From the Department of Medicine
Karolinska Institutet, Stockholm, Sweden

THE ROLE OF INTERFERON-REGULATED GENES IN THE IMMUNE SYSTEM

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Karolinska Institutet

Stockholm 2017

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Published by Karolinska Institutet.
Printed by E-PRINT AB

ISBN 978-91-7676-573-9

The role of interferon-regulated genes in the immune system

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i **CMM Lecture Hall**, L8:00, Karolinska Universitetssjukhuset, Solna

Friday February 17th 2017, at 9.00

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ABSTRACT

Type I interferons (IFNs) are potent inducers of the first-line defense against pathogens. Their activity leads to the up- and downregulation of a large number of genes with various effects on the immune system, including direct effects on the pathogens. Due to the strong response evoked by IFN signaling, the IFN pathway is tightly regulated by IFN stimulated genes to avoid detrimental effects of long-term exposure. If the IFN signaling pathway is not regulated properly, or for other reasons constantly activated, it can lead to interferonopathies and autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). Indeed, many therapeutics targeting the IFN pathway are currently in clinical trials. In contrast, type I IFNs are used to treat certain types of cancer, virus infections and multiple sclerosis. The complex role of type I IFN signaling in disease is not well understood and need further characterization for better therapeutic inventions.

The aim of this thesis was to identify genes that are regulated by type I IFNs and investigate their role in the immune system. To do this, we quantified the expression of interferon-regulated genes in sorted immune cells from patients with primary SS, and from individuals treated with IFNβ. Using global gene expression analysis on PBMCs as well as qPCR on sorted cells, we could identify several genes that were differentially regulated by type I IFNs in different immune cell populations. Among them were TRIM21 that was upregulated in T cells and B cells, BAFF that was upregulated in T cells, monocytes and neutrophils and miR-150-5p that was selectively downregulated in monocytes. The downregulated expression of miR-150-5p consequently led to an increased expression of its target c-Myb. In addition, monocytes from patients with SLE displayed an increase in c-Myb target genes. When investigating the regulation of BAFF and TRIM21, we found that both were regulated by members of the interferon regulatory factor (IRF) family. Specifically, both were upregulated by IRF1 and IRF2, and downregulated by IRF4 and IRF8. To further investigate the role of TRIM21 in the immune system, we generated *Trim21*$^{-/-}$ mice. These mice were prone to granulocyte infiltrations and developed symptoms of autoimmune disease after being triggered by metal ear tags. We found that TRIM21 negatively regulates the immune response by ubiquitinating IRFs, and that naïve *Trim21*$^{-/-}$ mice control the development of eosinophils in the bone marrow.

LIST OF PUBLICATIONS

I. Type I IFN reduces micro-RNA-150 levels in monocytes and activates a c-Myb transcriptional program
Alina Johansson, Maria Sjöstrand, Petra Bergman, Mohsen Khademi, Magnus Andersson, Fredrik Piehl, Ruxandra Covacu, Maja Jagodic, Alexander Espinosa
Manuscript

II. The expression of BAFF is controlled by IRF transcription factors
Maria Sjöstrand, Alina Johansson, Lara Aqrawi, Tomas Olsson, Marie Wahren-Herlenius and Alexander Espinosa
J Immunol 2016; 196:91-96; doi: 10.4049/jimmunol.1501061

III. Expression of the immune regulator tripartite-motif 21 is controlled by IFN regulatory factors
Maria Sjöstrand*, Aurélie Ambrosi*, Susanna Brauner, Jenna Sullivan, Stephen Malin, Vijay K. Kuchroo, Alexander Espinosa and Marie Wahren-Herlenius
J Immunol 2013; 191:3753-3763; doi: 10.4049/jimmunol.1202341

IV. Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway
Alexander Espinosa*, Valerie Dardalhon*, Susanna Brauner*, Aurélie Ambrosi, Rowan Higgs, Fransisco J. Quintana, Maria Sjöstrand, Maija-Leena Eloranta, Joan Ní Gabhann, Ola Winqvist, Birgitta Sundelin, Caroline A. Jefferies, Björn Rozell, Vijay K. Kuchroo and Marie Wahren-Herlenius
J Exp Med 2009; 206:1661-1671; doi: 10.1084/jem.20090585

V. TRIM21 controls the maturation of eosinophils in the bone marrow
Maria Sjöstrand, William Nyberg, Alina Johansson and Alexander Espinosa
Manuscript

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LIST OF ABBREVIATIONS

BAFF	B cell activating factor
cDC	Conventional dendritic cells
CDN	Cyclic dinucleotides
DDX41	DEAD-box helicase
DNA	Deoxyribonucleic acid
GAS	Interferon γ-activated site
IFN	Interferon
IFNAR	Interferon-α/β receptor
IKKi	IκB kinase i
IRF	Interferon-regulated factor
IRG	Interferon-regulated gene
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK1	Janus kinase 1
LPS	Lipopolysaccharide
MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation-associated gene 5
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor-κB
pDC	Plasmacytoid dendritic cell
pSS	Primary Sjögren's syndrome
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase
TLR	Toll-like receptor
TRIM	Tripartite-motif
TYK2	Tyrosine kinase 2

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1. INTRODUCTION

In the early 1950's Nagano and Kojima observed an anti-viral agent in rabbit skin infected with vaccinia virus (1). Isaacs and Lindenmann later discovered and named the factor causing this antiviral state "interferon" (IFN) (2). Not until 20 years later, Rubinstein and colleagues published the purification of IFN (3). In 1980, Taniguchi et al. and Mantei et al. published the first successful cloning of IFN (4, 5). IFNs turned out to be a family of proteins consisting of IFN type I, II and III. IFNα, IFNβ, IFNε, IFNκ and IFNω are collectively called type I IFNs, and they are the largest class of IFNs. Thirteen human genes that code for twelve different IFNα proteins, and only one single gene each that codes for the human IFNβ, IFNε, IFNκ and IFNω proteins, have been described. IFNγ is the only member of the type II IFN subclass, while type III is the most recently described subclass consisting of IFNλ1, IFNλ2 and IFNλ3. This thesis will address type I IFNs and its effector genes.

1.1. Type I IFN signaling

1.1.1. Classical signaling pathway

All type I IFNs share the same ubiquitously expressed receptor consisting of two chains, interferon-α/β receptor (IFNAR) 1 and IFNAR2. IFNAR1 constitutively associates with tyrosine kinase 2 (TYK2) and IFNAR2 with Janus kinase 1 (JAK1) (6, 7). Binding of type I IFNs to its receptor leads to the activation of the JAK-STAT (signal transducer and activator of transcription) signaling pathway (8, 9). After dimerization of the receptor subunits, TYK2 and JAK1 are activated followed by phosphorylation of intracellular tyrosine residues on IFNAR, creating docking sites for STATs. STATs are then recruited to the intracellular domain of the receptor and bind the docking site through their Src-homology 2 (SH2) domains followed by phosphorylation of STATs on tyrosine residues (10, 11). Phosphorylated STAT1 and STAT2 form a complex together with interferon-regulated factor (IRF) 9, called interferon-stimulated gene factor 3 (ISGF3), which in turn is transported into the nucleus where it can bind to a transcription factor binding site named interferon-stimulated response element (ISRE) and activate gene transcription (8, 10, 11). Type I IFN signaling can also active other STAT homo- and heterodimers which binds to the IFNγ-activated site (GAS) (12, 13).

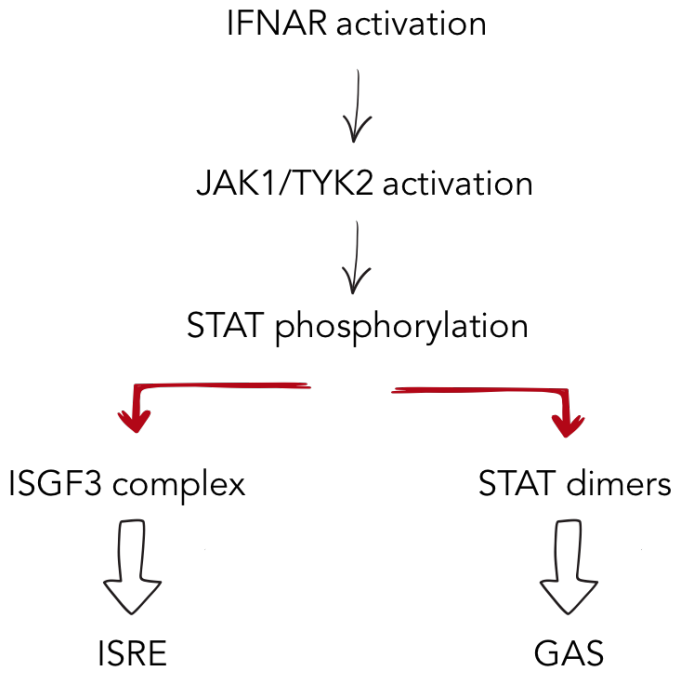

Fig. 1. Type I IFN signaling. Binding of type I IFN to its receptor leads to the activation of the JAK-STAT pathway in which JAK1 and TYK2 phosphorylates STATs. Phosphorylated STAT1 and STAT2 forms a complex together with IRF9 called ISGF3 that can bind to ISRE in the nucleus and activate gene expression. Phosphorylated STAT homo- or heterodimers on the other hand can bind to GAS-elements and activate gene expression.

1.1.2. Non-classical signaling pathways

IFNAR can also signal via other pathways than the JAK-STAT signaling pathway, particularly the p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways.

1.1.2.1. The p38 MAPK pathway

p38 is activated by an upstream phosphorylation cascade where each kinase act as a substrate for the other. p38 is phosphorylated by MAPK kinase (MAPKK), which is phosphorylated upstream by other kinases in the phosphorylation cascade. The activation of the IFN signaling pathway via p38 is not fully understood, but the G-protein Rac1 seems important and is activated by upstream kinase activity, possibly a JAK (14). The downstream effect of the p38 MAPK pathway ultimately leads to the activation of two serine kinases, MAPKAPK2 and MAPKAPK3. It is not known how p38 activates the transcription of interferon-stimulated genes (ISGs), however evidence so far indicates that the p38 MAPK pathway is essential for the transcription of ISGs (15).

1.1.2.2. The PI3K pathway

Members of the type I IFN family can induce the phosphorylation of insulin receptor substrate (IRS) 1 and 2, and PI3K associates with IRS, followed by activation of PI3K, in an IFN-dependent manner downstream of JAKs (13). Protein kinase C (PKC) δ is activated by IFNs and has been proposed to phosphorylate STAT1 on Ser727 (16). IFNγ-stimulated cells in which the PI3K activity has been inhibited cannot phosphorylate STAT1 on Ser727, leading to reduced levels of STAT1 transcripts (17). Taken together, these data suggest that the IFN-mediated activation of the PI3K pathway leads to the phosphorylation of STAT1 on Ser727 (13). The phosphorylation on Ser727 in STAT1 is not essential for STAT1 binding to ISG promoters, but for full transcriptional activation (18, 19).

1.2. The production of type I IFNs

Type I IFNs can be produced by all nucleated cells in response to the activation of pattern recognition receptors (PRRs). Cytosolic PRRs that recognize nucleic acids from viruses are the predominant receptors responsible for type I IFN production in most cells. The activation of some Toll-like receptors (TLRs) can however also induce type I IFNs in certain cell types such as macrophages and dendritic cells (DCs). One cell type is specialized in type I IFN production and produce large amounts after TLR activation, namely the plasmacytoid DC (pDC) (20).

1.2.1. MDA5 and RIG-I

Melanoma differentiation-associated gene 5 (MDA5) and Retinoic acid-inducible gene I (RIG-I) are caspase activation and recruitment domain (CARD) -containing PRRs activated by viruses. MDA5 and RIG-I sense different types of viruses due to their ability to recognize different RNA structures (21). They signal via mitochondrial antiviral-signaling protein (MAVS, also known as IPS-1, VISA and Cardif) and the kinases TANK-binding kinase 1 (TBK1) and IκB kinase i (IKKi) and activates IRF3 and IRF7 which in turn form homo- or heterodimers that enter the nucleus and bind to ISRE (22).

1.2.2. STING and cGAS

Stimulator of interferon genes (STING) is an immune signaling molecule associated with the endoplasmic reticulum (ER) that is activated by pathogen-derived or endogenous cyclic dinucleotides (CDNs) in the cytosol of the cell (23, 24). STING activation leads to transcriptional upregulation of several genes, including type I IFNs. Cytosolic DNA can activate cyclic GMP-

AMP synthase (cGAS) to produce CDNs, known as cytosolic guanosine monophosphate-adenosine monophosphate (cGAMP), in the presence of ATP and GTP (25). The CDNs in turn can activate STING, which forms a complex with TBK1 that is necessary for the delivery of TBK1 to the endolysosomal compartment where it phosphorylates IRF3 and nuclear factor-κB (NF-κB) (24, 26, 27). STING has also been proposed to work as a downstream signaling molecule of DNA PRRs, such as DEAD-box helicase 41 (DDX41) and IFNγ-inducible protein 16 (IFI16) (28).

1.2.3. TLRs and pDCs

Some members of the TLR-family can induce type I IFNs; TLR3, TLR4, TLR7, TLR8 and TLR9. Of these, only TLR4 is located on the cell surface while the others are endosomal. TLR4 is the only TLR known to induce type I IFNs in response to non-nucleic acid ligands, namely bacterial lipopolysaccharides (LPS). TLR3 and TLR4 can signal via the adaptor molecule TIR-domain-containing adaptor protein inducing IFNβ (TRIF) and TBK1 to activate IRF3 (29). Conventional DCs (cDCs) express TLR3, TLR8 and low levels of TLR2 and TLR4, pDCs however express high levels of TLR7 and TLR9. TLR7 and TLR9 respond to ssRNA and DNA respectively and are dependent on the adaptor protein myeloid differentiation primary response gene 88 (MyD88) for signal transduction. Only in pDCs, TLR7 and TLR9 are efficient type I IFN-producers due to their constitutive expression of IRF7 (30). The MyD88 and IRF7 complex is retained in the endosomal compartment and induces type I IFNs in a spatiotemporal regulatory manner (31).

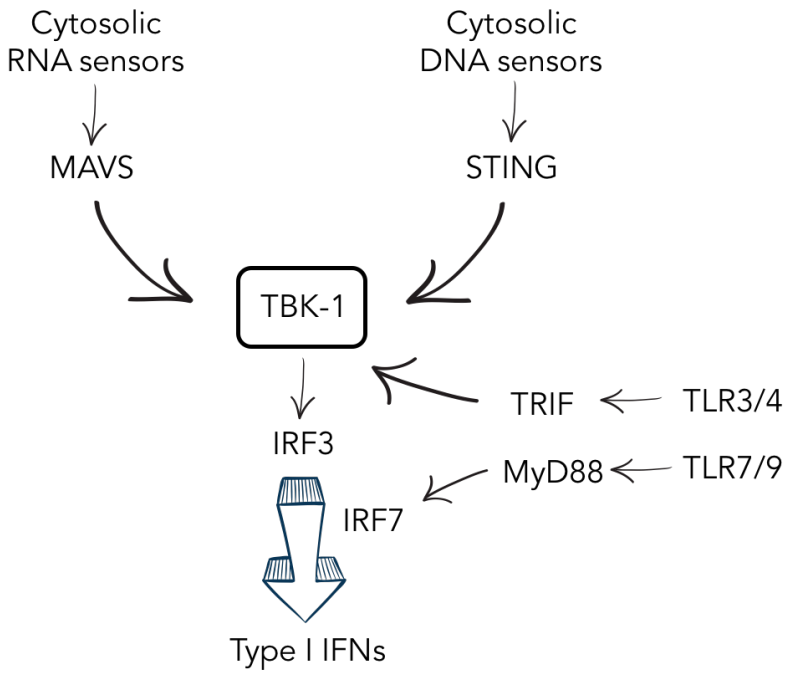

Fig. 2. Type I IFN induction. Cytosolic PRRs recognize RNA and DNA, and signal via MAVS and STING respectively as well as the kinase TBK1. TLR3 in the endosome, and TLR4 on the cell surface, can signal via TRIF and TBK1. TBK1 signaling ultimately leads to IRF3 activation and type I IFN production. Endosomal TLR7 and TLR9 can activate the MyD88 pathway upon type I IFN stimulation, and activate IRF7 exclusively in pDCs.

1.3. Interferon regulatory factors

IRFs are transcription factors acting under PRRs. The mammalian family of IRFs consists of 9 members, IRF1-IRF9, all containing a well-conserved DNA-binding domain that recognizes ISRE (A/GNGAAANNGAAACT) (10). IRF1, IRF3, IRF5 and IRF7 have been described as important for type I IFN gene transcription (32). The IRF1 gene was the first IRF reported to bind type I IFN gene promoters, however it seems redundant for type I IFN expression (33). IRF3 and IRF7 are key regulators of type I IFN expression. IRF3 is constitutively expressed while IRF7 is expressed in small amounts in all cells, and induced by type I IFN signaling via the ISGF3 complex (32). TBK1 and IKKi are the virus induced tyrosine kinases that activates IRF3 and IRF7 (34, 35).

In addition to IRF7, IRF5 can also be activated by the TLR7/TLR9-pathway, bind to MyD88 and subsequently initiate gene transcription (36). IRF4 has also been reported to bind MyD88, and has been suggested to compete with IRF5 to inhibit MyD88-dependent IRF5 activation in T cells, B cells and macrophages where IRF4 is mainly expressed (37, 38). IRF8 is also an immune-specific IRF but contrary to IRF4, it increases TLR9-signalling (39). IRFs form various heterodimers between each other (except IRF1 and IRF2), but can also form complexes with other proteins. IRF4 and IRF8 for an example, can act as transcription repressors by binding to ETS/ISRE domains when bound to ETS-family transcription factor PU.1 (32, 38).

Several IRFs are also regulating immune cell development, e.g. IRF4 is required for plasma cell differentiation, for differentiation of $CD4^+$ DCs, for Th2 differentiation and supports B cell development; IRF8 is required for the development of $CD8\alpha^+$ DCs and pDCs, stimulates macrophage differentiation, supports B cell development and promotes Th1 differentiation through macrophages and DCs (38, 40).

1.4. Interferon-regulated genes

Type I IFN signaling consequently leads to the up- and downregulation of a large number of genes (10). These genes are involved in numerous biological processes, such as anti-viral and anti-intracellular bacterial defense, immune regulation, apoptosis, cell differentiation etc. The regulatory mechanisms that induce ISGs are well-characterized and have been extensively described in this thesis. However, the mechanisms that leads to the downregulation of genes by type I IFNs is not well-characterized. miRNAs that are regulated by IFNs could play an important role in this downregulation of gene expression (41). Genes that are up- and downregulated following IFN-stimulation are collectively called interferon-regulated genes (IRGs). The Interferome (www.interferome.org) is a database collecting information from high-throughput experiments containing type I, II and III IRGs. IRGs in the Interferome database are identified from organisms or cells treated with IFNs, and defined as statistically up- or downregulated genes with a 2-fold change in expression. With these generous definition parameters, around 3000 genes are classified as IRGs in human and mouse (42).

1.4.1. ISGs in viral defense

ISGs can inhibit different stages of the virus life cycle. For an example, myxovirus resistance 1 (MX1) inhibits an early stage of the virus life cycle by forming ring-structures around nucleocapsids to trap them (43). Another example of an ISG that can inhibit viruses at an early stage is tripartite motif-containing (TRIM) protein 5α. This protein can bind to the retroviral capsid of HIV-1 and accelerate its cytoplasmic uncoating (44). TRIM5α is only one of many members in the TRIM family with anti-viral effects (45). Members of the 2'-5' oligoadenylate synthase (OAS) family on the other hand, can inhibit dsRNA viral replication by activating the endoribonuclease RNase L to degrade viral transcripts (43). During the late stages of the virus life cycle, its nucleic acids are packed into capsids before it exits the cell. Tetherin is a protein that inhibits virus budding by trapping virions on the plasma membrane (46, 47). Due to the multifaceted anti-viral response by type I IFNs, many viruses have developed mechanisms to inhibit IFN-signaling and ISGs (48).

1.4.2. ISGs and immune regulation

Since IFN-signaling leads to a strong immune response, the IFN pathway is tightly regulated by ISGs to avoid detrimental effects of long-term exposure. One example is suppressor of cytokine signaling (SOCS) proteins that inhibits JAK-STAT signaling by inhibiting JAK enzymatic activity (49). Another example are members of the TRIM protein family, in which many or potentially all

are E3 ligases that are involved in both the positive and negative regulation of the IFN response (50). E.g. TRIM21 can ubiquitinate and negatively regulate members of the IRF family and DDX41 (51-55).

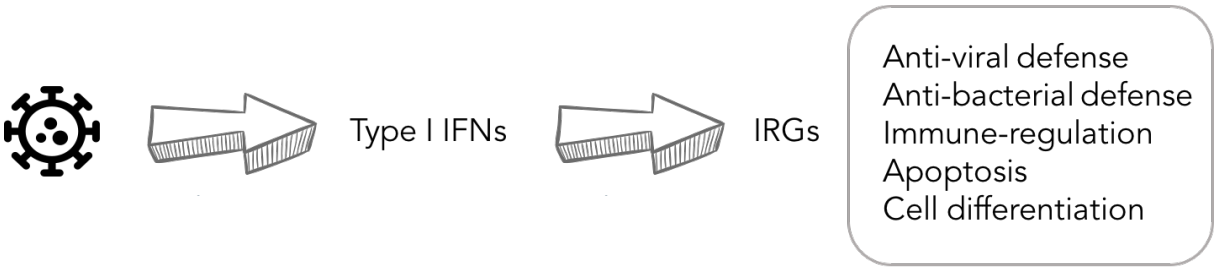

Fig. 3. Type I IFNs regulate numerous biological processes via IRGs. Infection by e.g. a virus, leads to the up- and downregulation of a large number of IRGs. These can in turn e.g. act in the direct defense against the pathogen, regulate the immune system, induce apoptosis or affect cell differentiation.

1.5. Type I IFNs in immune disorders and disease

Since its discovery, the role of type I IFNs in clearing viral infections has become evident. However, aberrant IFN-signaling has emerged as an important feature in several autoimmune and autoinflammatory disorders. In contrast, type I IFNs is successfully used to treat multiple sclerosis (MS) and certain types of cancer. The complex role of type I IFN signaling in disease is not well understood and need further characterization for better therapeutic inventions.

1.5.1. Type I interferonopathies

Genetically determined disorders driven by type I IFNs are collectively called interferonopathies. The type I IFN response can result due to several reasons; enhanced levels or abnormal chemical modifications of endogenous nucleic acids, constant activation or enhanced sensitivity of the type I IFN pathway in a ligand-independent manner, or defects in the negative regulation of the type I IFN pathway, either dependent or independent of nucleic acid sensing (56). Definitive proof that type I IFN is causing the pathology behind these diseases is lacking, however many of these diseases have clinical phenotypic overlaps (57). A few examples of type I interferonopathies are Aicardi-Goutiéres syndrome (AGS), systemic lupus erythematosus (SLE) and interferon-stimulated gene 15 (ISG15) deficiency. AGS was the first monogenic disorder described to have increased type I IFN activity, and some of the proteins in which the gene is mutated in AGS include DNA 3' repair

exonuclease 1 (TREX1), adenosine deaminase acting on RNA (ADAR) and MDA5 (58). TREX1 is a nuclease that target ssDNA and dsDNA and ADAR is a dsRNA-editing enzyme.

1.5.2. Pathological mechanisms behind SLE and SS

SLE is a complex systemic autoimmune disease where genetic background as well as environmental factors contribute to pathology. In only a small fraction of patients (<5%), a mutation in a single gene is thought to be causative for disease (59), and of those even less have mutations where evidence can predict an upregulation of type I IFNs (57). Therefore a very small proportion of SLE cases qualify as monogeneic type I interferonopathies as defined by Rodero and Crow (57). Nonetheless, many adult SLE patients and nearly all pedriatic SLE patients display an 'IFN signature' (60, 61). The IFN signature is defined by elevated levels of ISGs in the blood, and is observed in several systemic autoimmune conditions including Sjögren's syndrome (SS) and rheumatoid arthritis (RA) (62, 63). Type I IFN mRNA levels are hard to detect in healthy individuals, even after vaccination, and also in interferonopathies (57). Therefore, a screening for ISG expression levels is more informative.

SLE is characiterized by the autoantibody production against components of the cell nucleus and a wide array of clinical manifestations such as inflammation, vasculitis, immune complex deposition, fatigue and glomerulonephritis. Type I IFNs, mainly IFNα, seem to be an important mediator responsible for many of the immunological features leading to clinical disease in SLE patients (64). Some patients with virus infections and certain cancer types that are treated with type I IFNs develop SLE or symptoms of SLE, that in many cases are transient and ceases to appear when the IFN-treatment is discontinued (65-67). This strongly suggests that ISGs are in fact responsible for the pathology behind the development of SLE.

The main hypothesis as to how ISGs are induced in SLE suggests an important role of the endogenously activated TLR7/TLR9-induced expression of type I IFN in pDCs (68). In this model, RNA and DNA are released from apoptotic cells and accumulate due to reduced clearing of apoptotic cells in SLE patients. Anti-nuclear antibody- (ANA) RNA/DNA complexes are then endocytosed by pDCs and activate IFN-production via IRF7 (69). Type I IFNs in turn activate DCs and upregulate major histocompatibility complex (MHC) and costimulatory molecules leading to the activation of autoreactive T cells (70-72). They also induce the production of B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that promote B cell survival, differentiation and isotype switching (73).

SS is an autoimmune disease with some clinical overlap with SLE, such as autoantibodies towards TRIM21/Ro52 and the IFN signature. Also in this disease, the IFN-signature and consequently the upregulation of BAFF is proposed to have an important role in the pathogenesis (74). Patients with SS have a predisposition towards inflammation in exocrine glands leading to sicca symptoms, such as dry eyes and dry mouth. SS can occur in combination with other autoimmune diseases, such as SLE, and is then called secondary SS. If the patient is only diagnosed with SS, it is referred to as primary SS (pSS).

1.6. Type I IFNs in therapy

1.6.1. Targeting the IFN pathway

With the emerging evidence that type I IFNs play an important role in the development of immune disorders with an IFN-signature, a number of new therapeutic strategies targeting the IFN-pathway are in clinical trials. The only established treatment so far targeting the IFN pathway in SLE is hydroxychloroquine (HCQ) (75). HCQ is an antagonist of endosomal TLRs by reducing endosomal acidification. Two anti-IFNα monoclonal antibodies (mAbs) have completed phase II clinical trials for SLE, sifalimumab and rontalizumab (76, 77). Both studies showed promising results with decreased disease activity and a decrease in the IFN-signature compared to placebo. Both drugs appeared to be safe, however sifalimumab-treated patients more frequently had herpes zoster infections. Despite the promising results, none of these molecules were chosen for phase III studies. On the other hand, a phase II study on anifrolumab, a mAb blocking IFNAR2 and thereby inhibits type I IFN signaling, indicated a more efficacious outcome for SLE patients (78). This molecule has been chosen for a phase III clinical trial. In addition, several molecules targeting JAKs have been developed. Tofacitinib, targeting JAK1 and JAK3, is approved for clinical use in RA and is in phase I clinical trials for SLE (75). There are also several other therapeutics targeting different nodes of the IFN pathway, such as TLRs and pDCs, in early clinical trials (75).

Targeting the IFN response has so far showed promising results in some systemic autoimmune diseases and further strengthen the evidence that type I IFNs are the driving force behind these.

1.6.2. IFN as therapy

Due to the pro- and anti-inflammatory, anti-viral and anti-cancer properties of type I IFNs, it is used to treat certain cancers, viral infections and also MS. In cancer, IFN has both direct effects

on the cancer cells, such as anti-proliferative and pro-apoptotic effects, and indirect by enhancing the immune response towards the cancer cells. Cancer cells can develop an IFN-resistance by e.g. downregulating type I IFN genes and receptors, indicating that type I IFNs are important for killing cancer cells. However, IFN treatment has had varying success. Recent trials indicate that combining IFN treatment with other therapies might be more beneficial (79).

In MS, IFNβ was the first therapy to show clinical efficacy and is still the most common first-line treatment. However, 40% of the patients do not respond, or respond poorly, to IFNβ (80). MS is an inflammatory disease of the central nervous system caused by demyelination of the nerves. It is still not known how IFNβ improves the clinical outcome of MS, but its anti-inflammatory activities are thought to be responsible. In a mouse model for MS, experimental autoimmune encephalomyelitis (EAE), type I IFNs were shown to inhibit Th17 development and thus provide a possible clue to the beneficial effects of type I IFNs in MS (81). Interestingly, an IFN signature was identified in a subset of the most common type of MS, relapsing-remitting MS (RRMS), and an increased baseline level of ISGs was associated with a lack of response to IFNβ (82, 83). Thus, the IFN signature can be used to predict clinical efficacy of IFNβ treatment in MS patients.

The main drawback of using type I IFNs in therapy is the large number of severe side effects, including influenza-like symptoms, fatigue, neurological toxicities, anorexia, depression and leukopenia (84). Therefore, there is a great need of finding therapeutic options for treating these diseases.

2. AIMS

The overall goal of this thesis was to identify new IRGs and investigate how they are regulated and what their roles are in the immune system. The rationale behind this is that IRGs can help us understand the complex nature of type I IFN signaling and its role in immune disorders. To reach this goal, we set up these aims:

- Identify human IRGs that could play a role in disease (paper I, II, III, IV and V)
- Identify regulatory elements in selected IRGs and test their functionality (paper II and III)
- Investigate human material or knock-out mice to identify a role for the IRGs in the immune system and/or immune disorders (paper I, IV and V)

3. RESULTS

3.1. Differences in IFN signature between major immune cell populations

(Paper I, II, III, IV and V)

We aimed to identify genes that are regulated by type I IFNs and investigate their role in the immune system. To do this we quantified the expression of IRGs in sorted immune cells from patients with pSS, and from individuals treated with IFNβ.

Since many patients with systemic autoimmune disease display an IFN signature with elevated levels of ISGs, we collected blood samples from patients with primary Sjögren's syndrome (pSS) and healthy controls and performed a gene expression array (62, 85, 86). When comparing patients with an IFN signature to controls without an IFN signature, we observed that many well-known ISGs such as MX1 and OAS2 were upregulated in patients. We also observed that many members of the TRIM gene family were differentially regulated in patients.

Feng et al. describe that different cell populations can respond differently to IFN stimulation due to varying abundance of the epigenetic marker H3K9me2 on the ISG promoters (87). To investigate the cell-specific regulation of some of the genes we identified in the pSS patients, we collected new blood samples from pSS patients and controls with sicca symptoms and sorted the cells into $CD14^+$ monocytes, $CD3^+$ T cells, $CD19^+$ B cells and $CD15^+$ neutrophils. In the pSS patients, we could observe that the classical ISGs MX1 and OAS2 were upregulated in all cell populations. However, individual TRIM genes were differentially regulated only in certain populations. For an example, TRIM1 (MID2) was most prominently downregulated in $CD19^+$ B cells and to some extent in $CD3^+$ T cells (Sjöstrand et al., unpublished data). TRIM25 was upregulated in the same populations (Sjöstrand et al., unpublished data). To validate that these genes were differentially regulated as a result of IFN signaling and also to observe an acute IFN response rather than a chronic, we collected gene expression data in blood samples from patients with MS before and approximately 18 hours after *de novo* IFNβ (Avonex®) administration and sorted these cells into the same populations as in the pSS patients. We could confirm the upregulation of TRIM25 in only T cells and B cells (Sjöstrand et al., unpublished data). We could also see a clear induction of TRIM21 in only T cells and B cells. Additionally, we observed that

BAFF was upregulated significantly in T cells, monocytes and neutrophils and with the same trend in B cells, however not significant.

To identify miRNAs that are regulated by type I IFNs, we performed a TaqMan-based miRNA gene expression array (754 genes) on one MS patient with a strong response to IFNβ (based on MX1 and OAS2 expression levels), on T cells and monocytes before and 18 h after IFNβ administration. We identified a number of miRNAs that could potentially be regulated by type I IFNs, including miR-150-5p that was strongly repressed in monocytes. We confirmed the results from the initial miRNA profiling in all sorted populations from all patients included in the study and confirmed reduced levels of miR-150-5p selectively in monocytes.

Collectively, we could identify several genes that were differentially regulated by type I IFNs in different immune cell populations. Among them were TRIM21 that was upregulated in T cells and B cells, BAFF that was upregulated in T cells, monocytes and neutrophils and miR-150-5p that was selectively downregulated in monocytes. We chose to study these three genes in more detail: how they are regulated by type I IFNs and what their role is in the immune system.

3.2. Type I IFNs reduces microRNA-150-5p levels in monocytes and activates a c-Myb transcriptional program

(Paper I)

After identifying miR-150-5p as a miRNA that is downregulated after IFNβ administration in MS patients, we wanted to investigate how this occurs in more detail. We found that the transcript levels of pri-miRNA-150 were not affected by IFNβ, suggesting that the reduced levels of mature miRNA-150-5p were due to either decreased maturation of pri-miR-150 or increased release of miR-150-5p from monocytes. Since monocytes have previously been reported to secrete miR-150-5p, we wanted to test if this was the case in our patient samples (88). We therefore isolated plasma miRNAs from MS patients before and after IFNβ administration and observed a strong reduction of plasma miRNA-150-5p, indicating that IFNβ blocks miR-150 maturation rather than effect its secretion from monocytes.

There is evidence that the IFN signature displayed in patients with the systemic autoimmune disease SLE is driving the disease (89). We were therefore wondering if miR-150-5p was also reduced in SLE patients. Indeed, miR-150-5p was reduced in PBMCs and cells from CSF compared with healthy controls and MS patients with an inflammatory disease but without an IFN signature. miR-150 post-transcriptionally regulates c-Myb, a transcription factor important for hematopoiesis (90, 91). We then wondered if c-Myb target genes were over-expressed in SLE patients. Indeed, we could see that c-Myb target genes were over-represented in gene expression data from monocytes in pediatric SLE patients (GSE46907) (92).

In summary, we identified miR-150-5p as an IFN-regulated miRNA selectively down-regulated in monocytes. Additionally, we observed that in SLE patients with an IFN signature, miR-150-5p was downregulated leading to an activation of a c-Myb transcriptional program.

3.3. The ISGs BAFF and TRIM21 are controlled by transcription factors of the interferon regulatory factor (IRF) family

(Paper II and III)

Both TRIM21 and BAFF were upregulated in humans as a result of type I IFN signaling, prompting us to investigate the molecular mechanism underlying this. First we set out to determine if TRIM21 was also regulated by IFNs in mice. We stimulated a mouse T cell line (EL-4) and mouse splenocytes with type I and II IFNs and could observe that TRIM21 was indeed upregulated after the addition of IFNs. From now on, the studies of TRIM21 gene expression is on the mouse gene while the studies of BAFF expression is on the human gene.

To identify putative regulatory elements in the promoter region of these genes, we compared the promoter region of BAFF and TRIM21 between different species to find conserved transcription factor binding sites. In both genes, we could find a highly conserved IFN-stimulated response element (ISRE), TRIM21 containing three GAAA repeats and BAFF containing two GAAA repeats, upstream of the transcription start site. Importantly, we could not find a conserved IFNγ activated site (GAS) in neither of the two genes. To investigate if the identified ISRE sites were functional, we generated luciferase reporter constructs containing each of the ISREs. Since members of the IRF family are known to bind ISRE (93), we performed a luciferase reporter assay

after the co-transfection of the reporter construct together with a panel of IRFs into HEK293T cells. IRF1 and IRF2 could induce expression of both constructs while IRF4 and IRF8 blocked expression. IRF1 and IRF2 failed to induce expression of a mutated version of the reporter constructs where the core GAAA elements were altered into AAAA.

Since TRIM21 was also upregulated after IFNγ stimulation despite the lack of a GAS element, we speculated that IFNγ stimulated the expression of IRF1 which in turn would bind to the ISRE rather than the direct binding of STAT homodimers. To test this, we pre-treated EL-4 cells with cycloheximide (CHX) to block *de novo* protein synthesis and stimulated the cells with type I and II IFNs followed by analysis of IRF1, STAT1 and *Trim21* expression. CHX completely blocked the induction of nuclear IRF1 expression while STAT1 expression was only mildly affected. Importantly, *Trim21* induction was greatly reduced after CHX treatment indicating that IRF1 is required for optimal TRIM21 induction while STAT1 is not.

In summary, we concluded that TRIM21 and BAFF are true ISGs whose expression is regulated by members of the IRF transcription factor family.

3.4. TRIM21 is a negative regulator of innate immune responses

(Paper IV)

To understand the role of the ISG TRIM21 in the immune system, we generated *Trim21*-deficient GFP-reporter mice. Using the GFP-reporter to detect TRIM21 expression, we found that TRIM21 was primarily expressed in immune tissues and to some extent in endothelial cells. *Trim21*$^{-/-}$ mice appeared normal and had no differences in frequencies of the major immune cell populations. However, CD3^{+} T cells had an increased frequency of activated T cells (CD62L^{low}).

While there was no apparent immune phenotype in naïve mice, over 90% of *Trim21*$^{-/-}$ mice that were tagged with metal ear clips developed severe dermatitis with ulcerations and granulocyte infiltrations. *Trim21*$^{+/+}$ littermates on the other hand, had no reaction to the tag. Cells from draining lymph nodes and spleen of *Trim21*$^{-/-}$ mice produced IL-6, IL-12/IL-23p40, IL-21, IL-22, and IL-17. IL-17 was produced by CD4^{+} T cells, indicating that there were elevated levels of Th17 cells at the site of inflammation. IL-4 and IFNγ was however not increased in *Trim21*$^{-/-}$ mice compared

with *Trim21*$^{+/+}$ mice. The phenotype was also replicated with low doses of the contact-sensitizing agent oxazolone.

To investigate what caused the elevated levels of cytokines we aimed to find substrates ubiquitinated by TRIM21. The transcription factors IRF3 and IRF8 have previously been identified as targets for TRIM21 (52, 54). We hypothesized that IRF5 also might be a target of TRIM21 since it regulates the expression of e.g. IL-12/Il-23p40 and IL-6. Using ubiquitination assays, we observed that IRF5 is indeed polyubiquitinated by TRIM21. To further investigate the effect of TRIM21 on the activity of IRFs, we performed a GAL4 one-hybrid luciferase reporter assay. IRF3 and IRF5 were fused to GAL4 and tested for their ability to drive luciferase expression after binding to a 4xGAL4-luciferase reporter. When cotransfecting with TRIM21 into 293T cells, we observed a decrease in IRF transcription factor activity after simulating with TLR ligands poly(I:C) or CpG.

Since the IL-23-IL-17 pathway with a skewing towards the Th17 axis of the immune system seem to be activated rather than the IL-4-IFNγ pathway, we hypothesized that we could rescue the phenotype by crossing the *Trim21*$^{-/-}$ mice with IL-23p19$^{-/-}$ mice. Indeed, when the IL-23-IL-17 pathway was abolished, the *Trim21*$^{-/-}$ mice were no longer sensitive to oxazolone.

In summary, we identified TRIM21 as a negative regulator of innate immune responses by controlling the activity of IRF transcription factors downstream of TLR signaling.

3.5. TRIM21 controls eosinophil development in the bone marrow

(Paper V)

IFNα is successfully used to decrease eosinophil numbers in patients with hypereosinophilic syndrome (HES), indicating that one or several ISGs can control eosinophil homeostasis (94, 95). Since *Trim21*$^{-/-}$ mice are prone to granulocyte infiltration, we hypothesized that TRIM21 could be an ISG involved in regulating eosinophil numbers. To test this, we performed FACS on naïve *Trim21*$^{-/-}$ mice compared to *Trim21*$^{+/+}$ mice and observed increased levels of eosinophils in blood and tissues.

To investigate if this was due to increased eosinopoiesis, we performed FACS on bone marrow cells from *Trim21*$^{-/-}$ mice compared with *Trim21*$^{+/+}$ to identify and compare different stages during eosinophil development. There was no difference in levels of eosinophil progenitors nor immature eosinophils. However, *Trim21*$^{-/-}$ mice had increased levels of mature eosinophils.

In summary, we identified TRIM21 as a novel regulator of eosinophil development.

4. CONCLUSIONS

In this thesis, we set out to identify new IRGs and investigate their role in the immune system. We observed that BAFF, TRIM21 and miR-150 were regulated by type I IFNs and confirmed that BAFF and TRIM21 were true ISGs whose expression were controlled by members of the transcription factor family of IRFs. By generating *Trim21*-deficient mice we could conclude that TRIM21 regulates innate immune responses by controlling the activity of IRFs and that TRIM21 controls the homeostasis of eosinophils in naïve mice.

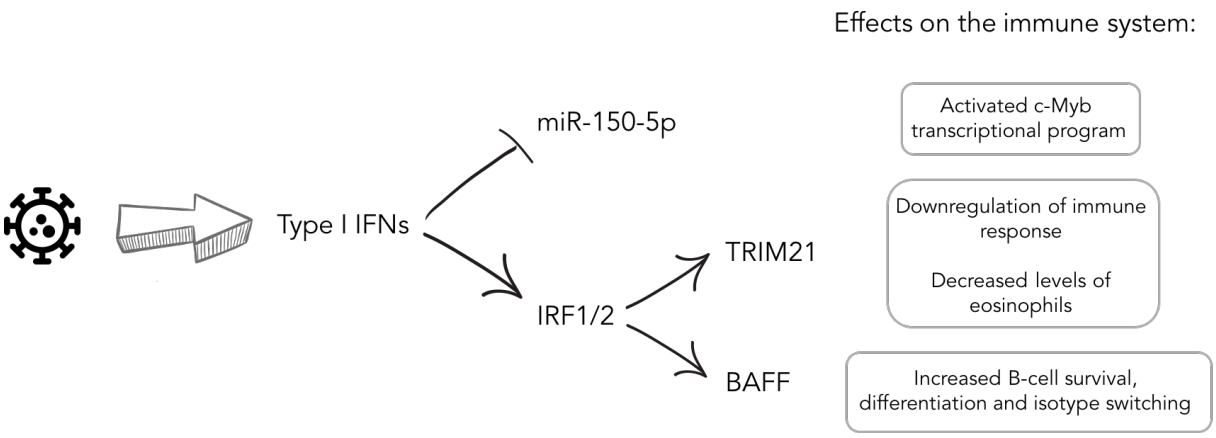

Fig. 4. Conclusions. Type I IFNs can reduce the levels of miR-150-5p in monocytes, consequently leading to an activated c-Myb transcriptional program. Type I IFNs stimulates the expression of BAFF and TRIM21 via the transcription factors IRF1 and IRF2. BAFF stimulates increased B cell survival, differentiation and isotype switching. TRIM21 is a negative regulator of the immune response by regulating IRFs. TRIM21 can also decrease the levels of eosinophils in the bone marrow.

5. DISCUSSION AND FUTURE PERSPECTIVES

IFN signaling play an important role in clearing viral infections and leads to the up- and downregulation of a great number of genes that both activates and negatively regulates the immune response to control for tissue damage. IFN signaling is very powerful and the fine-tuning of the response is therefore very important. The expression pattern of IRGs can differ between different viral infections and different autoimmune diseases (96). Probably due to factors such as genetics, different IFN-escape mechanisms in different viruses and the acute vs chronic state in viral infections compared with autoimmune disease. The most studied IRGs are genes involved in viral defense, such as MX1 and members of the IFIT family (97). In this thesis, we used different approaches to identify new IRGs that could possible play a role in human disease.

5.1. How should one choose important IRGs to study further?

The material chosen to identify IRGs (e.g. cancer cell lines or freshly isolated tissue) will greatly impact the results. Therefore, it is important to carefully select a system to identify relevant IRGs. Our aim was to identify IRGs in human and study their regulation and function (in mouse or human). We therefore chose to identify IRGs that were generally affected by type I IFNs in an *in vivo* human model. For this purpose, we chose pSS patients that naturally have elevated levels of type I IFNs, and MS patients before and after *de novo* IFNβ therapy. pSS patients are often untreated, thus reducing the risk of treatments impacting the gene expression analysis. In sorted cells from pSS patients, we sometimes had difficulties reaching statistical significance. The sorted cells were compared with persons with sicca symptoms and not healthy controls, this could explain the clearer results from the microarray on PBMC where pSS patients were compared with healthy controls. In sorted cells from MS patients, we compared the same patients before and after IFN-treatment. This is an optimal control since individual differences in basal expression levels will be accounted for. Another advantage with this material is that the sample after IFNβ administration was taken after approximately 18 hours, which makes it likely that the differences we observe in gene expression is due to a direct effect of IFNβ.

We first used a broad approach where we performed global gene expression analysis on PBMCs from pSS patients with an IFN-signature. We then used a more selective approach where we quantified selected IRGs in sorted immune cells from pSS patients. In addition, we sorted cells

from MS patients before and after IFNβ administration and determined the expression of selected genes by qPCR. The benefit of this approach is that we could look at cell specific regulation and that we are less likely to miss differentially regulated genes due to a "dilution effect" in PBMCs or due to possible lymphopenia in the patients (98). The downside is that we will select genes based on the data from the PBMCs and cannot screen for new genes. We identified a family of genes that were differentially regulated, the TRIM family. The fact that many members of the same family of genes are differentially regulated suggests that they are true IRGs. Indeed, they have previously been reported to be regulated by IFNs *in vitro* and many of the TRIM genes contain putative ISRE sites (99). Comparing a chronic IFN-signature in pSS patients with an acute in IFNβ-treated MS patients, we could confirm some of the TRIM genes expression patterns. In the MS material, we do not expect to identify genes that are differentially regulated due to pSS-related effects not caused by IFN-signaling. Comparing these two patient materials and selecting genes that are specifically differentially regulated in only one or the other could possibly lead to finding genes important for that specific disease, however in this thesis we chose to focus on genes that are generally regulated by type I IFNs. Also since the sorted cells were only analyzed by qPCR it was not possible to compare gene expression in an unbiased way. Since the TRIM family of genes were differentially regulated in both materials and since many TRIM genes are reported to regulate the immune system (50), we speculated that TRIM genes could be important IRGs in human disease. Interestingly, one of the TRIM genes that we identified, TRIM21, is an autoantigen in several systemic autoimmune diseases such as pSS and SLE. Little was previously known about its function in the immune system, we therefore chose to study TRIM21 in more detail. We also identified BAFF as differentially regulated in our material. Since BAFF has been reported to contain an ISRE (100), and since BAFF is targeted as therapy for SLE (101), we chose to study how it is regulated in more detail.

When identifying IFN-regulated miRNAs, we chose monocytes and T cells from one of the MS patients, before and after IFNβ administration. This patient was chosen based on a strong induction of MX1 and OAS1 expression. Choosing only one patient has its disadvantages since there is a huge variation between individuals due to differences in genetic background and environment. Since we were limited in how many arrays we could perform, we however reasoned that choosing one patient was better than pooling patients and risking diluting differences in gene expression. In this patient, we identified one miRNA that was strongly downregulated in monocytes, miR-150-5p. We could also confirm this by qPCR in monocytes isolated from all MS patients. It could also make sense to choose miRNAs that are differentially regulated in both T

cells and monocytes, since that would decrease the risk of choosing a false positive. We however found that since miR-150-5p was so strongly downregulated, made it an interesting target to verify and study further. miR-150-5p has previously been shown to regulate B cell differentiation by targeting the transcription factor c-Myb, making it an interesting gene to study in B cell driven autoimmune diseases such as SLE and pSS.

In this thesis, I have chosen a few IRGs from our patient samples to study in more detail based on what is previously known in the literature about these genes. In future studies, a more systematic approach could be considered where each of the identified IRGs could be knocked-down/out or overexpressed in cell lines and then, based on the *in vitro* data, a few of them could be selected for further studies *in vivo* in animal models. For this you would need a very specific question, e.g. which IRGs affects B cell differentiation? These IRGs could then be potential targets in B cell driven autoimmune diseases.

5.2. Can IRGs help us understand IFN-driven autoimmune diseases?

Patients treated with type I IFNs during e.g. viral infections or certain cancers sometimes develop symptoms of systemic autoimmune disease, which strongly suggests that IRGs are the driving force in these diseases (65, 66). Determining the role of IRGs in the immune system is important for understanding the pathology behind IFN-driven systemic autoimmune disease.

In paper I, we link type I IFNs with decreased levels of miR-150-5p and increased levels of its target c-Myb. miR-150-5p was specifically downregulated in monocytes after IFNβ administration in MS patients with a decrease in both cellular and circulating miR-150-5p. Monocytes have been reported to secrete miR-150-5p, however we cannot be certain if the observed decrease in circulating miR-150-5p is due to decreased release from monocytes or due to reduced levels of monocytes in response to the IFNβ-treatment (88). We also observed an increase in the miR-150-5p target c-Myb in SLE patients and an increased level of c-Myb target transcripts. miR-150-5p and c-Myb are therefore affected in both acute and chronic IFN responses. c-Myb has previously been reported to be overexpressed in SLE and c-Myb expression levels have been correlated to disease activity in SLE (102, 103). This could be a reflection of the IFN signature, which correlates with disease activity in SLE (60, 61). c-Myb is a proto-oncogene and is a key regulator of hematopoiesis (104). The fact that one or several proto-oncogenes are indirectly upregulated by type I IFNs is interesting and counterintuitive, since type I IFNs are used as cancer therapy (105).

Interestingly, patients with SS have an elevated risk of developing certain neoplasms, most commonly non-Hodgkin lymphoma (106). The chronic effects of IFN-signaling and c-Myb expression could then in part contribute to this. There is *in vitro* based evidence that circulating miR-150-5p could be taken up by cells and bind to c-Myb (88). Less circulating miR-150-5p could then potentially affect c-Myb expression in B cells. c-Myb has also been reported to block monocyte differentiation (107, 108). Monocytes from SLE patients have distinct features compared with healthy controls, such as increased sensitivity to apoptosis (109). Also, miR-150-5p levels are reduced in intermediate monocytes compared with classical and non-classical monocytes (110). Our data suggest that the aberrant features of SLE monocytes can in part be explained by the IFN-mediated decrease of miR-150 and the concomitant increase in c-Myb expression.

In paper III, we identify TRIM21 as an ISG and in paper IV we describe how TRIM21 regulates the immune response by negatively affecting the activity of members of the IRF transcription family. TRIM21 is also known as Ro52 and is an autoantigen in several systemic autoimmune diseases such as SLE and SS. Additionally, TRIM21 is overexpressed in SLE and pSS (111). The role of TRIM21 as a negative regulator of the immune system is hard to connect to the development of autoimmune disease, however, TRIM21 overexpression could simply be a "side effect" of the IFN-signature in these diseases. Autoantibodies directed towards the RING domain of TRIM21, isolated from patients with pSS, can indeed block the ubiquitinating activity of TRIM21 (112). However, it is not known if the autoantibodies will ever reach TRIM21 since it predominantly resides in the cytoplasm (113). As mentioned, we and others have reported that TRIM21 regulates members of the IRF transcription family. There are however conflicting reports on the outcome of the ubiquitination of IRFs by TRIM21. For an example, Yang et al. report that TRIM21 is essential to sustain IRF3 activity (114) while Higgs et al. report TRIM21-mediated degradation of IRF3 (52). Therefore, TRIM21 could regulate members of the IRF family differently in different situations. Hence, we cannot exclude that the ubiquitination of IRFs by TRIM21 could lead to the upregulation of certain genes important for the development of autoimmune disease.

We made an attempt to understand if the overexpression of TRIM21, without the simultaneous effect of other ISGs, could contribute to the pathology of autoimmune disease by generating a conditional knock-in mouse for TRIM21. We inserted cDNA encoding FLAG-tagged TRIM21 into the ROSA26 locus, enabling TRIM21 overexpression in all tissues in both embryonic and adult mice. To prevent constitutive overexpression of *Trim21*, a floxed stop cassette was introduced upstream of FLAG-TRIM21. In order for TRIM21 to be expressed the mouse has to be crossed

with another mouse expressing Cre recombinase under the promoter of choice. With this system, it is possible to control the expression of *Trim21* in a spatial and temporal manner, and to detect *Trim21*-overexpressing cells by green fluorescent protein (GFP) fluorescence. We crossed the mice with Vav1-Cre mice so that *Trim21* would be overexpressed in all hematopoietic cells, and could confirm successful recombination by GFP expression in immune cells. There were no apparent differences in the mice that were born compared with littermate controls. We immunophenotyped the mice and could not see any differences in immune cell populations. The mice were slightly less responsive to *in vivo* poly(I:C) stimulation (Sjöstrand et al., unpublished data). From these experiments, we could conclude that TRIM21 overexpression alone is not important for the development of autoimmunity in mice. TRIM21 could however play a role in mice genetically prone to develop autoimmune disease. This could be tested by crossing the overexpressing ROSA26-TRIM21 mice with disease prone mice or by inducing SLE using the pristane injection model.

Future studies on understanding the role of TRIM21 in autoimmune disease should focus on clarifying the outcome of IRF ubiquitination by TRIM21, and also look for new targets for TRIM21. The latter could be done for an example by using the BioID technique (115). In this method, a biotin ligase is fused to your protein of choice, e.g. TRIM21, and expressed in cells where it biotinylates endogenous potential interaction partners. The biotinylated proteins can then be isolated and identified.

5.3. IRFs and the regulation of ISGs

In study II and III, we find that TRIM21 and BAFF expression is controlled by members of the IRF family. IRFs are transcription factors acting downstream of PRRs such as TLRs and cytosolic PRRs (e.g. RIG-I and MDA-5). More specifically, we found that both TRIM21 and BAFF expression were upregulated by IRF1 and IRF2, and downregulated by IRF4 and IRF8 by binding to the two respective ISREs (TRIM21 containing three GAAA repeats, and BAFF containing two). We tested the ability of all other IRFs (with the exception of IRF6) one by one to induce expression of TRIM21 and BAFF, and for BAFF also a combination of IRF3, -5, -7 and -9 as they are known to heterodimerize, and they could not affect BAFF or TRIM21 expression. However, it is still possible that these other IRFs could affect TRIM21 or BAFF expression after proper activation by post-translational modifications (40, 116) while IRF1 and IRF2 can bind ISRE without post-translational modification (117-119). Since CHX treatment in EL-4 cells completely abolished

Trim21 expression after IFN stimulation, the ISGF3 complex containing IRF9 might not be able to induce *Trim21* expression, or induces it poorly.

IRF2 is generally known to repress IRF1 transcription activation by binding to the same site and blocking transcription (120). However, e.g. Oshima et al. has shown that IRF2 can activate gene expression of the same gene (*Il7*) as IRF1 in human (121). IRF1 and IRF2 are induced by type I IFNs. IRF1 is also strongly induced by IFNγ while our data show that IRF2 is not induced by IFNγ. IRF4 and IRF8 are not induced by type I IFNs, IRF8 however can be induced by IFNγ. It could be that in cells where IRF4 and/or IRF8 are expressed, such as T cells and B cells, it is important to keep TRIM21 and BAFF basal expression low, and to control the IFN-induced expression. This further supports the hypothesis that TRIM21 is important in T cell biology as suggested by Ishii et al (122). Our data also shows that neutrophils are the most potent inducers of BAFF after type I IFN stimulation, while the induced and the basal expression in T cells and B cells is much lower.

With these two studies, we provide further insight into how IRFs can control ISG expression. Both IRF1 and IRF2 are important for the IFN-induced expression of these genes while IRF4 and IRF8 provide a tight regulation, and are perhaps important for keeping the expression of certain ISGs lower in certain cell types. As mentioned, the ISGF3 complex is a weak inducer of *Trim21* gene expression, however all IRFs have the potential to bind ISRE. There are therefore additional regulatory mechanisms controlling the specificities or affinity for IRF binding to ISRE.

Future studies on how different numbers of GAAA repeats in the ISRE are important for the control of ISGs by IRFs could provide new insight in ISG regulation. Genes containing triple or double GAAA repeats in tandem, are more strongly induced by IFN than genes containing single or double GAAA repeats (123). Perhaps the number of GAAA repeats is also important for the negative control of certain ISGs by IRF4 and IRF8.

5.4. Is there a link between type I IFNs and the development of eosinophils in the bone marrow?

In study IV, we observed that *Trim21*$^{-/-}$ mice were prone to develop contact hypersensitivity with granulocyte infiltrations. In study V, we immunophenotyped naïve *Trim21*$^{-/-}$ mice and observed a double frequency of eosinophils in blood and elevated levels in tissues compared with *Trim21*$^{+/+}$

mice. Eosinophils have recently been linked to the Th17 pathway where GM-CSF induced by IL-23 leads to eosinophil accumulation and subsequent intestine inflammation as well as increased production of eosinophils in the bone marrow in a T cell transfer model of IL-23-driven colitis (124). We thus propose that TRIM21 can regulate the IL-23/Th17 pathway by ubiquitinating members of the IRF family. Interestingly, type I IFN has been shown to inhibit Th17-mediated inflammation in EAE, a mouse model for MS (81). In all, this suggests that there is a link between type I IFNs, TRIM21, the Th17 pathway and eosinopoiesis.

We did not observe an increase in the canonical cytokines IL-5 nor Eotaxin-1 in the *Trim21*$^{-/-}$ mice, indicating that TRIM21 regulates eosinopoiesis via non-canonical factors, either one or several cytokines or a molecule expressed on the cell surface acting via cell-cell contact. Since no other granulocyte population was affected, the factor must be specific for eosinophils, which leaves out a number of canonical cytokines involved in eosinophil development including GM-CSF. In study IV, we observed that TRIM21 is expressed in endothelial cells in naïve GFP expressing *Trim21*$^{+/-}$ mice. Endothelial cells contribute to the bone marrow microenvironment which is important for hematopoiesis, and these cells could therefore be potential producers of the TRIM21-regulated factor(s).

The increase of infiltrating granulocytes in the *Trim21*$^{-/-}$ mice suggests that the lack of *Trim21* could worsen the outcome in a model where one triggers hypereosinophilia e.g. asthma. This is however yet to be tested. In future studies, we will focus on finding the TRIM21-regulated factor(s) controlling eosinopoiesis in the bone marrow and what cells that are producing it. To find the factor(s), we will look in the bone marrow where the eosinopoeisis occurs rather than in sera. For an example, we could isolate mature eosinophils in the bone marrow and perform RNA sequencing and compare with immature eosinophils to find expression patterns that could result from known signaling pathways. To rescue the eosinophilic phenotype, we are crossing the conditional knock-in ROSA26-TRIM21 mice to *Trim21*$^{-/-}$ and Vav1-Cre expressing mice. Using the TRIM21 knock-in mice on a *Trim21*$^{-/-}$ background and crossing them to different Cre expressing mice could also help us to identify what cell that is producing the unknown factor. For an example, we could cross the mice with Tie2-Cre (Tek-Cre) where Cre is expressed in endothelial cells.

5.5. IRGs and clinical relevance

The more we know about the molecular mechanism behind a disease, the more likely it is to identify drug targets. And the more we know about the role of a potential drug target under normal conditions, the better we can predict toxic side-effects when targeting it for therapy.

Targeting the IFN pathway has shown promising results in clinical trials for SLE, however in e.g. MS, HES and certain cancers type I IFNs are administered as therapy. The fact that type I IFNs are the driving force in one autoimmune disease while improves the clinical outcome of another highlights the complexity of the IFN signaling response. Since type I IFNs are important for the defense of viral infections, blocking their function could lead to severe infections and potential reactivation of latent infections. Administering type I IFNs on the other hand, leads to a very strong immune response with the upregulation of a great number of genes causing severe side effects. It is not clear how type I IFNs are causing disease nor how they are beneficial in treating disease. In this thesis, we have identified IRGs and investigated their role in the immune system to better understand the IFN-response and its role in disease, and to potentially find new drug targets.

miR-150-5p mimics could potentially be delivered as a therapy in SLE to block B cell development and antibody production. However, due to the promiscuous nature of miRNAs, it is hard to predict toxic side-effects. miR-150-5p could on the other hand be used as a biomarker, as previously proposed by Bergman et al. (125). Follow-up studies on IFN-treatment outcome in MS patients could perhaps tell us if miR-150-5p can predict the response to IFNβ in individual patients.

BAFF is targeted for therapy in autoimmune disease due to its role in regulating B cell survival, differentiation, maturation, immunoglobulin class switching and antibody production (126). Anti-BAFF monoclonal antibodies (belimumab) is the first approved biologic for SLE by the U.S. Food and Drug Administration, and has shown promising results in phase II clinical trials for pSS (101, 127). In paper II, we study the molecular mechanism behind the regulation of BAFF and show that its expression is controlled by IRFs. The different IRFs have different expression patterns and control different sets of IRGs, e.g. our data from paper III shows that TRIM21 expression is upregulated by IRF1 and IRF2 and not by other IRFs. This would make IRFs interesting drug targets since one could target a specific axis of the IFN response. For an example, IRF7 is mediating the TLR-induced massive type I IFN production by pDCs in SLE. By targeting IRF7 instead of type I IFNs in general, type I IFNs could still be induced after viral infections. Unfortunately,

transcription factors are usually poor drug targets due to their structural properties. However, it might be possible to target proteins mediating post-transcriptional modification on IRFs.

As previously mentioned, type I IFNs are successfully used to decrease eosinophil numbers in HES patients who respond poorly to corticosteroids. This suggests that one or several ISGs can regulate eosinopoiesis. In study V, we identify the ISG TRIM21 as a negative regulator of eosinopoesis via one or several unknown factors. Identifying this factor could lead to a new drug target for HES that could replace type I IFNs which has many unbeneficial and severe side-effects.

6. ACKNOWLEDGEMENTS

During my years as a PhD student, I have had the pleasure of working with a number of amazing people.

First and foremost, my main supervisor **Alex**. Thank you for reading my thesis at 3 in the morning!! But most of all, thank you for making these 5 (6? Who's counting anyways) years so much fun, inspiring and educational. You have set the standard high for my future employers, and I hope that you will continue being my unofficial mentor :)

My co-supervisor **Marie**, you were the first to take me in to your lab and I am so happy you did! You are an inspiring scientist (and a very positive/optimistic one, I must say), and working in your lab has been a joy from day one. Also, I truly appreciate you taking your time helping me with future science career plans.

I also want to take this opportunity to thank **Lars K** and **Ingrid L** for making floor 4 such a great and vibrant place to work at.

Former and present members of the MWH/Espinosa group: First of all, the little **Espinosa crew**: **William**, you make the perfect Espinosa lab member: smart, passionate about science and a little bit disorganized. Looking forward to read your TRIM28-paper ;) **Alina**, it's been a pleasure having you at the lab. Good luck at your new job! (Also, I really, reeeeally miss your impeccable genotyping!!) **To the rest of the MWH group**: **Amina**, you are the backbone of this lab! And such a lovely, kind and sweet person. **Vijole**, thanks for always helping out, for baking wonderful cakes and for contributing so much to the great atmosphere at the lab. **Nånnis**, you are a little firecracker, so much fun! And a great scientist, you will do wonderful things I'm certain. (Also, have you named your daughter yet?!?) **Aurélie**, the French, not so French, yet very French French girl! We have to get a coffee soon (chocolate for you?) and talk about all the things I can't miss in NYC! **The MWH mama mafia**: Sabrina, Gudny-Ella, Malin and Amanda. **Sabrina**, my office roomie and friend! I'm so glad to have had the opportunity to share office with you and talk about everything from kids, apartments, happy days, crappy days, to occasionally science ;) **Gudny-Ella**, multi-talented de luxe! Thanks for all the great party stories and all the fun! **Malin**, please work full-time at the lab again?! Then again, I might not be there :/ Thanks for being the super sweet YOU that you are! **Amanda**, how great that you had a baby almost at the exact same time as me

so that we could hang out more! Let's plan for the next one?! *This is where I would have entered the emoji laughing so much that it's crying*. **Joanna**, I always enjoy talking with you! Funny and smart! **Marika**, thank you so much for helping out with all the Sjögren patients for my projects. And for bringing baklava now and then ;) **Åse**, we still miss you and your lovely stories! **The super crew: Rita**, thanks for Russian pancakes (yum), helping out with FACS when I had a meltdown at the lab, being super cool and so on :) **Jorge**, the scientist aka the DJ aka the traveler! Maybe you're up next from the MWH group?! Keeping my fingers crossed for you! **Nikos**, hard-working is the one word (or is it two?) that describes you the best! I hope that you achieve all the things you work so hard for, you deserve it. **Lauro**, first thing that comes to mind: amazing tiramisu! Keep up all your good work! **Lara M**, you are brave, picking a high-risk project for your PhD. But those are also the fun ones! Good luck! **Lara A**, my BAFF-partner! Wishing you all the best in your future science career! **Albin**, what an intense start you had at the lab!! But it just turned out to prove that science fits you like a glove. Wishing you all the best! **Vilija**, **Stina** and **Therese**, come visit more often!

To all the former and present colleagues at the Rheumatology unit, thank you so much for all these years! I would especially like to thank: **Lasse**, thank you for introducing me to CMM!! The rest of the **HMGB-1 group**, it's lovely hearing your laughter through the hallway! **Heidi**, cell room for life! **Julia**, I will never forget our night at the infamous Patricia!! **Joan**, you're up next! You will rock!! **Karin**, my preggo/baby mama companion! **Ferdinand**, thanks for all the fun times! **Stina**, **Gunnel**, **Lillemor** and all you lovely admin ladies, you have made my life so much easier. Thank you!! Last but not least, **Peter**, my dear friend. Thank you for being you!

My office-buddies: **Christina**, **Aase**, **Sabrina M**, **Sabrina R** and **Espen**!

To my second lab family, the Neuroimmunology unit! Rasmus, **Karl** and **Harald** – We will always have Montenegro; **Andre**, my dear friend; **Nada**, sweet lovely Nada – Looking forward to a lifetime of friendship with you guys!
To the people that left the lab: Ame, Raphael Saadiq with you will go down in history! **Alan**, Canada, eh? Come back to visit soon! **Mella**, my superwoman! I'm so glad I got to know you and your family! **Louise**, miss you! Come hang out with me and Alexa!

"Gänget med hänget" – **Mikkis**, my long-time partner in crime. How could I ever have loaded a gel if it wasn't for you?! **Petra**, my loud, lovely, cuddly hug bug! **Pernilla**, my funny, cool, fashion babe. Love you guys!!

My friends outside academia: Annika, Stella, Hanna, Fia. My girls – Forever and always! Love you!

And to my family, you're everything! Mamma, thank you for helping out with Alexa and for supporting me through this – but most of all, thank you for being my role model and the first love of my life! **David**, my kind, smart and generous brother. Thank you for encouraging me to study science and for helping me with math all those times! Love you and your girls! **Stefan** and **Rosanne,** I wish you guys were closer, miss you and love you!

My little dream-team: Roham and **Alexa**, I can't wait to see what the future holds for us! The two of you have my heart for life. I love you to the moon and back and then some!

Last but not least, thank you to all the patients who have contributed with invaluable samples for this work!

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