Evaluation of interferon-beta neutralizing antibodies in individual patients with multiple sclerosis based on phospho-specific flow cytometry

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Scientific environment

The work in this thesis has been carried out at the Neuro-immunology Laboratory and the Multiple Sclerosis Competence Center at the Department of Neurology, Haukeland University Hospital (HUS) and University of Bergen. Flow cytometry work has been carried out at the Hematology Research Laboratory (UiB), the Department of Immunology and Transfusion Medicine (HUS), and the Flow cytometry core facility (UiB).

Contents

SCIENTIFIC ENVIRONMENT……..2

CONTENTS……3

ACKNOWLEDGEMENTS…….....5

LIST OF ABBREVIATIONS……8

ABSTRACT…….9

LIST OF PUBLICATIONS……..11

1. INTRODUCTION……..12

1.1 Anti-drug antibodies……..12

1.2 Anti-interferon-beta antibodies in multiple sclerosis……13

1.3 Type I interferon-beta……...18

1.4 IFN-β signaling……..20

2. IMMUNOGENICITY EVALUATION IN IFN-β THERAPY……...24

2.1 *In vitro* identification and quantification of BAbs and NAbs ……...24

2.1.1 Binding antibody ELISA…….24

2.1.2 Neutralizing antibodies - CPE assay……...24

2.1.3 Neutralizing antibodies - Mx1 and MxA assasy…….26

2.1.4 Neutralizing antibodies - luciferase assay……...27

2.2 Evaluation of tests……..28

2.3 *In vivo* identification and quantitation of NAbs…….31

2.3.1 Mx1 induction assay…….31

2.3.2 Stats phosphorylation……...32

3. PHOSPHO-SPECIFIC FLOWCYTOMETRY……33

4. OBJECTIVES……..36

4.1. Multiplexed phospho-specific flow cytometry in immune cells……..36

4.2. *Ex vivo* IFN-β pathway activation and NAb effects in PBMCs and whole blood……..37

4.3. Analysis of IFN-β and NAbs in whole blood in vivo……...37

5. MATERIAL AND METHODS……...38

5.1. Background……...38

5.2. Phospho-specific flow cytometry…….39

5.3. INF-β ELISA of human sera……43

5.4. Type I IFN pathway-specific real-time quantitative PCR……43

5.5. Analysis, visualization and interpretation……44

5.6. Controls for phospho-specific flow cytometry…….45

6. RESUTLS……..47

6.1. Paper I - Multiplexed phosphoprotein analysis in immune cells……..47

6.2. Paper II - Flow cytometry and cell activation……..…….47

6.3. Unpublished data - Pathway specificity and Stats phosphorylation profiles in PBMCs……47

6.4. Unpublished data - IFN-β potency and pre-incubation effect……..49

6.5. Paper III - Immunogenic effects of recombinant interferon-beta therapy disrupt the JAK/STAT pathway in primary immune cells from patients with multiple sclerosis……51

6.6. Paper IV - Deficient phosphorylation of Stat1 in leukocytes identifies neutralizing antibodies in multiple sclerosis patients treated with interferon-beta……55

7. DISCUSSION……58

8. CONCLUSION……66

9. FUTURE PERSPECTIVES……68

10. REFERENCES……...73

11. APPENDIX……..85

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"For the mind does not require filling like a bottle, but rather, like wood, it only requires kindling to create in it an impulse to think independently and an ardent desire for the truth." —Plutarch, "On Listening to Lectures". (link)

“It is a miracle that curiosity survives formal education”. Albert Einstein

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Dedication

Vittoria De Cia and Pasquale Gavasso

List of abbreviations

BAb	binding antibodies
NAb	neutralizing antibodies
RRMS	relapsing-remitting multiple sclerosis
IFN-β	interferon-beta
MS	multiple sclerosis
CNS	central nervous system
MRI	magnetic resonance imaging
EAE	experimental autoimmune encephalomyelitis
MMP	matrix metallo proteinase
IFNAR1/2	interferon α/β receptors
Stat	signal transduction and activation of transcription
Jak	Janus kinase
Tyk	non-receptor tyrosine-protein kinase
ISGF3	IFN-stimulated gene factor 3 complex
IRF9	Interferon regulatory factor 9
ISRE	IFN-stimulated response elements
GAS	IFN-γ activated sites
ELISA	enzyme linked immunosorbent assay
CPE	cytopathic effect assay
Mx1	myxovirus resistant gene
MxA	myxovirus resistant protein
RT-qPCR	real-time quantitative PCR
SOCS	suppressor of cytokine signaling
SHPs	Src-homology 2 domain containing phosphatases
PCA	principal component analysis
PC	principal component
PLSR	partial least square regression
ANOVA	analysis of variance
MANOVA	multivariate analysis of variance
NU	neutralizing units

Abstract

Background: Recombinant human interferon-beta (IFN-β) is the most frequently used drug for treating relapsing-remitting multiple sclerosis (RRMS), a demyelinating disease of the central nervous system. It modulates the autoimmune state of patients by systemically affecting immune system functions. The treatment slows disease progression only in a subset of patients and treatment efficacy can be negatively affected if the patient produces neutralizing antibodies (NAb) to IFN-β. NAb in sera are quantified using *in vitro* bioassays; however, these assays do not reveal the immunogenic state of the patient and are not predictive NAb outcome.

Objective: Patient specific NAb assessment based on drug mechanism in primary cells from patients.

Method: A whole cell flow cytometry-based technique was developed to quantify the activation of the IFN-β signaling pathway by measuring the phosphorylation of signal transduction and activation of transcription family (Stat) molecules in primary immune cells and assess NAbs effects in individual patients. Peripheral blood mononuclear cells (PBMCs) and whole blood from patients with NAb were re-stimulated *ex vivo* with a serial dilution of IFN-β and levels of phosphorylation of Stat 1/3/4/5/6 transcription factors were quantified. To assess *in vivo* implications of NAb in an individual patient whole blood was collected before and after IFN-β injection, lysed, fixed, and analyzed for Stats activation with phospho-specific flow cytometry. RNA extracted from whole blood at the same time points was used to measure pathway specific gene expression changes in immune cells and sera was used to quantify IFN-β and NAb levels in blood after IFN-β injection.

Results: *Ex vivo* re-stimulation of PBMCs revealed that Stat signaling patterns were modulated in treated NAb-negative patients and inhibited in all treated NAb-positive

patients independently of NAb titers. Similar results were obtained when whole blood, rather than PBMCs, was re-stimulated *ex vivo*. *In vivo* results confirmed the inhibitory and modulatory effects of NAb on the Jak/Stat signaling pathway as assessed directly after INF-β administration. The Stat proteins affected by NAbs in PBMCs *ex vivo* were also responsible for much of the variation caused by NAbs *in vivo* after IFN-β administration. Levels of pStat1, of all Stat proteins, were highly correlated with NAb in both cases.

Conclusion: Immunogenic effects altered the response in primary cells even at low NAb levels, suggesting that results from cell line-based immunogenicity testing are not correlated with the immunogenic response in many patients. Based on the IFN-β responsiveness of primary cells three fundamental issues are addressed: 1. the mechanism of action of IFN-β, 2. the immunogenic effect of NAbs in individual patients, 3. underlying disease mechanisms.

List of publications

I. Gavasso S, Myhr KM, Vedeler C.

Multiplexed phosphoprotein analysis in immune cells. Acta Neurol Scand Suppl. 2006; **183**: 58-60.

II. Gavasso S.

Flow cytometry and cell activation. Methods Mol Biol. 2009; **514**: 35-46.

III. Gavasso S, Gjertsen B, Anderssen E, Myhr K, Vedeler C.

Immunogenic effects of recombinant interferon-beta therapy disrupt the JAK/STAT pathway in primary immune cells from patients with multiple sclerosis. Mult Scler. 2012; **18**(8): 1116-24.

IV. Gavasso S, Mosleth E, Marøy T, Jørgensen K, Nakkestad HL, Gjertsen BT, Kjell-Morten Myhr KM, Vedeler CA.

Deficient phosphorylation of Stat1 in leukocytes identifies neutralizing antibodies in multiple sclerosis patients treated with interfero-beta. (manuscript)

Introduction

1.1. Anti-drug antibodies

Biopharmaceuticals in use today are potentially immunogenic[1-3]. This means that at some point during therapy, the patient's own immune system can recognize the therapeutic agent as foreign and produce antibodies to the agent. Recombinant human proteins are used extensively to treat cancer and autoimmune diseases, and the effects of an activated immune response to the medication on efficacy of the drug are unpredictable[4-7]. The consequences of immunogenicity range from undetectable to severe. Responses are complicated, disease specific, and patient specific[8-10].

The biological systems used to produce recombinant human proteins for therapy range from bacterial to mammalian cells[7]. Even though only mammalian cells have the cellular machinery necessary to produce recombinant proteins that are the same as the human counterparts with regard to post-translational modifications, bacterial systems are widely used due to simplicity and low cost. In some instances, the amino acid sequence is changed relative to that of the wild-type protein. For example, changes may extend the half-life of the protein in the human body. A human protein that is altered either in amino acid sequence and/or post-translational modifications may be recognized as foreign by the immune system and induce a classical immune response. But what if the amino acid sequence is the same and post-translational modifications are in place? Why are these proteins immunogenic? This phenomenon may be explained by break of tolerance[5, 6, 11]. In addition to dosage, mode and frequency of administration are fundamental in break of tolerance. High frequency subcutaneous injections of recombinant proteins tend to be more immunogenic than low frequency and/or intramuscular injections, intravenous injections are least immunogenic.

Contaminants and vehicle media affect immunogenicity. Vehicle and storage conditions can induce aggregation, degradation, or precipitation of proteins. In such instances the bioavailability of the compound is reduced and the formed aggregates increase the likelihood of immunogenic effects in patients. Unlike these methodological problems that can be addressed and sometimes resolved, the genotype and the phenotype of a patient and the disease itself also contribute to immunogenicity. Currently we can only speculate why certain patients do or do not develop an immunogenic reaction or why, for example, cancer patients are less likely to mount an immune response to recombinant proteins than are patients with autoimmune diseases[12].

The possibility of severe adverse effects in patients treated with human recombinant protein mandates monitoring of immunogenic effects in patients. Ideally, this should be a functional test. Screening assays are used to detect any antibodies in sera of patients that bind to the drug with a certain affinity and are called binding antibodies (BAb). Cell line based bioassays are used to detect neutralizing antibodies (NAb) that interfere with the function of the drug.

1.2. Anti-interferon-beta antibodies in multiple sclerosis

In patients with relapsing-remitting multiple sclerosis (RRMS) treated with recombinant human interferon-beta (IFN-β), many factors have hampered international consensus on the NAb issue despite years of research. Identifying immunogenic effects of this pleiotropic immuno-modulatory drug in a disease with unclear pathogenesis is a challenge.

Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system (CNS). As the disease progresses destruction of the myelin sheath surrounding

neurons and axonal loss eventually lead to various neurological symptoms. The hallmark of the disease is focal inflammatory lesions in the brain and spinal cord of affected individuals, especially in the earlier phases of the disease. Ongoing inflammation in the CNS of patients is visualized by magnetic resonance imaging (MRI) and the contrast agent gadolinium. The images show myelin (fat) brighter than cerebrospinal fluid (water) and highlight areas of inflammation where the disruption of the blood brain-barrier has allowed the contrast agent to diffuse into the brain parenchyma. These *T1*-weighted gadolinium enhanced lesions are the most sensitive surrogate marker for inflammation in the CNS of a patient with MS (Fig.1). *T2*-weighted MRI images are used as surrogates for lesion accumulation in patients.

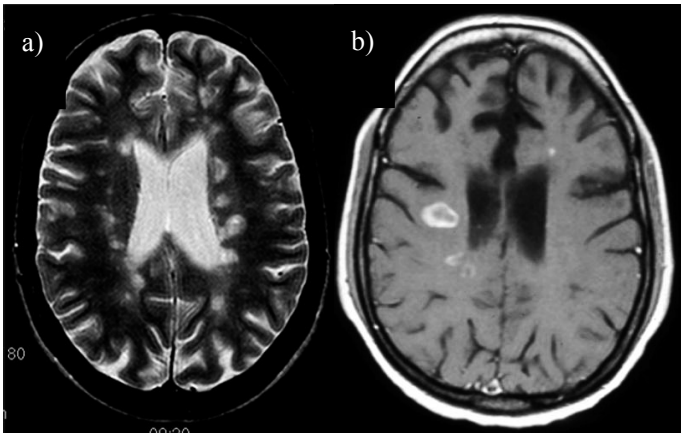


Figure 1 a) T2-weighted magnetic resonance imaging shows white matter lesions. **b)** T1-weighted contrast enhancing magnetic resonance imaging shows leakage of contrast through a disrupted blood brain barrier in MS.

Clinically, MS is variable and unpredictable. Inflammatory episodes with worsening of disease symptoms can be followed by complete or partial recovery and are termed relapses or exacerbations, or a chronic progressive course may take place with limited inflammation[13]. Based on clinical manifestations the course of MS can broadly be

divided into four main types (Fig. 2). A relapsing-remitting disease course shows phases of relapse that can last several weeks followed by symptom remission. These attacks or relapses are unpredictable. About 85% of patients start out with this form of the disease and in more than 50% of patients the disease eventually turns secondary progressive with accumulation of disability and little remission. Primary progressive multiple sclerosis is defined by a steady increase in disability without relapses while the progressive- relapsing form shows characteristic attacks and a steady increase in disability.

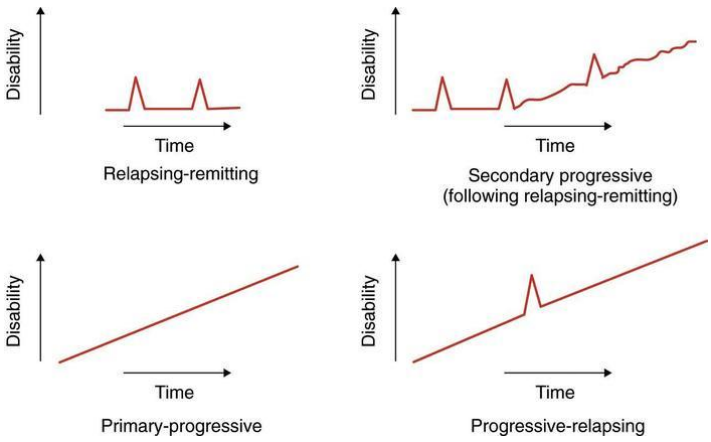


Figure 2 Schematic representations of the four major disease subgroups in MS

Therapies for RRMS focus on averting the immune-mediated damage to CNS tissue. The etiology of inflammatory focal lesions around blood vessels is unknown. Histochemistry of affected CNS tissue shows a perivascular accumulation of immune cells in inflammatory lesions. This phenomenon is accompanied by a disruption of the brain-blood barrier[14]. In active MS lesions, concurrent inflammation and increased permeability of the blood vessel wall cause edema. Immune cells are also observed in the brain parenchyma of MS patients. Inflammation in the brain of patients can be

extensive, and therefore it is not surprising that almost any cell of the immune system can be detected at one point in time in the CNS of affected individuals (Fig 3).

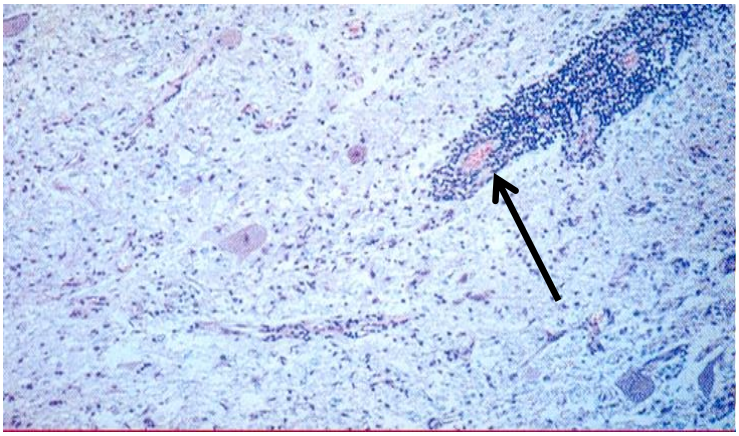


Figure 3 Image of a focal inflammatory lesion with perivascular immune cell infiltrates.

Based on the animal model for MS, experimental autoimmune encephalomyelitis (EAE), the disease is mediated by encephalitogenic T cells with highly inflammatory subtypes, Th1 and Th17. Interestingly, EAE can be induced by both cell subtypes, but only animals transferred with myelin-specific Th1 cells benefit from IFN-β therapy[15]. In MS, high serum levels of IL-17A, a proinflammatory cytokine secreted by Th17 cells, is associated with non-responsiveness to IFN-β treatment[16].

IFN-β therapy is the first-line treatment for RRMS. Based on clinical trials in 1990, IFN-β preparations reduced relapse rate by about 30% and significantly increased time to sustained progression[17-20]. Clinical findings were supported by a significant decrease in lesion load assessed by *T2*-weighted MRI and in new active lesions assessed by *T1*-weighted gadolinium-enhanced MRI. Studies have shown that up to

40% of MS patients can be defined as non-responders to IFN-β therapy[21, 22]. Clinical efficacy has to be determined over a minimum of one year of treatment since there are no available biomarkers for disease progression or for treatment efficacy. Complicating this issue is the fact that up to 40% of treated patients develop NAb to IFN-β during the treatment course[23, 24].

Unpredictability of the MS disease course (ranging from benign to severe), the lack of appropriate biomarkers for treatment efficacy, and the random development of immunogenicity to the medication makes this system a puzzle. Nevertheless, since NAbs do affect the clinical efficacy of IFN-β testing is recommended[25] (Fig. 4).

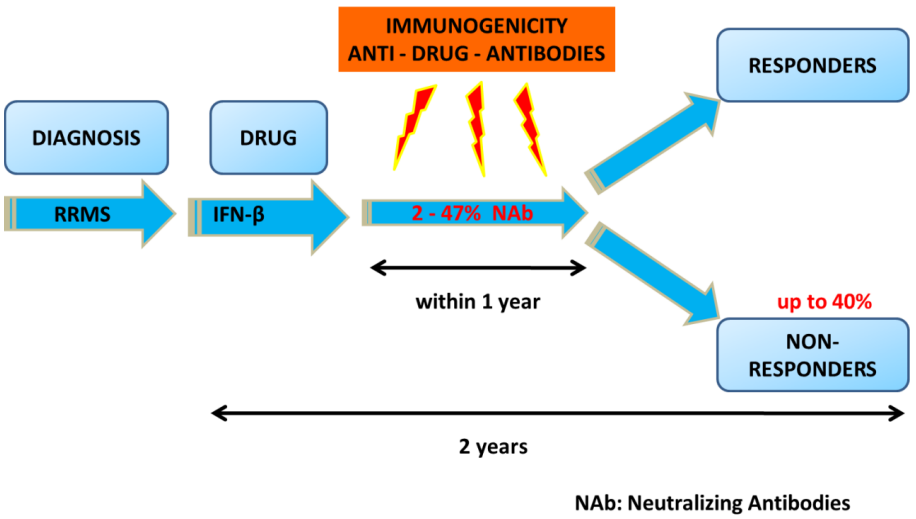


Figure 4 Clinical and biochemical parameters determine diagnosis of RRMS. Early treatment is recommended. IFN-β has partial effects in responders. Relapse rates average 0.5/year, and the gradual disease progression and accumulation of disabilities may require two years to identify responders and non-responders to IFN-β. Another factor affecting responders is NAb development that can interfere with therapy efficacy.

1.3. Type I interferon-beta

There are three types of interferons (IFN) that each signal through distinctive cell surface receptor complexes[26, 27]. Type I IFNs include a single form of IFN-β, 13 subtypes of IFN-α, and seven additional subtypes. Type II IFN is IFN-γ, and Type III IFNs include three subtypes. Type I and III IFNs are induced during a viral infection, and Type II INF-γ is involved in allergic response, tumor control, and response to intracellular pathogens. The cell type-specific and tissue-specific expression of cognate IFN receptors confers specificity[28-30]. IFNs have systemic modulatory properties that are not well characterized, and how cells respond to IFN can range from proliferation to apoptosis. Several recombinant human IFN-β preparations are available on the market today (Table 1).

Product name	Biological production System	Amino Acid length Molecular weight	Injection route	Injection Frequency	Dosing per year	Protein Amount [µg]	Activity in International Units [million]
AVONEX IFN-β-1a	Chinese hamster ovary	166 aa 22.5 kD	Intra-muscular	Once a week	52	30	6
REBIF IFN-β-1a	Chinese hamster ovary	166 aa 22.5 kD	Sub-cutaneous	Three times a week	156	44 or 22	12 or 6
BETASERON IFN-β-1b	Echerischia coli	165 aa 18.5kD	Sub-cutaneous	Every other day	182	250	8
EXTAVIA IFN-β-1b	Echerischia coli	165 aa 18.5kD	Sub-cutaneous	Every other day	182	250	8

Table 1 Various IFN-β preparations available for the treatment of MS.

The efficacy of the drugs in clinical trials is similar, but their immunogenicity varies greatly, ranging from 2 – 47 %[23, 24]. Intramuscular IFN-β-1a produced in mammalian cells and injected once a week is least immunogenic. This is not unexpected as the recombinant IFN-β-1a protein has an amino acid sequence identical to that of the human protein and is glycosylated. IFN-β-1b, which is produced in bacteria that do not have the glycosylation machinery and has a changed amino acid sequence, is more

immunogenic than IFN-β-1a. IFN-β-1a (Avonex®) is injected intramuscularly and given at lower doses and at longer intervals compared to the other drugs. The intramuscular low frequency mode of administration is less immunogenic compared to either subcutaneous and/or high frequency IFN-β-1a (Rebif®) injections. IFN-β-1b (Betaferon® / Extavia®) preparations are the most immunogenic preparations. This is not surprising, because they are produced in bacteria, have changed amino acid sequences relative to the human protein, and are injected subcutaneously; in addition, very high dose at a concentration that forms aggregates is necessary to reach biological activity similar to IFN-β-1a.

The rational for trying IFN-β in MS came from the hypothesis that the disease is caused by a viral infection. IFN-β is a potent agent that can prevent virus induced cell death and exhibits systemic pleiotropic effects. The mechanisms are poorly understood. In the case of MS, beneficial effects are added to the list as research progresses[31]. Serum of IFN-β treated patients has been shown to stabilize the brain-blood barrier *in vitro*[32, 33]. Positive effects of INF-β on the blood-brain barrier integrity and neutrophil infiltration have been shown in rats[34]. Interestingly, IFN-β blocks the pro-inflammatory disruption of endothelial tight junctions induced by IFN-γ[35]. In RRMS patients matrix metalloproteinase-9 (MMP) levels are increased in sera and cerebrospinal fluid relative to levels in healthy volunteers[36]. The MMP family of enzymes is involved in remodeling of the extra cellular matrix and the migration of immune cells[37, 38]. IFN-β treatment decreases MMP levels, and this decrease is associated with reduced clinical disease activity as shown by MRI[39]. INF-β has further been shown to affect the cytokine milieu in sera and CSF of patients[31, 40]. Beneficial effects have been attributed to a shift from pro-inflammatory cytokines to generally anti-inflammatory cytokines such as IL10 and IL4. IFN-β may even promote repair by stimulating production of factors that increase neuronal survival[40, 41].

1.4. IFN-β signaling

Many of the mechanisms of action of IFN-β proposed above counteract brain inflammation[31, 42]. This anti-inflammatory effect is supported by the findings of reduced edema in gadolinium-enhanced MRI images of treated RRMS patients that respond to IFN-β therapy. The systemic and specific cellular response to IFN-β is transduced and amplified by IFN-β binding to its cognate cell surface receptor. Depending on the phenotype (i.e. the responsive state of the cell) the signal at the cell surface is transduced into outcomes ranging from apoptosis to differentiation and proliferation. In humans, the heterodimeric receptor complex consists of two subunits named IFN-α/β receptor 1 and 2 or IFNAR1 and IFNAR2[28] (Fig. 5). Both Type I IFNs, IFN-β and IFN-α, signal through the same receptor complex, but may differ in affinity. IFNAR1 is a transmembrane receptor associated with kinase Tyk2 of the Janus family. IFNAR2c is a transmembrane receptor associated with Jak1 of the Janus family. Isoforms of IFNAR2 exist. A truncated isoform, IFNAR2b, lacks intracellular domains for kinase association and may act as a negative regulator. Isoform IFNAR2a is a soluble receptor[29].

The formation of the IFN-β receptor complex leads to conformational changes and auto-phosphorylation of the kinases[43, 44]. In this fully activated state, the kinases phosphorylate docking sites for signal transduction and activation of transcription (Stat) proteins, adaptor molecules, and signaling modifying molecules in the intracellular domains of IFNAR1 and IFNAR2. The Stats are phosphorylated and dimerize as hetero- and /or homo-dimers. Determination of signaling specificity depends on cell type and is probably achieved through organized signaling domains. Down-stream, this initial signal is integrated into many signaling pathways in so-called signaling nodes.

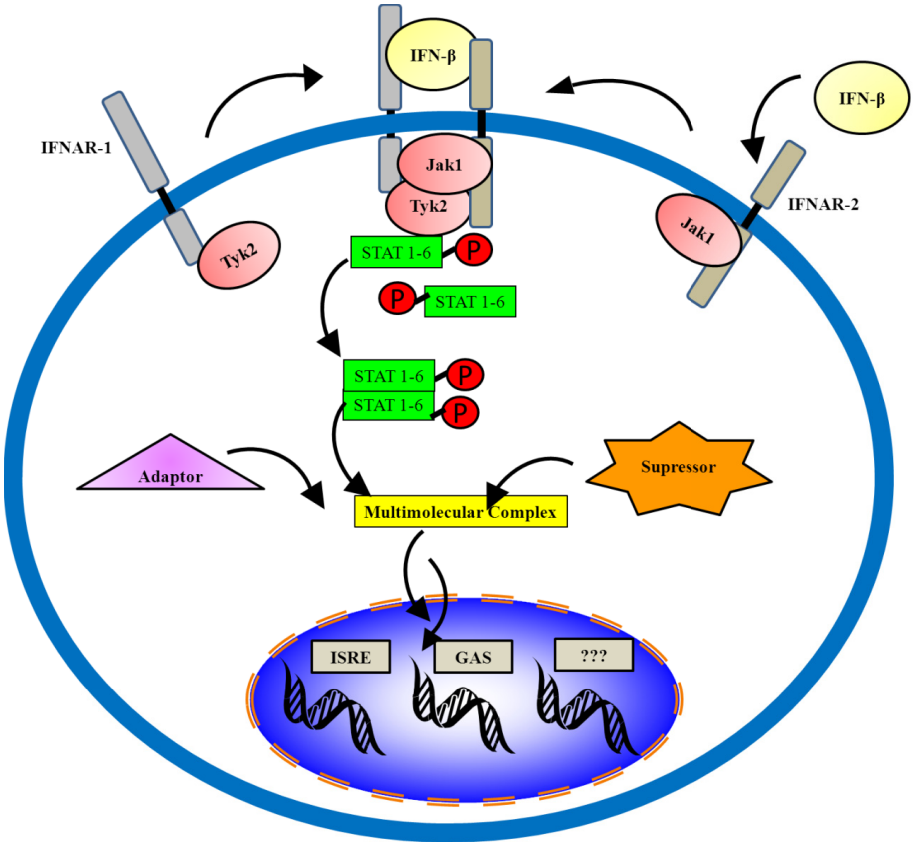


Figure 5 The Jak/Stat signaling pathway is activated by IFN-β binding to the subunit IFNAR-2 to form a signaling receptor complex with IFNAR-1. The fully activated IFN-β receptor complex phosphorylates Stats transcription factors.

Other molecules can form complexes with activated Stat dimers. The IFN-stimulated gene factor 3 complex (ISGF3), for example, is formed by Stat1 and Stat2 and IRF9. This activated complex translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) in promoter regions of IFN-inducible genes. Interestingly,

Type I interferon- activated signaling complexes can also bind to promoter sites of genes with γ activated sites (GAS) and regulate the expression of genes that are activated by Type II IFN-γ. Several other signaling pathways may be activated by IFN-β in a Stat-independent manner. These pathways include those involving the mitogen-activated protein kinases (MAPKs) and the PI3K pathway.

IFNAR1 and IFNAR2 are expressed in hematopoietic cells of both myeloid and lymphoid origin, in neurons, microglia, astrocytes, and oligodendrocytes, and in all known nucleated cells of the human body. IFN-α/β is induced by viral and bacterial products through activation of conserved pattern recognition receptors such as members of the Toll-like receptor family. Significant amounts of IFN-α/β are produced upon activation of plasmacytoid dendritic cells[45].

An interesting experiment by Prinz et al. showed that IFNAR activation in myeloid cells within the CNS of EAE mice in response to endogenous locally produced IFN-β reduces inflammation[46]. In MOG-induced EAE with adjuvant mycobacterium, an increase of local endogenous IFN-β production in the brain was measured in sick animals compared to animals before disease onset. There was no such difference in the blood. Mice deficient in IFNAR had a more severe disease course. By selectively deleting IFNAR the study showed that mice with deficient IFNAR in myeloid cells, macrophages, monocytes, microglia, and neutrophils had more severe disease. IFNAR activation reduced MHC class II expression and microglia activation and modulated the cytokine milieu. This study showed the importance of the brain innate immune response in CNS inflammation. Nevertheless, this finding may not be applicable to human disease. In a similar study where much less adjuvant mycobacterium was used, the results were markedly different and the innate immune response was not involved[47, 48]. This phenomenon where slightly different EAE induction protocols

lead to variable immune responses inducing disease shows how incredibly fine-tuned and distinct the immune system is.

The Stat family consists of seven members, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. Many cytokines and other signaling pathways lead to the activation of Stats in various cell types by phosphorylating tyrosine and/or serine, threonine and histidine residues[49, 50]. The functions of various Stats, alone or in combination and phosphorylated or un-phosphorylated, in different cell types under various stimulating conditions are poorly understood[50]. The mechanism by which Stats regulate transcription is unclear. A puzzling observation is that the same Stat complexes can both activate and repress gene transcription[51]. These opposed responses may be induced by negative regulators like suppressor of cytokine signaling (SOCS) and Src-homology 2 domain containing phosphatases (SHPs). Depending on the responsive state of a cell, chromatin remodeling and the presence of different adaptor molecules may further explain some of the variability observed in Stat signaling.

The intricacy of cell type differentiation state (i.e. cell phenotypic signaling and the downstream integration of many signals from the cell environment) is appreciated but not very well studied. There are gaps in our knowledge about how a cell integrates many signals from its environment to guide the cell machinery. The evolution in flow cytometry in the last decade has made this technique an important platform for the study of signaling pathways in single cells. It is one of the few available techniques that allow the study of signaling pathways in specifically identified cells within heterogeneous cell populations by measuring many parameters simultaneously[52]. To elucidate differences in Stat phosphorylation in response to stimuli phospho-specific flow cytometry is the perfect tool for multiplexed analysis in single cells.

2. Immunogenicity evaluation in IFN-β therapy

2.1. *In vitro* identification and quantification of BAbs and NAbs

2.1.1. Binding antibody ELISA

The European Medicine Agency (WC500003946) and several American agencies, including the FDA (UCM192750), have separately issued guidelines for the assessment of immunogenic reactions to biotechnology-derived therapeutic proteins. The agencies recommend high throughput screening assays for first line evaluation of BAb. These assays should be relative low cost and have high sensitivity in order to detect relevant levels of BAb with affinity to IFN-β. Immunoassays, such as enzyme linked immunosorbent assay (ELISA), are widely used for this purpose. These tests are based on antigen antibody interactions[53]. Assays are optimized for high specificity that maximizes detection of true positive samples.

2.1.2. Neutralizing antibodies - CPE assay

About 80-90% of RRMS patients treated with IFN-β develop BAbs and, depending on preparation, 2-48% develop NAbs[23, 24, 54, 55]. Thus, the next step is to test the positive samples from screening assays for neutralizing capacities. Such cell line based tests are more elaborate, but provide crucial information regarding whether the antibodies from a treated patient interfere with the functionality of IFN-β. The World Health Organization has recommended the cytopathic effect assay (CPE) for NAb testing to IFN-β[56] (Fig. 6). This assay is based on the 1957 observation by Isaac and Lindmann that a substance secreted by cells can protect cells from virus-induced death[57-59]. This secreted substance turned out to be IFN-β/α. In the CPE assay, the human lung carcinoma A549 cell line is challenged by a virus, encepholomyocarditis

virus, in the presence of IFN-β and sera containing various amounts of antibodies to IFN-β. A stain like crystal violet is used to detect intact live cells, and these cells are quantified by either counting the cells or eluting the stain and quantifying the absorbance. The titers are calculated according to the Kawade formula[60-62] . By definition calculation of titers are based on the patient sera dilution that will neutralize the activity of the IFN-β used in the assay from 10 to 1.

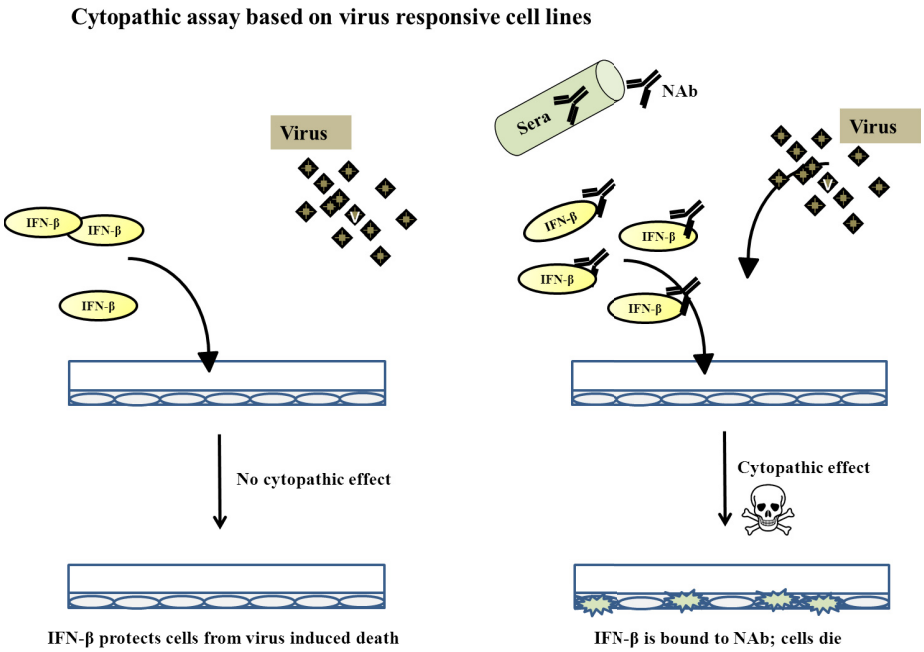


Figure 6 Schematic representation of the CPE assay. This assay is based on the protective properties of IFN-β against virus-induced cell death. In the presence of IFN-β cells only die if NAbs disrupt its protective effect.

2.1.3. Neutralizing antibodies - *Mx1* and MxA assays

The myxovirus resistant *Mx1* gene and the MxA protein are induced specifically by Type I interferons and are used as biomarkers for IFN-β activity (Fig. 7). The MxA bioassay for NAb detection measures MxA induction in a Type I IFN responsive cell line, human lung carcinoma A549. In a sandwich ELISA two MxA protein specific antibodies are used for binding, one for coating the plate that captures MxA in sera and one biotinylated antibody for detection[63]. To detect the bound protein-antibody complexes, streptavidin-HRP is used; the solution changes color when an appropriate substrate is added. The change in optical density is measured with a spectrophotometer and is proportional to the amount of MxA protein in sera. To determine the NAb titer the Kawade formula is used.

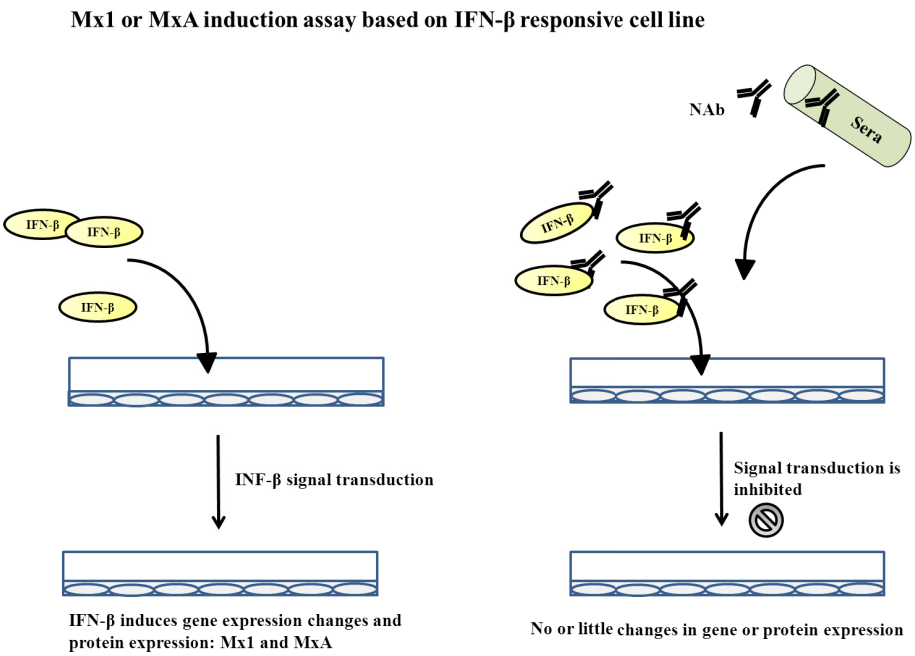


Figure 7 The *Mx1* and MxA induction assay. An IFN-β responsive cell line is stimulated with IFN-β. Cells respond by increasing *Mx1* and MxA levels. If NAb are present in patient sera, the signal transduction is inhibited and less or no transcription and/or translation will take place. MxA protein is usually quantified with an ELISA and *Mx1* mRNA by RTqPCR.

The same bioassay is used to measure *Mx1* gene expression changes after IFN-β stimulation of cells[64, 65]. For this purpose total RNA is extracted, reverse transcribed, and *Mx1* gene expression measured by real-time quantitative PCR (RT-qPCR). The properties of real-time PCR allow the use of relative quantification by the ΔΔCt method (Applied Biosytems Bulletin 2).

2.1.4. Neutralizing antibodies - luciferase assay

Many countries in Europe are implementing a luciferase assay for measuring NAb titers; this is a standardized assay that will make it possible to compare NAb results between laboratories[66]. The luciferase assay uses a human fibro-sarcoma cell line (HT1080) transfected with a plasmid containing the cDNA for luciferase under the control of the early Type I IFN inducible 6-16 promoter. When IFN-β binds to its receptor the signal is transduced and the activated transcription factor complex binds to the promoter region of the plasmid and initiates transcription and translation of luciferase. If appropriate substrate is added the enzyme luciferase catalyzes a reaction that emits light. The response to INF-β stimulation can be quantified with a photometer and is proportional to the IFN-β concentration used for stimulation of cells (Fig. 8). In the presence of NAbs, less luciferase is produced and therefore less

light is emitted. Results are reported in Tenfold Reduction Units (TRU)/mL as per World Health Organization recommendations.

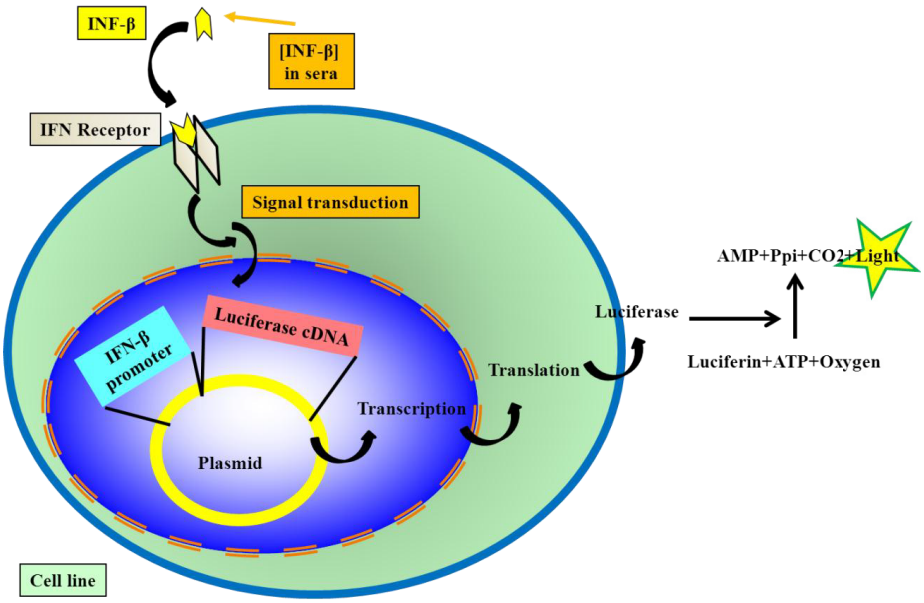


Figure 8 Graphic representation of the luciferase assay.

2.2. Evaluation of tests

No matter which cell line based assay is used, inter-laboratory variations are of great concern[67-69]. The assays include many steps that are susceptible to the introduction of variation. Efforts to standardize assays have not been productive for many reasons, and approaches and techniques are still debated. This is not only a concern for IFN-β therapy in MS. More and more recombinant human biomolecules are reaching clinical trials, and entities responsible for patient health and care are working on issuing not

just recommendations for monitoring adverse effects and immunogenic reactions to a drug but also on issuing solid procedures for companies and healthcare institutions.

An important obstacle in clinical settings is the fact that NAb measurements based on cell line systems are not correlated with clinical outcomes in some cases. The immune system of every individual is different, depending not only on the genetic material inherited but also on previous encounters with pathogens and on symbiotic organisms. Therefore a drug may act similarly in individuals regarding therapy but the biochemistry/metabolism may be affected quite differently. I believe it is this difference in phenotype that explains why some people may develop severe complications due to drug-induced immunogenicity whereas others ease through therapy showing no adverse involvement of the immune system.

A further concern is the BAb versus NAb issue. Is it possible that the immune system, with its array of possible combinations to make antibodies that recognize different epitopes on IFN-β, will only produce antibodies with low affinities and directed towards epitopes that do not interfere with the signaling of IFN-β? IgGs are about eight times the molecular weight of IFN-β, 160kDa and 20kDa, respectively, and signaling is expected to be affected by this size disparity. Many researchers argue that the difference between NAb and BAb is methodological. BAb assays are designed for sensitivity whereas NAb assays are designed for specificity and sensitivity. In addition, antibody binding, like receptor binding, is affected by temperature and pH, and these two variables are highly correlated. Differences measured in *in vitro* BAb and NAb assays may be explained by such variables as temperature and pH since most assays are done at room temperature. One must determine the effect of NAbs in a treated patient *in vivo* when possible or in *ex vivo* assays that reflect the conditions in a human body as closely as possible (Fig. 9).

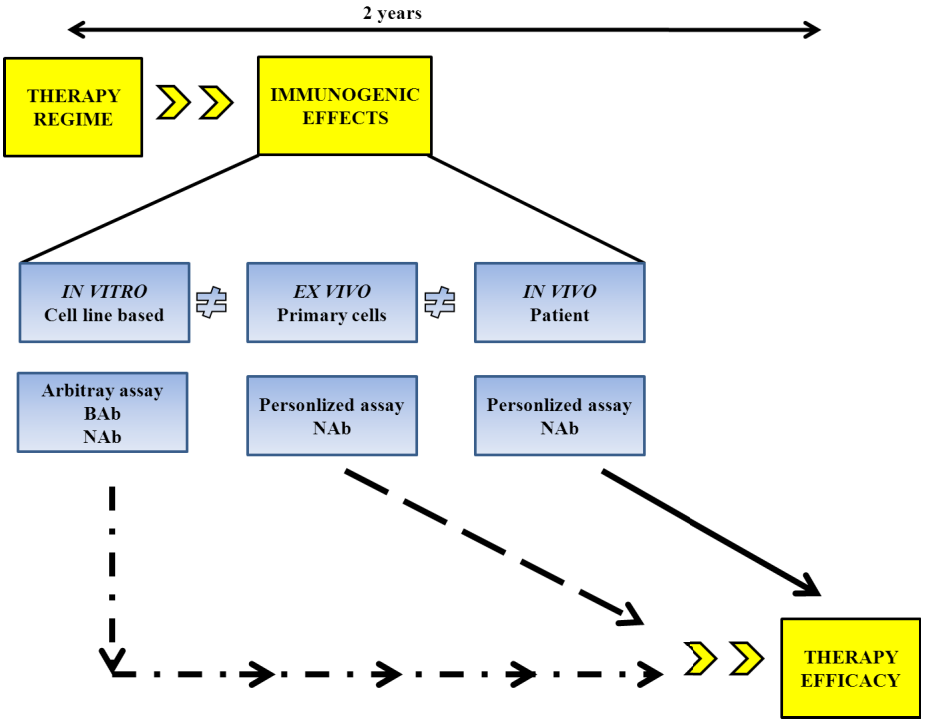


Figure 9 Cell line-based research is hardly ever directly transferable to the complex processes in whole organisms. Nevertheless, these assays are valuable indicators of therapy-associated problems like NAb development. *In vivo* assays are the most informative, but can only truly predict therapy success if the biomarkers identified have mechanistic values both for the disease and the therapy. The *ex vivo* approach could provide a simpler assay compared to *in vivo* analysis but may still provide crucial mechanistic information needed to make therapy decisions in individual patients.

2.3. *In vivo* identification and quantification of NAbs

2.3.1. *Mx1* induction assay

In vivo biomarkers for the bioactivity of IFN-β have been evaluated. Hundreds of genes are induced in a cell-type specific manner after IFN-β injection. Studies have shown that gene expression changes induced by IFN-β are highly variable and patient specific and that there is no perfect gene for the *in vivo* evaluation of IFN-β efficacy[70-72].

Type I IFN specific gene expression of *Mx1* has been validated as an *in vivo* biomarker[73, 74]. *Mx1* mRNA levels reach the highest values between 4-12 h post injection. However, expression levels of *Mx1* vary in patients before IFN-β injection, and the actual induction levels vary as well. Nevertheless, an assay for NAb evaluation has been proposed where RNA is extract from whole blood of patients before injection and again 12 h after IFN-β injection. *Mx1* gene expression is evaluated by RT-qPCR[75]. In the presence of NAb, expression may be completely blocked or substantially decreased, but not in all cases[76-79]. van der Voort et al. argue that one sample taken 12 h post injection may suffice for NAb evaluation[80]. Hesse et al. showed that NAb-positive patients, with NAb titers that blocked the expression of *Mx1*completely had no significant induction of any other genes assessed by microarray *in vivo*[81]. This study strengthens the value of *Mx1* expression as a biomarker for NAb evaluation in INF-β therapy.

As of today, no assay exists that satisfies the needs of the medical and pharmaceutical community. The latest article where international experts have convened and

extensively discussed the NAb issue was published in Lancet Neurology 2010[77]. They clearly state that NAb is an issue in therapy.

2.3.2. Stats phosphorylation

This thesis shows that it is possible to measure NAb effects in single immune cells from treated patients with phospho-specific flow cytometry (Paper III[82] and VI). NAbs disrupted the IFN-β/Stats signal transduction at the cell surface. Based on these proof of concept studies a personalized NAb assay may be available.

3. Phospho-specific flow cytometry

Phospho-specific flow cytometry (phospho-flow) is fast becoming the central platform for signaling pathway and signaling network analysis[52, 83-85]. Quantification of many parameters simultaneously is possible with the recently developed flow cytometers. Most parameters measured are based on emitted light from fluorescent molecules attached to a detection entity such as an antibody. The technique exquisitely combines the powerful identification of specific cells inherent to flow cytometry with analysis of intracellular activated signal transduction pathways[86-88]. Immune cell subtypes are identified by the cluster of differentiation (CD) markers, but any other cell type may be identified if markers are available. A central post-translational modification for the activation of signaling molecules is phosphorylation; phosphorylation allows transduction of chemical energy into signal propagation in cells (Paper I)[52]. Highly specific monoclonal antibodies are available that will only recognize and bind to the phosphorylated forms of signaling molecules. Many such phospho-specific antibodies, tested for specificity and selected and optimized for phospho-specific flow cytometry are commercially available. This technique offers incredible opportunities to study primary cells of patients. Signaling molecules in specific cell subtypes within heterogeneous populations can be analyzed and quantified. In a relative short amount of time, measurements on multiple proteins are collected simultaneously in thousands of cells at the single cell level (Fig.10).

In terms of clinical translation, phospho-specific flow cytometry in primary cells may link cellular processes to physiological processes in the disease state (i.e. link drug mechanisms at the single cell level to clinical outcomes). In immunology, phospho-specific flow cytometry may be the direct link to biochemistry. Not only is it possible to study specific cell subtypes and, for example, their activation states, but one can collect information on intracellular process. Analysis of many signaling pathways

simultaneously may reveal the drift of the immune state from the homeostatic state seen in healthy people to that of the pathological state.

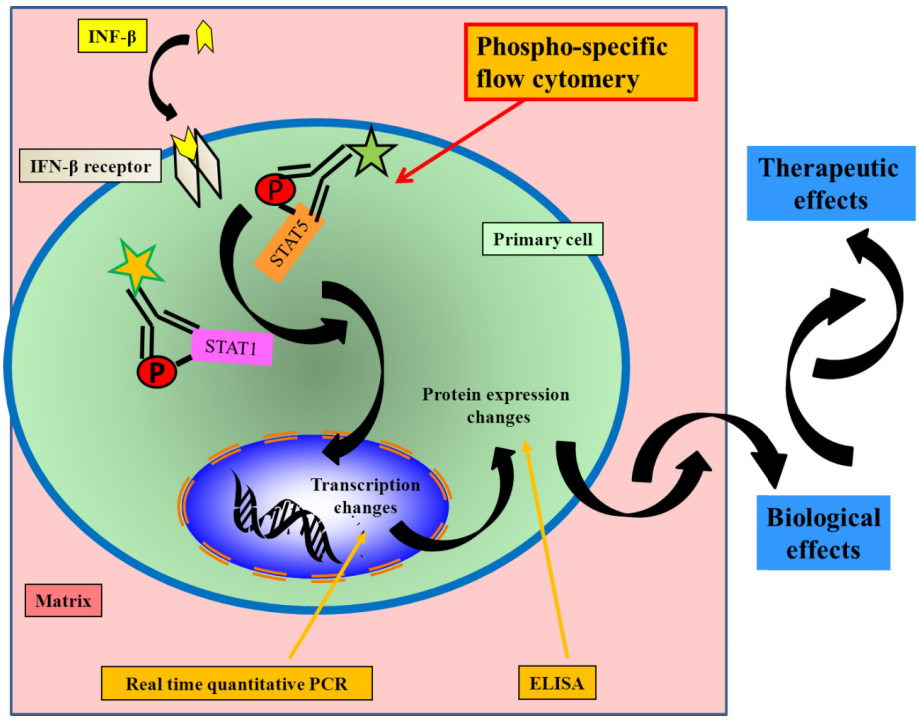


Figure 10 Schematic representation of the IFN-β/Stat signaling pathway. In phospho-specific flow cytometry, antibodies that only bind to phosphorylated forms of Stats are used to quantify pathway activation.

Signals from outside the cell are relayed via receptors to machineries within cells that will direct how a cell responds to that specific input. Since a cell in any tissue is in constant communication with its environment, many signals have to be integrated to assure an appropriate response at the cellular level, and pathways that are distinct at the cell surface may be relayed in signaling nodes. This downstream processing of information in signaling nodes allows a cell to adjust to its environment. In immune cells, stimuli at the cell surface can produce opposite effects in cells depending on cell type and activation state. In patients with immune-mediated diseases, it appears unlikely that a single biomarker will be able to relate clinical symptoms to mechanisms of the disease. Nevertheless, identifying relevant mechanistic biomarkers that also reflect the drug action are imperative to truly monitor therapy efficacy in the clinic. Many articles have been published on this topic showing that many researchers acknowledge the potential of this method[83-85, 89-92].

4. Objective

The purpose of this thesis was to establish phospho-specific flow cytometry analysis of primary immune cells and adapt this technique to the study of immunogenicity in MS patients treated with IFN-β and to explore the possibility of a personalized test.

The major questions we asked were:

1. Can phospho-specific flow cytometry be adapted to the study of signaling in primary immune cells of patients with immune-mediated diseases undergoing therapy?
2. Can we adapt the method to the study of the IFN-β Jak/Stat signaling pathway in PBMC subtypes?
3. Can we identify and quantify the effects of NAbs on the Jak/Stat signaling pathways in PBMCs?
4. Can we adapt the method to whole blood?
5. Can we measure Stat activation *in vivo*?
6. Will the same Stat protein correlate with NAb levels *ex vivo* and *in vivo*?

4.1. Multiplexed phospho-specific flow cytometry in immune cells

- Review literature and scientific approach
- Optimize and test a protocol for serum-free cryopreservation
- Optimize and evaluate signaling pathway quantification of the Jak/Stats pathway in primary immune cell subtypes with phospho-specific flow cytometry
- Test technique and Jak/Stat pathway quantification with a panel of pro-inflammatory Th1 and anti-inflammatory Th2 cytokines

4.2. *Ex vivo* IFN-β pathway activation and NAb effects in PBMCs and whole blood

We wanted to test the hypothesis that NAbs affect signaling networks and cause a specific phenotypic signaling dysfunction in immune cells. We further hypothesized that the inappropriate response to IFN-β stimulation could be detected and quantified by single-cell flow cytometry, both in PBMCs and whole blood, and that specific phosphorylated Stat protein levels correlated with NAb effects.

- Evaluate and optimize technique for NAb effect quantification
- Design and run experiment and analyse

4.3. Analysis of IFN-β and NAbs in whole blood *in vivo*

We wanted to test the hypothesis that Stat phosphorylation could be quantified *in vivo* with phospho-specific flow cytometry and that a NAb effect could be detected *in vivo*. Furthermore we wanted to test whether phospho-specific flow cytometry data correlated with gene expression changes and levels of IFN-β measured in blood and whether the same Stat proteins identified in PBMCs *ex vivo* correlated with NAb effects *in vivo*.

- Test *in vivo* technique
- Optimize time of sample collection and sample processing
- Recruit patients and define logistics for *in vivo* sampling
- Run experiments and analyse

5. Material and methods

5.1. Background

Ethical approval and patient consent was obtained for experiments carried out in this thesis. Until 2006, sera from patients treated with IFN-β were routinely analyzed by a BAb screening ELISA and a cell-line based NAb bioassay using an MxA ELISA for detection of protein induction after stimulation with INF-β. In 2007, the MxA protein specific antibody was no longer available, and our laboratory turned to RT-qPCR to measure not protein induction of MxA but *Mx1*mRNA in the same bioassay system[65].

Over the years the meaning of the term "NAb positive" has changed. Until 2009, patients were categorized into 4 groups according to serum NAb titers: NAb negative (≤ 20 neutralizing units (NU)), NAb low (20-180 NU), NAb medium (180-300 NU), and NAb high (> 300 NU). It was not unusual to measure very high NAb titers (> 1000 NU) in sera of patient. After 2009, based on the Mx1 RTqPCR assay, patients were categorized in 3 groups according to NAb titers: NAb negative if the titer value was ≤ 20, NAb low-medium if the titer value was between 20-300, and NAb high if the titer was ≥ 300. For NAb-positive patients no systematic or national recommendations for testing or treatment options were available. Fortunately, in September 2011, the National Norwegian Health Directorate in collaboration with The Norwegian Multiple Sclerosis Competence Center released recommendations for NAb testing and for how to proceed with treatment if patients are persistently NAb positive (http://helsedirektoratet.no). Testing for NAb in Norway is centralized and done at the Neuro-immunology Laboratory, Department of Neurology, Haukeland University Hospital, Bergen.

5.2. Phospho-specific flow cytometry

The methods used in this study are described in detail in the publications (Paper II-IV[52, 82, 88]). Briefly, the main steps are described below with some additional information.

The actual steps in phospho-specific flow cytometry are simple, but the idea and concept are beautifully complex. With the advent of monoclonal antibodies that could recognize specific post-translational modifications on signaling molecule, such as phosphorylation on specific residues, and the single-cell analysis capability of flow cytometers, a tool for signaling pathway analysis was available that could identify cell subtypes and analyze biochemical processes within single cells simultaneously. In Paper I[52] and Paper II[88] of this thesis we published the scientific bases to pursue this method and worked out a detailed protocol for the analysis of the Jak/Stat signaling pathway in primary immune cells by phospho-specific flow cytometry including IFN-β[52, 88]. Figure11 schematically represents every step of the procedure that I will discuss in more detail below. The vast amounts of data regarding biochemical processes occurring within cells that can be produced with this technique are a challenge for analysis and data representation.

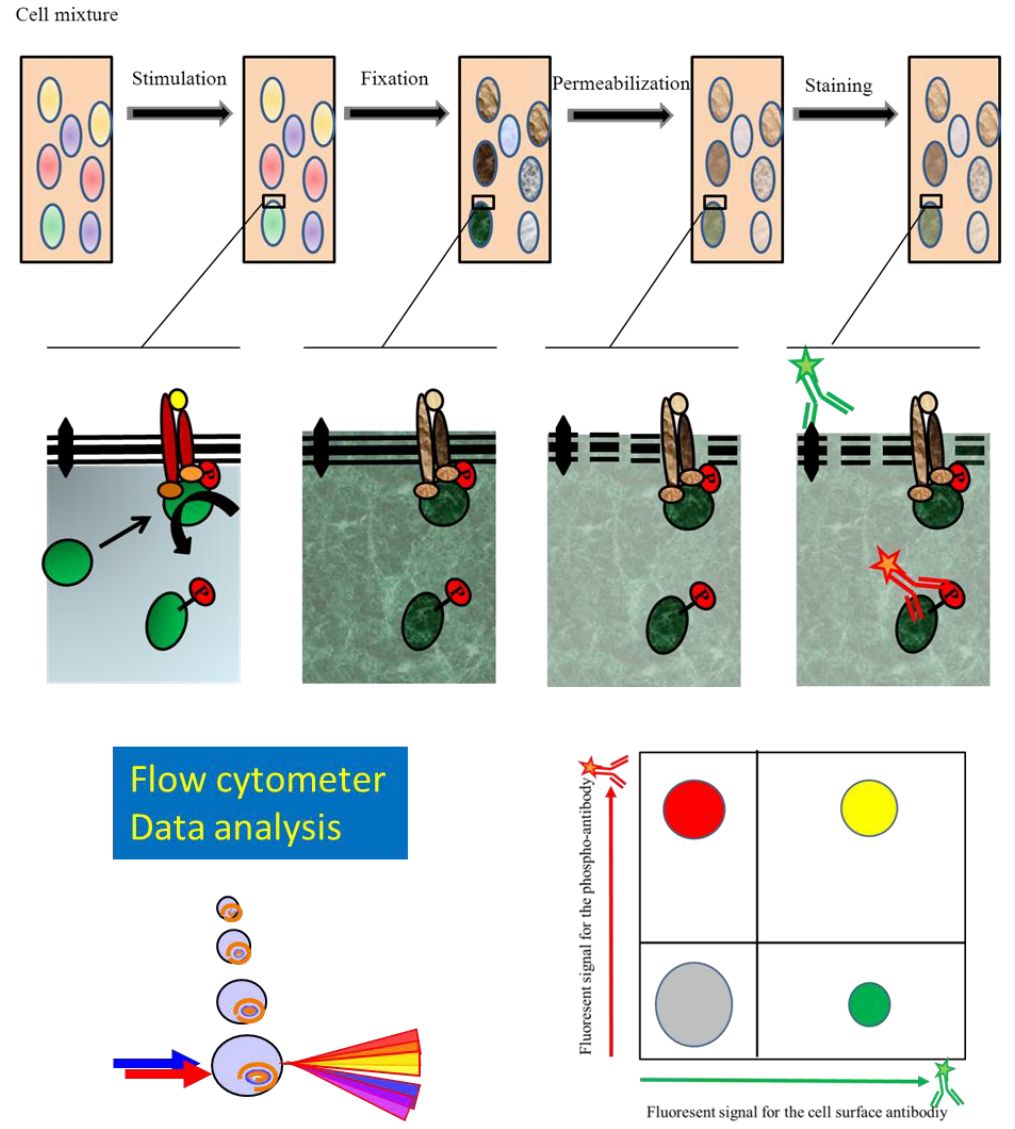


Figure 11 Schematic representation of the steps from stimulation to single-cell analysis of fluorescence. A pathway is activated, the signal frozen by fixation and intracellular antigens made available by permeabilization. A suitable antibody cocktail with specific fluorescent markers allows quantification of signals and identification of cell types at the single-cell level.

The technique starts with the care of the primary cells of interest[88]. The issue of cryopreservation and thawing of cells is of great concern not only for stem cell therapy techniques, but also for research. Many slightly different protocols are available for cryopreservation. The freezing rate between 0 and -20° C has been shown to be central for cell viability. In part of this study, the plan was to use cryopreserved cells from IFN-β-treated patients with NAb and their autologous sera[82]. Fetal calf serum varies from batch to batch and may contain uncharacterized molecules that can affect signaling. In order to standardize the cryopreservation step as much as possible, we used serum-free freezing media.

Patients were asked to take the last IFN-β injection at least 12 hours before the scheduled PBMC and sera sample collection. This time frame follows recommendations and assures little interference of the medication with NAb tests[77]. Sterile conditions were essential to hinder bacterial growth during the resting period for cells prior to stimulation. Contamination with bacteria and other compounds can activate the Type I IFN systems and other pathways in cells and had to be avoided.

Avonex (IFN-β-1a) (Biogen Idec) was used for stimulation in all experiments. The potency of different preparations was similar, but the main concern for this study was the rationale that Avonex is the preparation used in our laboratory for the bioassay to determine NAb. Of technical concern is that in addition to differences in potency, the amount of NAb that binds to different preparations varies[93]. Avonex and Rebif have a ratio of 1:1, and Betaferon has a ratio of 1:3, which may be explained by aggregate formation in Betaferon preparations. This issue remains to be assessed for NAb tests based on *ex vivo* re-stimulation of PBMCs or whole blood.

Primary cells are inherently sensitive to temperature, pH, and osmolarity fluctuations. Therefore the cells are perfused with warm paraformaldehyde directly added to the stimulation matrix. The concentration of paraformaldehyde affects epitope detection by antibodies. For immune cells, low concentrations of paraformaldehyde between 1-2% are recommended.

Many protocols are available for permeabilizing cells. In flow cytometry and histochemistry conditions usually include either ethanol or methanol[88]. Both of these substances dehydrate the cells and cause protein denaturation and can destroy epitopes recognized by antibodies. Methanol makes nuclear proteins accessible, which is perfect for detection of nuclear Stats. The denaturing properties of methanol are an advantage when using antibodies that are directed against linear epitopes, which is often the case for monoclonal antibodies. Methanol is very effective in denaturing proteins and is used ice cold to reduce the damage to the epitopes of interest.

Antibody specificity and titration is a must in phospho-specific flow cytometry.

Collection and cryopreservation of PBMCs from patients takes time and resources. We therefore determined if NAb effects could be quantified in whole blood (Paper IV).

Ethical approval and consent from patients were obtained for *in vivo* experiments. Preliminary experiments showed that phosphorylation of Stats after IFN-β injection in patients was generally low compared to the phosphorylation potential seen in re-stimulation of whole blood and peaked around 4 hours after IFN-β injection. Storage

of sample in methanol for such weak signals is not recommended and we had better results with storage in PBS at -80 °C. What exactly happens in the body after subcutaneous or intramuscular injection of IFN-β is unclear and bizarrely patient specific. In fact, the time to peak levels of IFN-β in serum varied considerably, from 1 to 16 hours[94, 95], and was dependent on drug preparation and patient. In preliminary experiments we therefore determined the best time to collect samples for phospho-flow analysis of Stats. The following time points were evaluated for detection of signaling *in vivo* before and after IFN-β injection: 0, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, and 24 h. A signal with phospho-flow could be detected around 2 h – 8 h post-injection.

5.3. INF-β ELISA of human sera

In order to evaluate the injection of INF-β itself we measured the concentration in sera. In some cases it has been observed that the injection of IFN-β can be biologically ineffective, especially in patients with frequent dosing schedules[96]. To compare results between patients we run all samples on one plate and calculated the concentrations from the same standard curve. Sample 112 had to be diluted to be within the range of the standard curve.

5.4. Type I IFN pathway-specific real-time quantitative PCR

As mentioned above, some of the IFN-β injections can be biologically ineffective for other reasons than NAb[97]. Besides IFN-β concentrations we therefore also measured down-stream gene expression in order to evaluate the whole signaling pathway from sera to gene induction. The plates included *Mx1* gene which is a promising biomarker for NAb evaluation *in vivo* and allowed us to compare Stats activation and gene expression based on the experience with the expression of this gene and NAbs.

5.5. Analysis, visualization and interpretation

Measurements of fluorescence emission in phospho-flow are commonly given as changes in average fluorescence emission between unstimulated samples that represent the basal phosphorylation levels of particular signaling molecules and stimulated samples that represent the increase in phosphorylation after activation of the pathway by the stimulus. Medians or means from such phospho-flow data are based on single-cell measurements of thousands of cells and are solid numbers for further statistical analysis. Multivariate data is difficult to visualize for interpretation purposes. The *ex vivo* PBMCs study, for example, produced over 200 dose response curves, but all data points – over 3000 of them – could be visualized and important variables identified with principal component analysis (PCA). PCA is a powerful tool to visualize and analyze multivariate datasets. PCA is a representation of the variability in a dataset based on a reduced set of variables termed principal components (PC). The largest variability is attributed to the first PC and each successive PCs accounts for less of the variability than the previous one. The visualization of samples in PCA in score plots lets one identify patterns within the data; for example, patients that respond similar to a treatment cluster and are easily recognizable. The variables in PCA are visualized in the loading plots and have to be interpreted together with the corresponding score plots. Variables represent vectors and are interpreted as such. Variables on opposite sides are inversely correlated and variables close to the origin contribute very little to the clustering seen in the scores plots. The Hoteling T^2 test and the fifty-fifty MANOVA can be used to test for significant differences between the clusters identified in the PCA. In Partial Least Square regression models (PLSR), the predictability of a variable or several variables in a matrix Y is tested based on an X matrix of variables. The covariance between X and Y is maximized in PLSR. Prediction models may be a useful tool for treatment evaluation in biomedicine. ANOVA and fifty-fifty MANOVA were scripted in R software. PCA and PLSR were run partly in software R (http://www.r-project.org/)

and partly in Unscrambler (CAMO). FACS data were analysed in Cytobank (www.cytobank.org).

5.6. Controls for phospho-specific flow cytometry

The standard controls for flow cytometers today are bead based. The BD Cytometer Setup and Tracking Beads (CS&T) were used to monitor performance of BD digital flow cytometers. The CS&T kits contain beads of different size and fluorescent intensities and are instrument set up specific. These controls identify optimal signal to noise ratios for every photomultiplier tube and reports generated visualize laser problems.

For analogue instruments, like the BD FACS Calibur, Spherotech Rainbow Calibration particles were used that monitor instrument performance in a manner analogous to the CS&T beads, but the operator has to manually generate plots. The beads in this kit are of similar size with different fluorescent intensities and are instrument set up specific.

BD Calibrite beads were used for fluorescent spillover compensation. For both the analogue and the digital instruments, the compensation matrix was calculated with either the instrument software for automatic compensation set up or the software provided by FlowJo or Cytobank. In the setup phase of the experiments, spill over matrices based on the beads were verified with PBMCs stained with fluorescent conjugated antibodies. Instument settings were adjusted to allow for optimal separation of positive and negative cells. In any experimental run, single-stained cells were run as well as beads.

The unstimulated sample in phospho-specific flow cytometry is the negative control for phosphorylation induction in the pathway of interest. In each experiment, an unstimulated sample is always run for every stimulus used. Experiment-specific internal controls can be assessed for each experiment separately. For example, in PBMC stimulation with IFN-β, B cells never responded to stimulation by phosphorylating Stat4 on tyrosine. In certain of our experiments, we used cryopreserved PBMC aliquots of one healthy person in each run to evaluate variation. In the *ex vivo* PBMC experiments, cells were re-stimulated in autologous sera and serum-free media at the same time. This approach provided the essential positive control for the stimulation in sera where NAbs were suspected to influence signaling.

In *in vivo* experiments, whole blood of the patient was re-stimulated *ex vivo* with IFN-β, and unstimulated and stimulated whole blood served as a positive control to evaluate the procedure. The negative control was the sample taken before injection of IFN-β. The controls mentioned above suit phospho-flow analysis in primary cells quite well.

6. Results

6.1. Paper I - Multiplexed phosphoprotein analysis in immune cells.

The scientific bases and hypothesis to pursue the work in this thesis is published here[52].

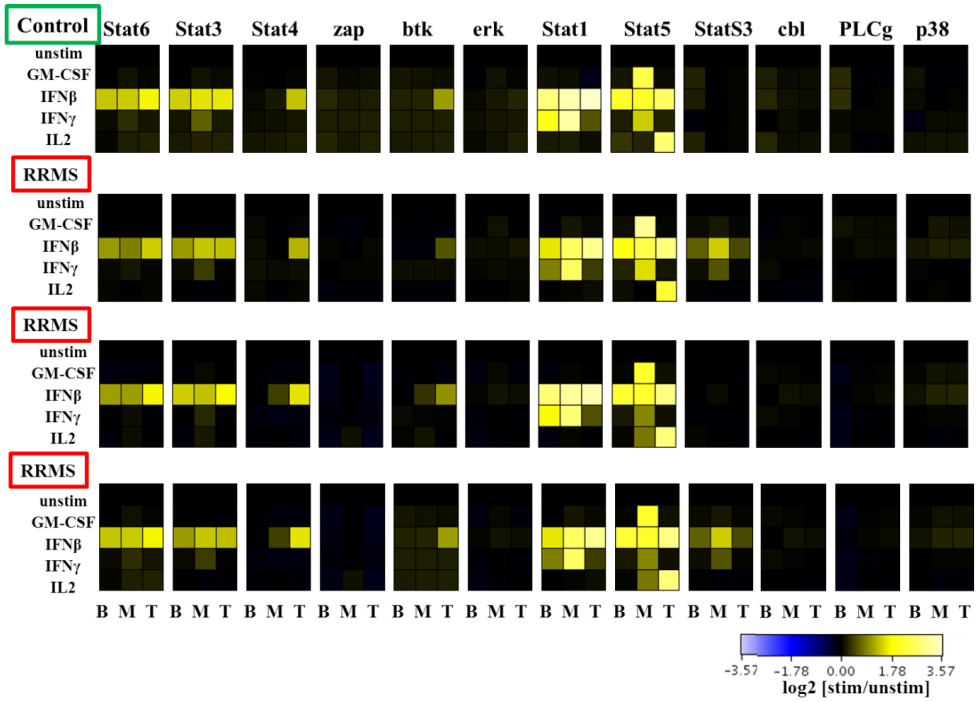
6.2. Paper II - Flow cytometry and cell activation.

In preliminary phospho-specific flow cytometry experiments we tested the method, and optimized the procedure. The standardized protocol was published here and we adhered to it minutely to minimize operator variation [88].

6.3. Unpublished data - Pathway specificity and Stats phosphorylation profiles in PBMCs

In set-up experiments we tested many intracellular phospho-specific monoclonal antibodies to various signaling molecules to assess the pathway specific activation of stimuli, especially IFN-β. A typical pro-inflammatory Th1 and anti-inflammatory Th2 panel of cytokines known to signal through phosphorylation of Stats was tested. Antibodies to proteins in other signaling pathways were included in the study to assess specificity of Stats activation by the stimuli.

We proceeded to test a subset of this panel in untreated relapsing-remitting MS patients and healthy individuals. Assays were carried out using serum-free support media to evaluate Stat signaling in MS patients. The results showed that untreated RRMS patients had immune cell responses similar to healthy controls, but some variation was present that encourages further studies. This approach ensured that we were not measuring pathway defects inherent to the disease when signaling was tested in the presence of NAb (Fig. 12, unpublished data).


Control
Stat6
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Stat4
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cbl
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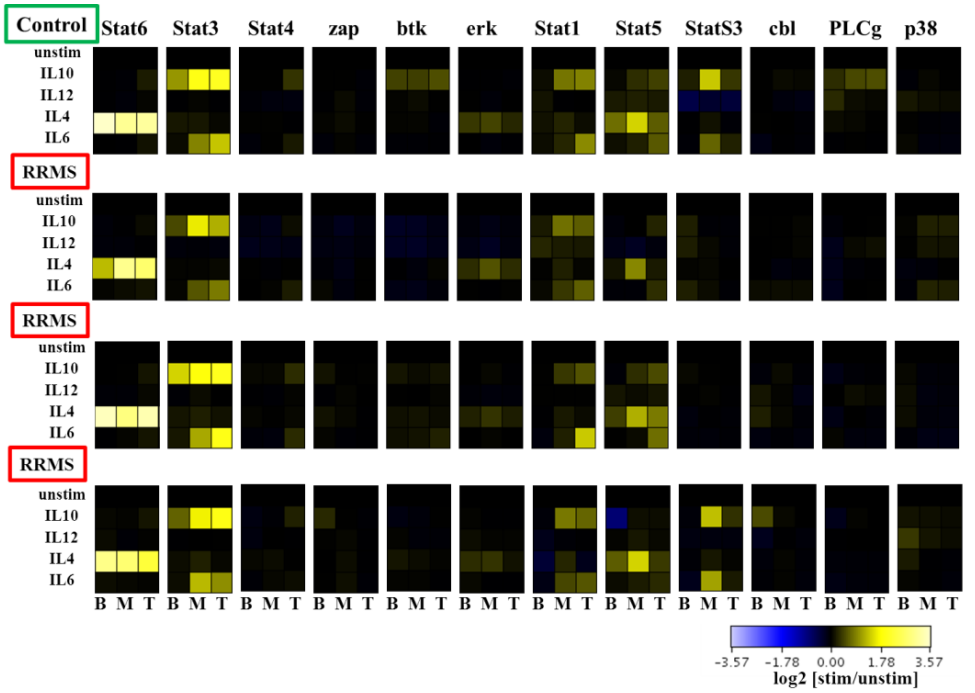
Control
Stat6
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-3.57 -1.78 0.00 1.78 3.57
log2 [stim/unstim]

Figure 12 The heat maps show the phosphorylation and thereby activation of indicated signaling molecules in columns and the stimulation agent in rows. The value of the unstimulated sample (unstim) is set to 0 (black) and fold changes are shown according to the colors on the log2 bar. Abbreviations: p, phosphorylated; zap, protein tyrosine kinase Zap-70 of the Syk family is involved in mediating T cell activation; btk, Bruton's tyrosine kinase belongs to Btk/Tec family of cytoplasmic tyrosine kinases and plays a role in B cell development; ERK, extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) are mitogen activated protein (MAP) kinases with over 160 known substrates; cbl, the c-Cbl proto-oncogene encodes a E3 ubiquitin-protein ligase; PLCg, phosphoinositide-specific phospholipase C gamma; p38, p38 mitogen-activated protein; GM-CSF, granulocyte macrophage colony-stimulating factor; B, $CD20^+$ B cells; M, scatter gated monocytes; T, $CD3^+$ T cells.

6.4. Unpublished data - IFN-β potency and pre-incubation effect

Recombinant IFN-β-1a and IFN-β-1b are the Type I IFNs used in MS therapy. To evaluate whether these two molecules activate primary cell subtypes similarly we stimulated PBMCs with a serial dilution of the same concentrations (in IU/ml) prepared from the two medications. Figure 13 (unpublished data) shows the activation of several Stats in response to stimulation with IFN-β-1a (Avonex) or IFN-β-1b (Betaferon).

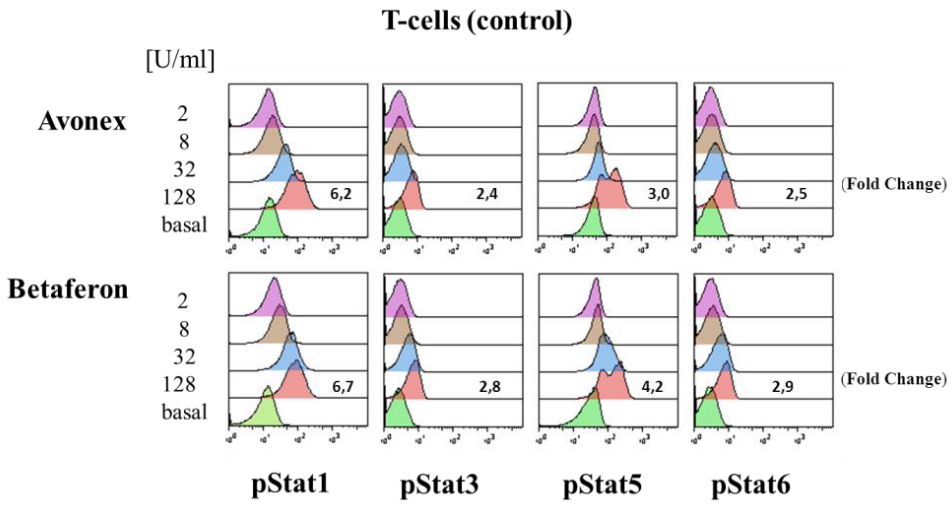

Figure 13 *Ex vivo* re-stimulation of PBMCs with IFN-β-1a (Avonex) or IFN-β-1b (Betaferon) with indicated concentrations in IU/ml for 15 minutes. The overlaid histograms show the activation of several Stats in $CD3^+$ T cells. Basal/unstimulated samples are shown in green.

In some procedures for NAb evaluation sera is incubated with IFN-β prior to testing in bioassays. We tested whether pre-incubation of IFN-β with sera affected activation of Stats in PCMCs of patients (Fig. 14, unpublished data). Incubation marginally affected phosphorylation of Stat proteins. The dynamics of signaling in such complex matrices as sera is not understood, and it is important to keep the procedures rigorous regarding time. In order to streamline the protocol we decided that 1h incubation allowed cells to reach a representative equilibrium in sera. This time period should also minimize IFN-β adsorption to surfaces and aggregate formation [98].

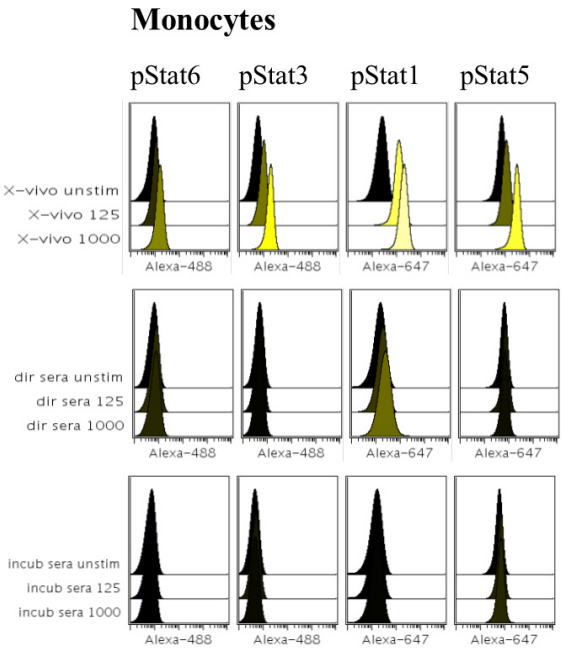

Figure 14 PBMCs of an MS patient with high NAb titers were either directly re-stimulated *ex vivo* in serum-free support media (X-vivo) or autologous sera (dir sera). In the same assay autologous sera was incubated for 1 h with either 125 or 1000 U/ml IFN-β and then added to the cells for re-stimulation for 15 min. Data from monocytes, which strongly respond to IFN-β stimulation, are shown.

6.5. Paper III - Immunogenic effects of recombinant interferon-beta therapy disrupt the JAK/STAT pathway in primary immune cells from patients with multiple sclerosis.

In this experiment we wanted to assess the impact NAbs have on the responsiveness of the IFN-β Jak/Stats pathway in PBMCs of MS patients with various NAb titers[82].

Data from 14 subjects were included for analyses and PBMCs of every individual were re-stimulated with a serial dilution of IFN-β both in serum free support media and in autologous sera. For detailed experiment overview refer to Figure 1 in Paper III[82]. We measured levels of phosphorylation of six Stats transcription factors in three immune cell subtypes.

The most interesting result of this experiment was that in all treated MS patients that were NAb positive the signal of IFN-β at the cell surface was basically turned off compared to the controls in the same cells of the same patients (i.e. the re-stimulation in serum free media). This finding let us to propose an assay that would not be based on arbitrary cut-offs but on a simple yes or no outcome. In addition, Stat1 phosphorylation showed the highest degree of activation compared to the phosphorylation of other Stats proteins. We argue that it may be possible to determine the IFN-β concentration where a Stat1 signal should be present in all NAb negative patients and turned off in NAb positive patients. Phosphorylation of Stat1 appears optimal because our data indicates that if Stat1 is turned off in NAb positive patients all other Stats proteins are shut down as well.

We further hint at a possible treatment response since we found that the treated patients that were NAb negative had a unique signaling signature.

The PCA of all data shows some interesting aspects of the NAb system (not shown in paper). Re-stimulation of PBMCs of NAb positive patients in serum-free support media showed that the cells of treated RRMS patients responded to IFN-β stimulation in absence of sera containing NAbs (yellow circle; Fig. 15). In these same patients the response to IFN-β stimulation in autologous sera depended on the presence or absence of NAb but not NAb titer (red circle). Treated NAb-negative patients formed a cluster

for both re-stimulation in sera and media, but were more similar to the cluster of patients and controls where cells were re-stimulated in serum-free media (squares). PBMCs from untreated MS patients and controls are very similar for the both the re-stimulation in serum-free media and autologous sera (green roundels and squares). Evidently sera containing NAbs has a significant effect on the responsiveness of the Jak/Stat pathway at any measurable *in vitro* titer.

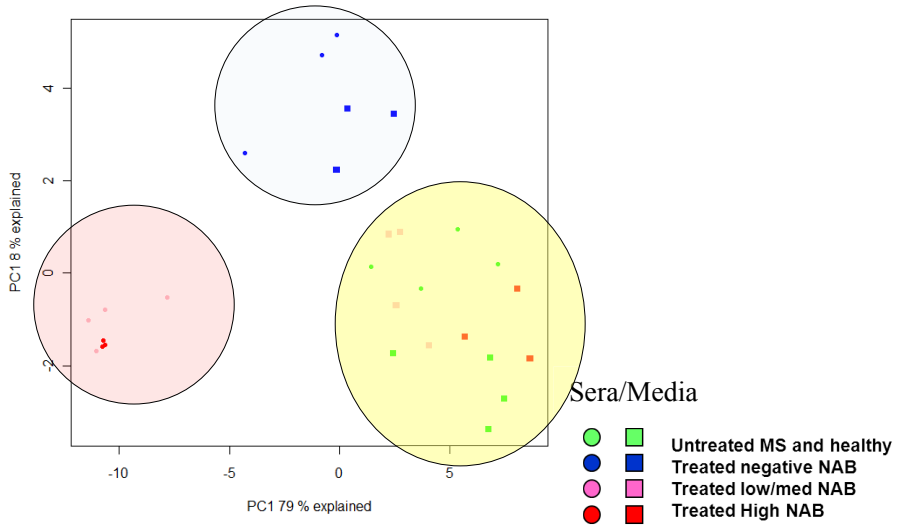

Figure 15 PCA of data for re-stimulation in serum-free media and autologous sera. Notice RRMS patients that are NAb positive form a tight cluster independently of NAb titers (pink and red roundels). This PCA also shows that re-stimulation of PBMCs in media, in which no patient sera containing NAbs or other inhibiting factors are present, results in clustering for all patients and controls (green, pink, and red squares). Treated NAb-negative patients clustered somewhat separately indicating a possible treatment effect compared to untreated patients (blue squares and roundels).

In set-up experiments we determined that Stat activation/phosphorylation by IFN-β stimulation in PBMCs followed a typical dose-response curve. *Ex vivo* re-stimulation

of PBMCs both in support media and autologous sera showed dose response curves for the stimulation with a serial dilution of IFN-β; signaling could be measured starting at 15-30 IU/ml and the plateau was reached at 1000-2000 IU/ml. The effects of sera containing NAbs on the responsiveness of the Jak/Stat signaling was readily detectable with phospho-specific flow cytometry. An example for phosphorylation of Stat1 (pStat1) is shown in Figure 16. Interestingly, the inhibition of pStat1 phosphorylation in PBMCs due to the presence of patient sera containing NAbs could be overcome with high doses of IFN-β in samples from most patients. Extremely high amounts of IFN-β, at levels that probably would be toxic in a human body, were needed in some cases to overcome the inhibition.

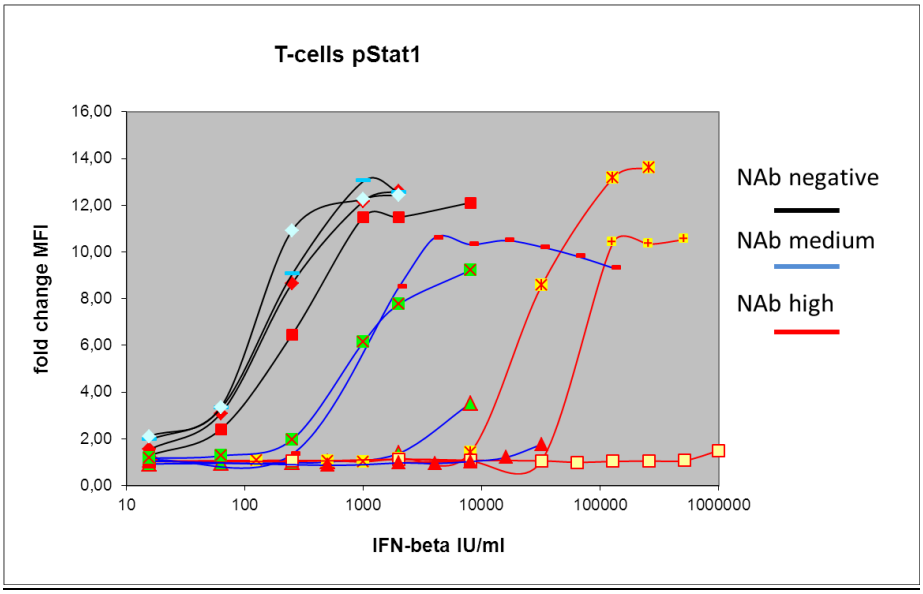

Figure 16 Dose response curves of PBMCs of RRMS patients with various NAb titers and NAb-negative patients. The re-stimulation with IFN-β was done in autologous sera. Notice the IFN-β concentrations range from 10 to 1,000,000 U/ml!

One patient with a very high NAb titer barely responded to IFN-β stimulation even at 1,000,000 IU/ml.

6.6. Paper IV - Deficient phosphorylation of Stat1 in leukocytes identifies neutralizing antibodies in multiple sclerosis patients treated with interferon-beta

In this experiment we used phospho-specific flow cytometry to assess the activation of the IFN-β Jak/Stats pathway directly after IFN-β administration in patients. Six IFN-β treated patients were included in this study. For detailed experimental overview refer to Figure 1 in Paper IV. We measured phosphorylation levels of six Stats transcription factors in seven immune cell subtypes in whole blood drawn from patients before and at several time points after IFN-β injection. In order to assess the entire pathway after IFN-β administration in patients from appearance of IFN-β in sera to phosphorylation of Stat transcription factors to gene induction we measured levels of IFN-β in sera and gene expression changes in whole blood mRNA.

The most interesting result of this work is that one can actually measure the phosphorylation of Stats transcription factors directly after IFN-β administration in patients. To our knowledge only very few studies have attempted this *in vivo* approach. In addition a NAb effect could be measured in NAb positive patients and similarly to the results in Paper III[82] the effect was seen in the turn off of Stat1 phosphorylation. We identified a NAb positive patient with peculiarly high IFN-β levels in sera. Interestingly, the phospho-data could identify this patient as different and we speculate that the *in vivo* responsiveness of the Jak/Stat pathway may identify patients with abnormal responses to IFN-β other than NAbs. Many IFN-β specific genes were induced by drug injection and significantly affected by NAb titers.

However, phosphorylation of Stats proteins was more predictive of NAb titers than gene expression changes.

Interestingly, *ex vivo* re-stimulation of whole blood from patients with different NAb titers clearly revealed the inhibition of the IFN-β Jak/Stats pathway similarly to the effects seen in Paper III[82]. This results indicates that phospho-flow in whole blood could be used for NAb assessment instead of PBMCs (Fig 17).

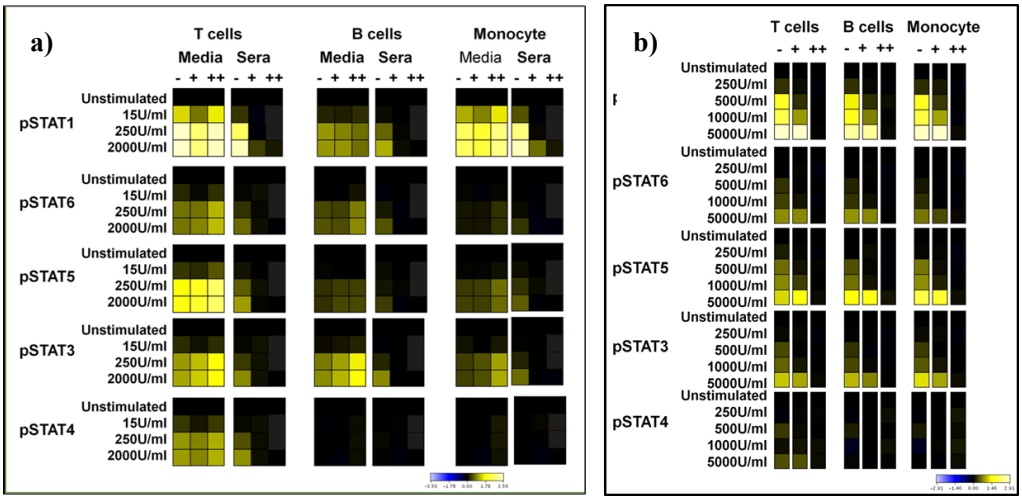

Figure 17 Heat maps of IFN-β re-stimulation dose-response curves in patient immune cells. a) for re-stimulation of PBMCs in media and sera and b) for re-stimulation in whole blood. – treated NAb negative; + NAb low/medium; ++ NAb high.

The Stats phosphorylation experiment *in vivo* was compared with gene induction profiles in whole blood. These measurements were also used as a pathway activation control for the phospho-flow analysis. Genes measured in this assay are established IFN-β response genes. Studies have already shown that gene expression is influenced by NAb *in vivo* in patients. However, variability between patients is very high even before IFN-β stimulation. In RT-qPCR there are several steps that cannot be

controlled sufficiently. RNA integrity and quantitation can be a problem. Reverse transcription efficiency can vary considerably as well. In comparison to phospho-flow in single cells within heterogeneous immune cell populations in blood, RT-qPCR is technically more challenging and time consuming and is also more expensive. Time of sample collection is crucial for gene expression measurements and may differ for every gene. The levels of the *Mx1* mRNA, for example, should be analyzed 4-12 hours after IFN-β injection for maximal response. In the PCA of gene expression data it is therefore not surprising that some time points are not clustered in the correct NAb class (Paper IV, Figure 2b). Notice, time was significant for gene expression in the fifty-fifty MANOVA.

We used partial least-square regression (PLSR) to find a model that fit our data and current knowledge and that can predict the behavior of Stat signaling patterns in future samples. Modeling is an iterative process and adjustments and choices are made during optimization for the task at hand. Since antibodies are costly, we were interested in reducing the set of variables that could be used to predict NAb titers. The analysis began with identification of variables that are highly correlated with NAb titers. From there sub-models with fewer variables were explored. We did not have a large dataset to work with but the models fitted the phospho-flow data well. Ideally we should have new data to validate this model, but as this was not the case, our model was validated by taking out all the measurements for one patient one at a time. The best fitting model for NAb prediction based on PLSR included pStat1 in monocytes and T cells or lymphocytes and pStat3 in monocytes ($r= 0.97$).

In the appendix supplement Figure 1 shows the 2D plots of the 3D plot presented in Figure 2a in Paper IV. Some additional PLSR models are shown in supplement Figure 2-4.

7. Discussion

The approach in this thesis is based on the paradigm that states that molecular response to treatment correlates with therapy efficacy. Based on this statement we studied the mechanism of signal transduction activation of IFN-β to assess IFN-β response in primary cells of treated RRMS patients and to evaluate NAb effects. In addition, a mechanistic approach to drug evaluation may offer insight into the pathogenesis of the disease and discover biological markers to guide therapy and estimate prognosis [52].

There are currently no validated biomarkers for disease progression in RRMS and no biomarkers to identify responders and non-responders to IFN-β therapy. However, based on the data presented in this thesis it may be possible to tackle the immunogenic aspect of this complex therapy system based on the responsiveness of the IFN-β signaling pathway in primary cells from treated patients.

The production of antibodies that neutralize IFN-β can be a substantial part of treatment failure in MS patients. Sometimes significant adverse consequences are readily visible in clinical management. However, in many cases adverse effects of NAb production go undetected for a long time. In the pivotal studies in 1990 of IFN-β therapy in RRMS patients, investigators measured NAb titers in sera of patients. Over the next twenty years of clinical IFN-β experience, the issue of NAbs has been debated, and the latest consensus on how to deal with NAbs in clinical settings was published in *Lancet Neurology* in 2010. The recommendations are based on a complete approach to the NAb issue including clinical status, *in vitro* NAb titer measurements and *in vivo* gene expression analysis. However, these are only recommendations and every treating physician is left to deal with the issue in his or her current settings including accessibility to assays. The intricacy of NAb evaluation in clinical settings is based at least partially on the fact that the natural course of the

disease cannot be distinguished from NAb effects and generalized rules are therefore difficult to apply in individual patients.

MS is described as an immune-mediated disease of the central nervous system caused by a combination of genetic and environmental factors in susceptible individuals. IFN-β is an immune-modulatory drug with poorly understood function and NAbs may mimic some aspects of the natural disease progression. The homeostasis of the immune system in treated RRMS patients is affected by exogenous factors, by modulations caused by IFN-β therapy and by the possible immunogenic reaction resulting in NAbs. In some patients it is impossible to separate the natural disease progression from treatment efficacy and immunogenicity with currently available tests.

Biomarkers for NAb evaluation are being sought, and with advances in flow cytometry and signal transduction analysis in single cells a promising tool to study the effect of NAbs in primary cells of patients is available. The signal transduction pathway of IFN-β is well characterized from the signal initiation at the cell surface to signal propagating proteins and gene expression.

The basic premise of this thesis was to use activation of the INF-β/Stat pathway at the cell surface to evaluate NAb effects. IFN-β regulates hundreds of genes in a cell-type specific manner and may induce opposite outcomes from proliferation to apoptosis. The complexity of the pathway increases with signal propagation and so does our current knowledge of the processes. The early induced gene expression changes after IFN-β stimulation are rapid, but other changes take hours or days to appear[99, 100]. Some genes are expressed transiently whereas others are expressed over days, and how a cell ultimately responds is not well understood. In addition, the continuous systemic administration of IFN-β in RRMS patients leads to an immune state that may be patient specific. The results in this thesis show that it is possible to measure NAb effects on the responsiveness of the IFN-β/Stat pathway after receptor activation at the cell surface in single cells within heterogeneous immune populations of patients without separating the cells (Paper II)[52].

NAb consistently inhibited and/or modulated the IFN-β signal transduction from the cell surface, both *ex vivo* in PBMCs and *in vivo* after IFNβ administration in patients (Paper III[82] and Paper IV). NAb evaluation focused on an individual approach where a patient's phenotype and disease characteristics were integrated into the analysis with the use of primary cells of the treated patient. Even though genetic and disease parameters are largely unknown they are reflected in the responsiveness of the patient's cells to IFN-β [52].

NAb-positive patients clustered in the PCA of the *ex vivo* dose-response data collected in autologous sera, and the clustering was independent of NAb titers per se (Paper III[82]). The results show that any titer measured in sera of patients inhibited the responsiveness of immune cells to IFN-β re-stimulation compared to the re-stimulation control in media (no patient sera) and also to untreated patients and healthy individuals. The differences in signal induction between NAb-positive patients and untreated or healthy individuals were highly significant, and upon re-stimulation with 250 IU/ml the IFN-β/Stat pathway was basically turned off in all NAb-positive patients (titer ranged from 17-6930 NU/ml).

The cutoffs used in *in vitro* assays mandated that a patient with a NAb concentration of 17 NU/ml be in the NAb-negative group. However, the inhibition of the IFN-β/Stats pathway in primary cells from this patient at 250 IU/ml of IFN-β shows that the signaling pathway is affected by very low levels of NAbs, and at this re-stimulation concentration the data of this patient clusters with all the other NAb-positive patients. At a re-stimulation concentration of 1000-2000 IU/ml of IFN-β the inhibition of such low NAb titers is overcome, and this patient clusters with the treated NAb-negative patients. The inhibitory effect of various NAb titers can be compensated by high re-stimulation doses of IFN-β as was shown for other patients. At a re-stimulation concentration of 1000 IU/ml of IFN-β, treated NAb-negative and NAb-high patients can clearly be separated whereas some of the patients with NAb titers in the lower ranges show some activation of the pathway. However, the signal

induction in these patients is about 50% of what is measured in treated NAb-negative patients.

For the stimulation in autologous sera the final sera dilution was 1:1. This was due to practical reason, but we speculate that the results of Paper III[82] would be even stronger in terms of total inhibition of the Jak/Stats pathway by NAbs at IFN-β concentrations that would normally induce maximal signal if sera were not diluted.

The results showed that pStat1 was a significant variable for clustering of patients according to NAb class and that measuring pStat1 in all cell subtypes may be redundant. Ideally a biomarker for NAb effects would require one measurement in one cell subtype at one re-stimulation concentration such as pStat1 in monocytes at 1000 IU/ml. To have a robust test, however, it may be necessary to measure phosphorylation of pStat1 at two re-stimulation concentrations, 250 IU/ml and 1000 IU/ml, in one cell subtype.

Only 15 patients were included in this study, but despite, the results of this proof of concept study regarding NAbs are highly significant. Our data indicate that a patient-specific NAb test in primary cells may be developed that is not dependent on arbitrary cut-offs but rather is based on whether or not the cells of the patient respond to IFN-β re-stimulation at certain concentrations.

No clusters were identified in the PCA of the data collected in serum-free media. This showed that sera containing NAbs is a major factor contributing to the inhibition of the IFN-β/Stat pathway. In addition the re-stimulation in artificial serum-free media allowed us to detect whether the IFN-β/Jak pathway is functional in that particular patient when sera containing NAbs was removed from the stimulation condition.

Before doing a larger study it was important to determine whether the pStat proteins measured *ex vivo* could also be identified and have similar meaning *in vivo*. Even though it was not possible to include many patients with various NAb titers in this study, the effects of NAb on the responsiveness of the IFN-β Jak/Stat pathway were measurable and evident (Paper IV). The variable pStat1 in several immune cell

subtypes was highly correlated with NAb class. The PCA was able to distinguish a patient with a low NAb titer as well. However, this patient did not cluster with the other NAb-positive patients. The peculiarity of this patient goes beyond NAb antibodies. The IFN-β levels in this patient were about four times as high before the IFN-β injection as those of other patients and were in the range of what can be measured in HIV infection (personal communication from the ELISA vendor). The *in vitro* NAb assay measured a titer of 1:40 before injection, and this went down to negative post-injection and was barely detectable after 8 hours. A reliable NAb status was probably difficult to determine in this specific patient at time of sample collection. The high amount of IFN-β in the system sequesters some of the NAbs and thus makes measurement unreliable. The PCA of the *in vivo* assay identifies this patients as different, and in the PLS analysis this patient was marked as a possible outlier for some measurements. The *in vivo* assay could therefore also be valuable in detecting patients with an abnormal response to IFN-β for other reasons than NAb.

The degree of Stat1 phosphorylation in blood cell subtypes after IFN-β administration *in vivo* was relatively high compared to other Stats as well. In NAb-negative patients, consistent and stable increases were measured over several hours in many blood cell subtypes. Compared to *ex vivo* re-stimulation with high doses of IFN-β the changes were small (in the range of two fold); the fold changes in PBMCs were 10 to 12 in T cells and monocytes, respectively. Notably, Stat1 phosphorylation was significantly reduced or shut down in many cell subtypes in patients with high NAb titers *in vivo*. Monocytes were more affected by NAb than T cells and showed marked inhibition of Stat6, Stat5, and Stat3 phosphorylation when Stat1 was inhibited. Individual patient variation certainly explains some of the differences seen in Stat activation *in vivo*. Stats are activated and regulated by many signaling molecules and a Stat signaling map in healthy individuals has not yet been determined. After IFN-β injections in healthy individuals, serum concentrations and gene expression inductions have been measured, but Stat activation has not. At this stage we can only compare Stat signaling within patients and evaluated NAb effects relative to treated patients without NAbs.

One of the major concerns in testing the signaling pathway of a biopharmaceutical in patients' cells is the time of sample collection. This is not only the case for *in vivo* studies where the effects of a medicine in a single patient are the desired measurement variables, but also for sample collection that is taken from patients for later analysis. *In vitro* assays and *ex vivo* assays are influenced by time of sample collection. For *in vitro* NAb evaluation, the consensus is that sera should be collected a minimum of 12 hours after the last injection to avoid binding of the injected IFN-β to NAb, which confounds NAb titer measurements. Some studies even suggest a wash out period to insure that IFN-β is completely metabolized. This issue needs careful consideration in any assay.

The time of sampling issue has clearly been shown in gene expression studies *in vivo*. First of all, timing is crucial for signal detection. The optimal time for detecting *Mx1* induction *in vivo* is between 4-12 hours post-injection of IFN-β. For detection of Stat phosphorylation by phospho-flow we observed a consistent signal between 2 and 6 hours after IFN-β injection. To measure both gene expression and Stat phosphorylation we took samples between 4 and 8 hours. A study published in 2011 by Zula et al. also looked at Stat phosphorylation after IFN-β injection in RRMS patients[101]. Samples of the eight patients included in this study were taken between 30 minutes and 150 minutes post injection. They used a different protocol for whole blood phospho-flow published by Chen et al. that is based on a two-step staining procedure[102]. After fixation, red cells were lysed and stained with cell surface markers. Only then were the cells permeabilized with methanol and stored over night at -20° C. The next day the cells were stained for intracellular phosphorylated Stat proteins. The readout of their analyses was percentage positive cells. Their results were extremely variable between patients. For pStat1 in monocytes, for example, the percentage of positive cells ranged from 0% to 22% for samples taken at the same time points. They explain this phenomenon by variation between patient responses. Another explanation is that their multi-step procedure and the methanol incubation interfered with consistent phospho-epitope analysis. In the one step assay used in this thesis, a consistent and similar change in median fluorescent intensities was observed

for all NAb-negative patients 2-4 hours post-injection. However, since components of the pathway itself are up-regulated by IFN-β it cannot be excluded that some of the Stat proteins levels are increased four to eight hours after administration. Nonetheless, a diagnostic test must be robust, and a stable measurement is desirable as measured for Stat1 phosphorylation levels in cells of NAb-negative patients. The intricacies of the *in vivo* response in individual patients remain to be elucidated and confirmed.

The importance of distinct homeostatic compartmentalized body fluids (matrices) surrounding cells is shown by the active maintenance of barriers such as the blood-brain barrier and distinct circulatory systems in the body such as cerebrospinal fluid, blood, and the lymphatic system. The blood circulation is the medium for transport of many proteins via carrier molecules, of signaling molecules that may be in their pre-active forms, and for systemic signaling flow. The matrix of immune cells can be very different from person to person and will influence immune cell function. Soluble receptors present in blood and a myriad of other compounds that are patient-specific influence signaling. For example, evidence is accumulating that the interferon signaling system plays a role in the disease and in response to therapy[29, 103-106]. Down-regulation of the fully functional receptor isoform (IFNAR-2c) and up regulation of the soluble isoform (IFNAR-2a) has been shown in RRMS patients treated with INF-β[104]. Interestingly, levels of endogenous IFN-β are elevated in sera of RRMS patients that are not responding to IFN-β therapy compared to responders[107]. Since phospho-flow has the potential to incorporate environmental aspects found in sera of patients with preconditioned response capabilities of cells, it may be possible to delineate an optimal treatment response signaling pattern pinpointing suitable patients for INF-β treatment and identifying non-responders.

Since the matrix surrounding cells directs signaling in cells, the conditions of stimulation are pertinent to analysis of signaling pathways. Serum contains all the compounds found in blood except blood cells and coagulation factors. This matrix reflects patient blood composition characteristics individually and is perfect for the analysis of NAbs. In *in vitro* assays this matrix is sequentially diluted with support

media to determine NAb titers. However, dilution of a patient's sera may not accurately reflect the patient's physiological state. By changing the matrix composition, artifacts may be introduced that will affect signaling outcomes in cells. Phospho-specific flow cytometry is a suitable technique to analyze the impact of NAbs in primary cells in their own matrix - sera or whole blood.

Cell-type specific Stat activation is of interest not only for NAb evaluation but also for evaluation of IFN-β response in patients to determine treatment efficacy. We may or may not have been looking at the most informative cell subtypes in our study. IFN-β receptors are widely expressed in human cells of all types. Optimally a cell type involved in the disease process would be the best choice since that cell type would be chosen because of its biomarker properties of disease progression. Unfortunately there are no such biomarkers for the disease progression in RRMS. Based on the immune-mediated component of MS and the immune-modulatory effects of IFN-β, we assumed that immune cells were the best option. For a diagnostic test, non-invasiveness is important, and blood cells are easily accessible, can be cryopreserved, and can be shipped for analysis to specialized centers.

The analysis PCA and PLSA used in the *in vivo* study are powerful methods to discern signal from noise. Many of the variables varied little or not at all in a cell type-specific manner. The measurement for some of these Stat variables may be noise and be in the lowest range of what is possible with phospho-flow in this setup. I believe that models to predict clinical outcomes based on the biochemistry of a drug at the single cell level will have great potential in biomedicine.

8. Conclusion

Immunogenicity of recombinant human proteins in biomedicine is a known complication of this type of therapy, but effects on efficacy are unpredictable. In MS, an inflammatory disease of the central nervous system, the immunogenic effect in IFN-β therapy is observed in *in vitro* assays and titers of anti-IFN-β neutralizing antibodies in patient sera have been reported. These assays do not reveal the biological effect of an immunogenic response to treatment in single patients, however. Here, the immunogenic effects of IFN-β therapy in primary immune cells from IFN-β-treated MS patients were evaluated using a multiplexed flow cytometry-based technique. In the presence of IFN-β neutralizing antibodies, the biological signaling response in primary cells was severely disrupted or modulated. Our data challenge the current rationale of basing therapy decisions on *in vitro* titer data.

The idea and concept to use the phospho-flow technique to interrogate immune cells of immune-mediated diseases such as MS and to monitor immune-modulating drugs in a personalized approach was published in Paper I[52].

In Paper II[88] we published a detailed protocol for the analysis of signaling pathways in PBMS based on phsopho-specific flow cytometry (and unpublished data is shown in the results section). We showed that pathways can be activated and signaling molecules quantified specifically for the IFN-β/Stats pathway. We established a routine protocol for serum free cryopreservation of PBMCs that was used for patient sample collection.

In Paper III[82] we showed a highly significant NAb effect on the responsiveness of the Jak/Stats pathway in PBMCs that can be quantified based on the method published in Paper II[88]. The protocol was specifically optimized and tested for NAb evaluation. We proposed a test that could be patient specific and independent of NAb titer cut offs.

Paper IV (manuscript) showed that it is possible to quantify the activation of the Jak/Stat pathway *in vivo* in patients treated with IFN-β and that NAb effects can be quantified with phospho-specific flow cytometry. We further show that the same Stats proteins have biomarker potential both *in vivo* and *ex vivo.* We postulate that Stats phosphorylation may be a more reliable marker for NAb effects than gene expression changes.

9. Future Perspectives

During the last decade the scientific and medical community has published data based on large clinical MS trials to find commonalities between patients in disease mechanisms and drug responses. These trials have produced considerable insight and generated exciting hypotheses. However, the etiology and pathogenesis of MS is largely unknown. What triggers an exacerbation or why the disease ranges from benign to progressive remains elusive.

A range of immune-modulatory properties are attributed to the beneficial effects of IFN-β in RRMS patients, especially the anti-inflammatory effects. New drugs attempt to take advantage of the beneficial effects of IFN-β in MS treatment. For example, Natalizumab is a recombinant humanized IgG4κ monoclonal antibody produced in murine myeloma cells with a human IgG framework and murine complementarity determining region that binds to specific integrins expressed on all leukocytes except neutrophils. The block of binding of these ligands to their receptors of the vascular cell adhesion molecule family (VCAM-1) expressed on vascular endothelial cells, prevents the transmigration of leukocytes across the endothelium (e.g. brain-blood barrier) into inflamed parenchymal tissue (e.g. the brain). In this case leukocytes accumulate in the blood. Intravenously administered Natalizimab is immunogenic and in about 10% of patients NAb appear[108]. Another new medication, Fingolimod, acts by sequestering leukocytes in the lymph nodes so that these cells cannot reach the brain. Fingolimod is a sphingosine analogue that when phosphorylated prevents leukocyte adhesion to endothelial cells in the lymph nodes. No data are available for Fingolimod given in tablet form. Oral drugs may be less immunogenic than subcutaneous injections, but the possible immunogenicity of drug metabolites is not well studied. Antibodies to drug metabolites may contribute to tissue-specific damage, and tests for this type of adverse immune reaction do not exist[109, 110].

Many new drugs are in the pipeline for approval in MS treatment, and the monitoring of anti-drug antibodies is recommended. The introduction of a mechanistic anti-drug test already in pre-clinical and clinical trials would increase the likelihood to detect such antibodies. In the early phase of clinical drug assessment results from such test could be used to adjust treatment but also to discover biological markers for treatment efficacy.

The issue of immunogenicity will not disappear from biomedicine based on our current understanding of the immune system. *In vitro* tools to predict immunogenicity of drugs are being developed, but the formation of anti-drug antibodies in any given patient will be difficult to predict. It is important to develop tools that will identify the formation of anti-drug antibodies early or even before treatment start with techniques that are patient specific. In RRMS it is virtually impossible to evaluate immunogenic reactions without actually measuring anti-IFN-β antibodies of the binding and neutralizing nature since biomarkers for disease progression are not available. In addition, the RRMS disease spectrum ranges from benign to severe, which indicates that disease subtypes exist that may or may not benefit from specific therapies. A predictive biomarker for disease progression could solve many problems regarding immunogenicity and therapy in RRMS.

IFN-β treatment failure in RRMS may mirror the variable pathological patterns of the disease. It has been suggested that RRMS is not a single disease but a collection of syndromes that cause demyelination. This heterogeneity may also relate to the response variability seen during IFN-β treatment. Several studies implicate the Type I IFN signaling pathway in treatment failure and possibly disease subtypes of MS. In RRMS, a subset of non-responders to IFN-β therapy expresses high concentrations of both the endogenous IFN-β and IL-17F before treatment starts [15]. Patients may differ in T cell profiles, and the authors hypothesized that IFN-β may be beneficial in patients with a Th1-mediated disease and may even be harmful in patients with a Th17-mediated inflammatory disease. IFN-β does not act independently in the human body. The yin and yang of cytokine signaling culminates in opposing effects in

different contexts and has been shown not only in divergent diseases such as cancer and autoimmunity, but also in closely related diseases such as MS and neuromyelitis optica[12, 111-113]. IFN-β is beneficial in MS, but exacerbates neuromyelitis optica [114, 115]. The tool developed in this thesis could be used to study disease-specific cell subsets and signaling pathways involved in disease mechanisms and potentially identify signaling patterns that are associated with response to therapy.

A study based on microarray technology showed that non-responders and responders to IFN-β could be differentiated by their Type I interferon-induced gene expression signature before treatment start[105]. Non-responders had increased levels of endogenous IFN-β activity in sera and lower induction of Type I IFN inducible genes in treatment-naïve PBMCs compared to responders. Upon treatment basal phosphorylation (activation) of Stat1 and the cell surface expression of IFNAR1 were elevated in monocytes of non-responders compared to responders. Another study measured the mRNA expression of the different IFN-β receptor isoforms before and during therapy and found that the isoform IFNAR-2c which is the full length transmembrane-receptor, was predictive of NAb development[104]. *Mx1* is a type I IFN inducible gene and is frequently used as a biomarker of IFN-β bioactivity in blood of patients. The lack of expression of this gene is associated with the occurrence of relapses even in the absence of NAb that inhibit *Mx1* expression in patients. Further, SNPs in genes encoding components of the Jak/Stat signaling pathway have been found significantly associated with MS[116]. For example, the SNPs in the kinase Tyk2 and in Stat3 may affect the signal transduction[117-119]. Of therapeutic importance is the fact that the Type I IFN signaling pathway is not only implicated in disease modulation in IFN-β-treated MS patients, but that the endogenous pathway may also define subtypes of MS disease and possibly explain the variable response seen in patients. In terms of clinical applicability the tool developed in this thesis may link cellular processes to physiological processes in disease state and therapy in MS[83]. Identifying Type I IFN signaling signatures in health, disease, and treatment based on the tool developed in this thesis may potentially be used in treatment evaluation based on predictive IFN signaling models that incorporate genetic factors like SNPs and

phenotypic variability between patients. Biomarkers could then be selected that represent pathogenesis as well as immunogenicity.

The tool developed in this thesis may be used in other medical fields where biopharmaceuticals are used. For example, in therapeutic antibody treatment in rheumatoid arthritis (RA) the injected drug is immunogenic. Several antibody preparations against TNF-α are used in the clinic to inhibit the activation of the TNF-α signaling and thereby down-regulate the inflammatory reaction. The immunogenic reaction to these TNF-α preparations is known, but as in MS, clinical practice is not based on mechanistic assays for treatment response[120-124]. Anti-TNF-α-antibodies are measured with ELISA and radio-immunoassays in sera of patients. However, the assays are not standardized. It appears that history repeats itself also in the anti-drug-antibody issue in this treatment regime. The current assays have little value regarding the inhibition of the activation of the TNF-α pathway *in vivo* in a specific patient. The disease is variable and biomarkers for RA progression are not available. The inhibitory effect of TNF-α treatment can be assessed with phospho-flow in immune cells. Immune cells express the TNF-α receptor and are involved in the pathogenesis of RA. The signal from the cell surface can activate various pathways, NF-κB, the MAPK, and the apoptotic pathway. Signal transduction molecules are modified in the propagation of the signal, and several monoclonal antibodies to these modified molecules are available for phospho-flow. In MS, and probably also in RA, *in vitro* assays are not informative enough to make therapeutic decisions possible in single patients. Based on the vast experience with IFN-β in MS treatment, a mechanistic approach to TNF-α evaluation in single patients may save the medical community time and money and above all benefit patient health.

The characterization of many disease-associated signaling signatures simultaneously at the single cell level is now possible with the latest technology. CyTOF technology, based on a combination of flow cytometry and mass spectrometry, allows simultaneous quantification of e proteins and phosphorylation sites in single cells[125].

The technology will improve our understanding of biochemical process within cells and could potentially be used in diagnostic and therapy.

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