulations

To assess the precision of the established assay; PBMCs from 3 separate donors were isolated and cryopreserved. Subsequently on 3 separate occasions over a 1 month period, PBMCs for each donor were thawed and processed as described in the method. Data of measured MFI for each phospho-protein under each stimulation condition was normalized against the corresponding unstimulated controls for each phospho-protein, cell type and donor. The coefficient of variation (CV; 100 ∗ standard deviation/mean) was calculated for the 3 replicates for each normalized phospho-protein MFI measurement in each cell type and stimulation agent. The results generated were grouped for each phospho-protein, cell type and stimulation agent and are shown in Fig. 3B. The data indicates excellent precision of measurements; with robustness of the assay also shown to be dependent on cell type and phospho-protein measured, as well as stimulation agent. The assay was shown to be more precise in measurements of pERK 1/2, pNF-κB, pStat1 (Y701), pStat3 (Y705) and pStat3 (S727) than pStat4, pStat1 (S727), pP38 and pStat5 with median CVs of 3.36%, 4.62%, 5.81%, 3.56%, 5.05%, 3.98%, 3.16%, 2.80% and 4.79%, respectively, with 95% of the results having CVs within 10% (pERK 1/2, pNF-κB, pStat1(Y701), pStat3(Y701), pStat3(S727)) and 15% (pStat4, pStat1(S727), pP38, pStat5). CVs for the triplicate phospho-protein measurements of T cells (2.88%) and NK

cells (3.64%) were lower than those of monocytes (4.76%), CD3 + CD56 + cells (4.61%) and B cells (4.73%). 95% of the results had CVs within 10% for CD3 + T cells and CD56 + NK cells and 13% for monocytes, CD3 + CD56 + cells and CD20 + B cells. Additionally, assay precision was shown to be greater using cytokine based stimulation (median CVs of 3.84% (IFNα), 4.27% (IFNγ), 3.8% (IL-10), 2.88% (IL-2), and 3.64% (IL-6)) than LPS (5.62%) or PMA (4.94%). Still, 95% of the results had CVs below 10% (IFNα and IL-6), 12% (IFNγ, IL-10, IL-2 and PMA) and 15% (LPS).

4. Discussion and conclusion

The monitoring of phosphorylation in signal transduction pathways is increasingly being seen as a relevant tool in the treatment of disease with multiple research groups showing its promise in the monitoring (Huang et al., 2011) and prognosis (Brown et al., 2015) of disease, as well predicting and monitoring therapeutic treatments (Everson et al., 2014, Gavasso et al., 2014). Phosphoflow cytometry which can quantitate the level of phosphorylation in numerous phospho-protein targets simultaneously at a cell type-specific, single cell level provides an ideal tool for these research endeavors. Accurate measures require well tested and validated panels showing consistent and reproducible results. Here we presented 3 panels for an optimized 8 color multiplexed phosphoflow assays for the analysis of cryopreserved PBMC samples. The assays incorporates monoclonal antibodies specific for phosphoproteins ERK 1/2, NF-κB p65, Stat1 (Y701), Stat1 (S727), Stat3 (Y705), Stat3 (S727), Stat4, Stat1 (S727), P38 and Stat5. These targets were chosen as their activation is involved in numerous immune responses. Moreover, associations have been shown with dysregulation of intracellular signaling molecules involved in immune responses and autoimmunity (O'Shea and Plenge, 2012). The assay incorporates markers for common cell lineages in peripheral blood - T cells, B cells and NK cells; while monocytes were identified using their scatter properties. Additionally the assays utilize a 9× barcoding matrix allowing up to 9 samples to be measured simultaneously as 1 sample. This significantly reduces antibody consumption and flow cytometry acquisition times, significantly reducing the cost and time required to process and measure multiple samples, while additionally increasing data robustness. The use of heparin as an anti-coagulant was shown to be superior in retaining PBMC responsiveness compared to citrate. Citrate strongly affected NK cell responses to stimuli in multiple kinases, while CPT based isolation methods were associated with higher basal phosphorylation. Because of the varied response in PBMC subtypes we recommend prior testing before committing on blood collection methodology. Care should be taken to assess the isolation procedures effect on responses by different cell type. Additionally the resting period prior to PBMC stimulation should be optimized with regards to isolation protocol used to minimize basal signaling. As an example to illustrate the strength of this method we measured multiple phospho-proteins under multiple stimulation conditions simultaneously within a single barcoded sample in triplicates for 3 different donors. The assay showed excellent inter-assay precision over a month long period with CVs calculated against process triplicates of normalized MFI of the phospho-proteins displaying median CVs of under 10% when grouped by cell type, stimulation agent and phospho-protein measured, while the CV derived from each normalized triplicate measurement did not exceed 20%.

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Errata for
Flow cytometry based analyses as a tool in biomarker discovery for patient stratification in primary Sjögren's syndrome

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Thesis for the degree philosophiae doctor (PhD)

at the University of Bergen

R.A. Davies

(signature of candidate)

(signature of faculty)

14 Feb. 2017

Errata

Page 19, Figure 2

Figure 2 legend last sentence reads "*Figure adapted from Deenick and Tangye (2007) [26].*", should read *"Figure reprinted with modification by permission from Macmillan Publishers Ltd:Immunol Cell Biol (Deenick and Tangye [26]), copyright (2007)."*

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Page 51, Table 1

Late sentence in footnote for table 1 reads "Table from Vitali *et al.*, 2002 [140]." Should read "Table adapted by permission from BMJ Publishing Group Limited [*Ann Rheum Dis*, Vitali *et al.* [140], 61(6), 554-8, 2002]"

Reference 58

Reference 58 is listed as "Miyagi, T., S.H. Lee, and C.A. Biron, *Intracellular staining for analysis of the expression and phosphorylation of signal transducers and activators of transcription (STATs) in NK cells.* Methods Mol Biol, 2010. **612**: p. 159-75." Should be "Miyagi, T., M.P. Gil, X. Wang, J. Louten, W.M. Chu, and C.A. *High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells.* J Exp Med. 2007. **204**:2383–2396.