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CELLULAR AND MOLECULAR FACTORS IN THE PATHOGENESIS OF SYSTEMIC AUTOIMMUNITY AND COMORBIDITIES

Margarita Ivanchenko

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Cellular and Molecular Factors in the Pathogenesis of Systemic Autoimmunity and Comorbidities
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Margarita Ivanchenko

Principal Supervisor:
Marie Wahren-Herlenius, MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Co-supervisor(s):
Susanna Brauner, MD, PhD
Karolinska Institutet
Department of Clinical Neuroscience

Malin Hedlund, MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Alexander Espinosa, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Eva Baecklund, MD, PhD
Uppsala Universitet
Department of Medical Sciences
Division of Rheumatology

Opponent:
Joanne Reed, PhD
Garvan Institute of Medical Research
Division of Immunology

Examination Board:
Professor Camilla Svensson, PhD
Karolinska Institutet
Department of Physiology and Pharmacology

Docent Aleksandra Antovic, MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Docent Olof Hultgren, MD, PhD
Örebro University
School of Medical Sciences
Department of Microbiology and Immunology

The public defense will take place in the CMM lecture hall (L8:00) on Friday 24th of May at 9.00 at the Karolinska University Hospital, Solna

The truth is out there. But so are lies.

– Dana Scully, The X-Files

ABSTRACT

Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) are systemic rheumatic autoimmune diseases having a major impact on patient's wellbeing. Besides fatigue, pain and direct damage to exocrine glands in SS, and various target organs, such as skin, joints, kidneys and brain in SLE, these diseases confer an increased risk of lymphoma development and can cause an autoimmune condition in the foetus of pregnant patients, termed neonatal lupus.

Several cell types and cytokines contribute to the autoimmune process, with central roles for B cells and autoantibodies. Mature B cells are attracted to the sites of inflammation by chemokine CXCR5. Recent studies identified *CXCR5* as a susceptibility gene in SS, and a decrease in expression of CXCR5 on circulating B cells in SS patients, however, the link between the polymorphisms in the *CXCR5* locus and B cell trafficking wasn't addressed. We therefore explored the expression of the chemokine on lymphocytes in peripheral blood and minor salivary glands in SS patients. Confirming the previous findings, our results demonstrate reduced CXCR5 expression in circulating B cell pool, coinciding with the accumulation of CXCR5-positive cells in the salivary glands of the patients. We conclude that the observed decrease in CXCR5 expression in SS results from relocation of CXCR5-highly-positive B cells from blood stream to the salivary glands.

T follicular regulatory (T_{FR}) cells also express CXCR5, and the frequency of circulating T_{FR} was suggested a biomarker of degree of tissue inflammation and autoantibody production in SS and rheumatoid arthritis. We could confirm the elevated frequencies of T_{FR} in the blood of SS patients, and show that most of them have a naïve phenotype.

B cells are producers of the SS hallmark autoantibodies against Ro and La, that have been used for diagnostic purposes for decades. In this thesis we investigated the anti-inflammatory and anti-proliferative properties of TRIM21/Ro52, one of the targets of anti-Ro. We studied the role of Trim21 in the homeostasis of mouse B cells, and the relevance of TRIM21 as a prognostic marker in diffuse large B-cell lymphoma. In Trim21 knockout mice, B cells had a higher rate of proliferation, follicular B cells were expanded and higher levels of both IgM and IgG were generated after immunization with both thymus dependent and independent antigens engaging the B cell receptor. In accordance with these data, maintained TRIM21 expression was associated with a better prognosis in diffuse large B-cell lymphoma, independently of subtype and underlying autoimmune disease.

Another severe comorbidity in SS and SLE affects foetuses and newborns of the patients. Maternal Ro and La autoantibodies are transferred to the developing foetus, and in 2% cases this leads to a congenital heart block (CHB), a part of neonatal lupus. Here, we studied signs of inflammation in the cord blood of neonates exposed to maternal anti-Ro/La. We observed an elevation of serum type I interferon (IFN) levels and an IFN-gene signature in autoantibody-exposed neonates. Besides, neonatal PBMC proved to be capable to produce IFNα *in vitro*, suggesting a possible foetal origin of the cytokine. Further, we show increased frequencies of circulating natural killer cells, and presence of type II IFN in the sera of some of these newborns. These novel findings contribute to the understanding of the mechanisms behind CHB.

In summary, we connect genetic variants of the *CXCR5* gene locus to expression patterns and cell compartmentalization in SS, demonstrate a role of the autoantigen TRIM21 in B cell homeostasis and function and present evidence for innate immune activation in Ro and La autoantibody-exposed newborns.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Diminished CXCR5 expression in peripheral blood of patients with Sjögren's syndrome may relate to both genotype and salivary gland homing**
Aqrawi LA, Ivanchenko M, Björk A, Ramirez Sepulveda JI, Imgenberg-Kreuz J, Kvarnström M, Haselmayer P, Jensen JL, Nordmark G, Chemin K, Skarstein K, Wahren-Herlenius M
Clin Exp Immunol. 2018 Jun;192(3):259-270

II. **FoxP3$^+$ CXCR5$^+$ CD4$^+$ T cell frequencies are increased in peripheral blood of patients with Sjögren's syndrome**
Ivanchenko M, Aqrawi LA, Björk A, Wahren-Herlenius M, Chemin K
Clin Exp Immunol. 2019 Jan 10, doi: 10.1111/cei. 13244 (epub ahead of print)

III. **The Sjögren's syndrome-associated autoantigen Ro52/TRIM21 modulates follicular B cell homeostasis and immunoglobulin production**
Brauner S, Ivanchenko M, Thorlacius GE, Ambrosi A, Wahren-Herlenius M.
Clin Exp Immunol. 2018 Dec;194(3):315-326

IV. **Reduced expression of TRIM21/Ro52 predicts poor prognosis in diffuse large B-cell lymphoma patients with and without rheumatic disease**
Brauner S, Zhou W, Backlin C, Green TM, Folkersen L, Ivanchenko M, Löfström B, Xu-Monette ZY, Young KH, Møller Pedersen L, Boe Møller M, Sundström C, Enblad G, Baecklund E, Wahren-Herlenius M
J Intern Med. 2015 Sep;278(3):323-32.

V. **Type I IFN system activation in newborns exposed to Ro/SSA and La/SSB autoantibodies *in utero***
Hedlund M*, Thorlacius GE*, Ivanchenko M, Ottosson V, Kyriakidis N, Lagnefeldt L, Tingström J, Sirsjö A, Bengtsson A, Aronsson A, Gemzell-Danielsson K, Rönnblom L, Bergman G, Espinosa A, Sonesson SE, Eloranta ML, Wahren-Herlenius M.
Submitted

VI. **Cytotoxic lymphocytes and type II interferon in Ro/SSA and La/SSB autoantibody-exposed newborns at risk of congenital heart block**
Ivanchenko M, Thorlacius GE, Hedlund M, Ottosson V, Ossoinak A, Tingström J, Sonesson SE, Chemin K, Wahren-Herlenius M
Manuscript

*Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ABC	Age-associated B cells
ABC (DLBCL)	Activated B cell-like DLBCL
AIA	Activation-induced apoptosis
ANA	Anti-nuclear antibodies
AU	Arbitrary units
AV block	Atrioventricular block
BAFF	B cell activating factor
BcR	B cell receptor
BM	Bone marrow
Breg	B regulatory cells
CD	Cluster of differentiation
CHB	Congenital heart block
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CSR	Class-switch recombination
CXCR	CXC chemokine receptor
DEG	Differentially regulated genes
DLBCL	Diffuse large B-cell lymphoma
DN	Double negative cells
EGM	Extraglandular manifestations
FACS	Fluorescence-activated cell sorting
FCM	Flow cytometry
FDC	Follicular dendritic cells
FL	Follicular lymphoma
G-CSF	Granulocyte colony-stimulating factor
GC	Germinal center(s)
GCB (DLBCL)	Germinal center B-cell-like DLBCL
HCQ	Hydroxychloroquine
HD	Healthy donors
HGT	Hypergeometric test
HLA	Human leukocyte antigen
IFN	Interferon
IFNAR	interferon-α/β receptor
IL	Interleukin
IRF	Interferon regulatory factor(s)
ISG	Interferon-stimulated gene(s)
ISRE	Interferon-stimulated response element

KO	Knock-out
La/SSB	Sjögren's syndrome antigen B
LESA	Lymphoepithelial sialadenitis
LN	Lymph node(s)
M-CSF	Monocyte colony-stimulating factor
MALT	Mucous-associated lymphoid tissue
mDC	Myeloid dendritic cells
MedFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MZ	Marginal zone
MZL	Marginal zone lymphoma
NHL	Non-Hodgkin lymphoma
NK	Natural killer cells
NLE	Neonatal lupus erythematosus
PBMC	Peripheral blood mononuclear cells
PC	Plasma cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cells
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RING	Really interesting new gene
Ro/SSA	Sjögren's syndrome antigen A
SG	Salivary glands
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism(s)
SS	Primary Sjögren's syndrome
T_{FH}	T follicular helper cells
T_{FR}	T follicular regulatory cells
Th	T helper cells
Th17	T helper 17 cells
TLR	Toll-like receptor(s)
TNF	Tumour necrosis factor
T_{reg}	T regulatory cells
TRIM21	Tripartite motif-containing protein 21

1 INTRODUCTION

1.1 Systemic autoimmune diseases: Sjögren´s syndrome and systemic lupus erythematosus

Primary Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) are systemic autoimmune diseases with an incidence of 3-10 cases per 100,000 person-years in Sweden each.[1,2] SLE is characterized by a heterogeneous clinical picture, and may have a severe course, primarily due to kidney, heart and nervous system damage. SS, which has a substantial clinical, genetic and immunological overlap with SLE, although often with a milder disease course, primarily affects exocrine organs, leading to oral and/or ocular dryness (sicca symptoms). Major extraglandular manifestations (EGM) include fatigue, myalgia, arthralgia and hematological involvement, and can have a severe impact on patients' lives.[3] SS is mostly not a life-threatening disease, however the risk of developing non-Hodgkin B-cell lymphoma is up to 16 times higher than in the general population.[4-6] In SLE, this risk is 3–5 times higher than that of the general population.[5,7]

The main diagnostic hallmarks of SS are xeropthalmia and xerostomia, confirmed by histological and functional tests, as well as autoantibody positivity (details are discussed in the following section).[8] In SLE, patients display autoantibody positivity, decreased levels of complement proteins C3c and/or C4 and involvement of one or two target organs.[9,10] The diversity of manifestations motivated the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) to develop classification criteria for SS and SLE, in order to stratify patients into more homogenous groups. One important advantage of the updated criteria is refined patient grouping for research purposes. The latest versions of the criteria with special focus on early SS and SLE were established in 2016 – 2017.[9,11,12]

In addition to classifications, disease activity indexes (ESSDAI, SLEDAI and other) and patient reported indexes (ESSPRI, SLAQ and other) were designed to assess disease severity and activity as well as facilitate personalized management strategies.[13-15]

1.2 Autoantibodies

The concept of autoimmune diseases infers the involvement of adaptive immunity, and in systemic autoimmune diseases a central role is traditionally given to autoantibodies, produced by B cells. Interestingly, disease-specific autoantibodies can sometimes be detected years before the appearance of the first clinical symptoms of the disease.[16] One of the first serological tests developed was for the detection of anti-nuclear antibodies (ANA).[8] It is commonly performed using a model cell line, HEp2, and patient autoantibodies are detected with immunofluorescence.[17] A positive result does not identify any particular antibody specificity, but the pattern of immunofluorescence often indicates the presence of several

types of autoantibodies. ANA titer of ≥1:320 is suggestive of SS,[3] and ≥1:80 – of SLE.[12] The umbrella term of ANA typically includes: anti-dsDNA, anti-Sm, anti-RNP, anti-DFS70, sp-100, PML, Ro/SSA (anti-Ro52 and anti-Ro60), La/SSB and more. Anti-dsDNA antibodies are included in the classification criteria of SLE[12] (present in 70 – 80% of patients),[18,19] and Ro/SSA antibodies in the criteria for SS (present in 40 – 75% of patients).[8] La/SSB were previously a part of the criteria for SS, but were omitted in the latest version after statistical modelling demonstrating they did not add to the precision of the criteria.[20]

Ro/SSA, La/SSB and the possible role of one of their targets, TRIM21/Ro52, in the pathogenesis of SS and SLE and comorbidities are discussed further in this thesis.

1.3 Interferon in Sjögren's syndrome and systemic lupus erythematosus

In SS and SLE and other systemic autoimmune conditions (such as systemic sclerosis and idiopathic inflammatory myositis) the interferon system is often continuously activated, and these diseases are frequently referred to as interferon-driven.[21] Indeed, accumulating evidence suggests that the autoantibodies to ribonucleoproteins, paired with a decreased ability of clearing cellular debris, triggers IFNα production in pDC and other cells.[22] The elevation of levels of type I IFN, a cytokine which has its primary function in protection against microbes, has broad direct and indirect consequences for the immune cell homeostasis. Below, I have chosen to highlight the effects most relevant for the current studies.

Altered expression of multiple cell-surface molecules

Type I IFN regulates function, survival and location of immune cells, among other ways, by modifying cell surface molecule expression. The upregulated molecules include activation markers CD69, CD86, CD25, PD-1, MHC class I molecules, chemokines CXCR3, CXCR4, CXCR5 and many other.[23,24] In contrast, type I IFN downregulates membrane-bound IgM.[25] The data describing the role on the expression of CD95 (FAS) are controversial. On one hand, it was shown that type I IFN downregulates CD95,[25] however immunoglobulin class switched memory B cells in SLE are CD95-positive.[26] MHC class II receptors in B cells do not seem to be regulated by type I IFN alone, but are upregulated upon synchronic BcR signalling.[25] In monocytes and DCs on the other hand, IFN is sufficient to upregulate HLA-DR.[27,28]

Effects on B cell fate decisions

In absence of other stimuli, the only effect of type I IFN on resting B cells is prolonged survival.[25] Except for downregulation of CD95, IFN-dependent B cell survival is mediated through upregulation of the anti-apoptotic molecules BCL-2 and BCL-XL.[23,25] Enhanced B cell proliferation was observed upon simultaneous stimulation with type I IFN and anti-IgM, which was further increased upon addition of CD40 antibody or IL-4.[25] Finally, the upregulation of transcription factors BLIMP and XBP, induced by type I IFN, drives B cells towards differentiation into plasma cells (PC).[23] Taken together, these effects result in intensified production of antibodies, including those with autoreactive potential.[29]

Intracellular signalling component expenditure

Type I IFN signalling is negatively regulated at both transcriptional and post-transcriptional levels. One of the main transcription repressors of interferon-stimulated response element (ISRE)-containing genes is IRF2.[30] Multiple IFN-induced proteins specifically target cytosolic nucleic acid sensors RIG-1 and MDA5 and signalling component downstream them (MAVS, STING).[28] Also, IFNAR subunits themselves can be downregulated.[31] A prominent negative regulation mechanism at the protein level is ubiquitination. There is a variety of IFN-induced E3 ubiquitin ligases that target products of interferon-stimulated genes (ISG): A20, an interesting and potent inhibitor of NFκB and TNFα signalling, and a tumour suppressor,[32,33] RNF125, IFI35,[28] as well as multiple TRIM family members.[34] Of these, TRIM21 has been of our particular interest, as it was first described as an autoantigen in SS and SLE.[35,36] These signalling repressors are themselves ISG, and are therefore expressed, at least partially, in a dose-dependent manner. However, the chronic nature of the signalling pathway activation in SS and SLE might have unexpected consequences, e.g., insufficiency of negative regulators. Additionally, their broad specificities and targets outside IFN pathways makes it difficult to predict the long-term effects of chronic exposure to type I IFN.

Besides rapid upregulation during infection, low concentrations of type I IFN are constantly present in the primary and secondary lymphoid organs.[25,28,30] This basal expression is controlled by the transcription factors IRF3 and IRF7, which allow rapid IFN self-amplification when infection occurs.[30] Lymphocytes deprived of basal IFNAR pre-stimulation require more time for activation.[24,30] Interestingly, in bone marrow-derived myeloid cells, low IFNβ expression is controlled by M-CSF and AP-1 transcription factors, and has an antiproliferative effect.[28,37]

Type II IFN

The role of IFNγ in SS and SLE is less well established. Detection of IFNγ in serum requires high sensitivity assays, thus the presence of circulating type II IFN in SS and SLE has become apparent only recently. Up to 30% of patients have detectable levels of this potent cytokine in blood, however the significance of this finding is not entirely understood.[38,39]

1.4 TRIM21/Ro52

Tripartite motif-containing protein 21 (TRIM21) or Ro52 is a RING-dependent E3 ubiquitin ligase,[40,41] and it is best known as a negative regulator of TLR signalling.[42,43] It is predominantly expressed in cells of hematopoietic lineages. Besides the first identified ubiquitination targets IRF3, IRF7, IRF5 and IRF8 downstream of TLR,[43-47] TRIM21 was suggested to interact with BCL-2,[48] TRAF6,[49] IKKb,[50] FLASH, DAXX,[51] DDX41,[52] C-FLIP and C/EBPα.[53] In human, the TRIM21 gene maps to 11p15.4 and contains seven exons; the full-length transcript yields 475 aa product. In mouse, the Trim21 gene is located at 7E3, contains eight exons (six are coding), and encodes a protein of 462 aa. Polymorphisms in the

TRIM21 gene have been associated with SS and SLE,[54,55] although GWAS projects have not been successful in replicating the association.[56-58]

TRIM21 was discovered as an autoantigen, and is targeted by autoantibodies in 50 – 70% of patients with SS,[59] in up to 98% cases of autoimmune hepatitis,[60] 35 – 40% of SLE cases,[61] and in 10 – 20% of RA, 36% of polymyositis and 20% systemic sclerosis patients.[62] Autoantibodies towards Ro52/TRIM21 and an unrelated protein Ro60 are traditionally denoted Ro/SSA autoantibodies. The main epitope of Ro52 is situated within the central coiled-coil region of the protein, but it is anti-Ro52 antibodies binding the RING domain of TRIM21 that can block its ubiquitinating activity *in vitro*,[44] however, whether the autoantibodies inhibit the intracellular enzyme *in vivo* remains unclear.

Two *Trim21* knock out mouse models were generated independently in 2009.[44,63] Both research teams used a GFP reporter, but their vectors targeted different exons of Trim21. The construct designed by Yoshimi *et al*. removed the expression of virtually the whole Trim21 mRNA, while Espinosa *et al*. used a vector which theoretically enabled the expression of a truncated protein. While in the former model, only a mild phenotype was observed,[63] the latter mice developed an autoimmune-like condition provoked by metallic ear-tags. This autoimmune phenotype included severe dermatitis, nephritis, hypogammaglobinaemia (mainly IgG1 and IgG2) and ANA production as well as splenomegaly and lymphadenopathy.[44] This phenotype is thought to be mediated, at least partially, by IL23/IL17,[44] however, Th17 cells, differentiated from *ex vivo* naïve *Trim21*$^{-/-}$ CD4 cells, displayed a gene signature of non-pathogenic subset of Th17.[64] The phenotypical differences between the two *Trim21*$^{-/-}$ mouse models were explained by the compensational upregulation of other proteins of TRIM family in mice generated by Yoshimi, but not in those from Espinosa *et al.*[65,66]

1.5 Immune cell aberrations in Sjögren's syndrome and systemic lupus erythematosus

1.5.1 B cells

The role of B cells in SS and SLE is illustrated by multiple findings related to humoral immunity: hypergammaglobulinemia and elevated levels of immune complexes and autoantibodies, B-cell infiltration of the inflamed tissues and increased risk of B-cell lymphomas.[67,68] Taken together with the abundance of B cell activating factor (BAFF) and IL-21, these phenomena underline the importance of examining B cell subsets in SS and SLE.

BAFF and IL-17A overproduction

BAFF is directly upregulated by IFNα,[29,69] and, in turn, promotes Th17 production, by several cell types. This cytokine trio creates a positive feedback between T and B cells within the germinal centers (GC) and also recruits monocytes and mDC.[21,29] BAFF is one of the master regulators of B cell differentiation. It is critical for the survival of immature B cells at the T2 stage,[70,71] as well as mature naïve and memory cells including autoreactive.[71,72] This makes BAFF availability a risk factor for a tolerance breakdown,[73] and, indeed, BAFF knock-

in transgenic mice develop a lupus like disease.[74] BAFF acts mechanistically through the NFκB pathway.[75] Other notable effects of the type I IFN–BAFF–IL17 axis are the promotion of differentiation of activated T cells towards Th17[21] and IL-21-producing T_{FH},[76] and the reinforcement of the monocyte differentiation into mDC.[29]

Naïve, marginal zone and class-switched memory B cells

In both SS and SLE findings typical in the circulating B cell population are a higher frequency of naïve B cells and a lower frequency of both CD27-positive compartments, class-switched memory and marginal zone (MZ) B cells.[26,67,68,76] For other B cell subsets (plasma cells, plasma blasts, double negative, transitional B cells) aberrations vary between the patients[26,68] and become apparent exclusively in clinically pronounced cases, e.g. accompanied by EGM.[76] Nevertheless, the decrease in the percentage of circulating MZ B cells has repeatedly been observed in both SLE and SS.[26,67,68,76] MZ subpopulation is also called unswitched memory, natural effectors or IgM memory cells. These cells are long-lived, have a history of antigen encounter and can expand and undergo BcR editing without interacting with T cells.[77] Microarray data from circulating MZ B cells[68] and upregulation of their surface molecules such as CD95 (FAS), CD80 and CD86[26] suggest that MZB are activated in SS and SLE.[68,78]

Interestingly, MZ B cells in SS and SLE overexpress the chemokine receptors CXCR3, CXCR4 and CXCR5, and were shown to home to the sites of inflammation, e.g. salivary glands (SG).[26,76] This MZ subpopulation displayed signs of antigen encounter (proliferation and somatic hypermutation, SHM) and comprised up to 50% of autoreactive cells in blood in SS patients.[67] Moreover, a part of MZ B cells from healthy donors stimulated *in vitro* with CpG, temporarily expressed activation-induced deaminase (AID), underwent class-switching (as well as SHM)[5,71] and rapidly differentiated into antibody-producing cells. Whether this occurs also *in vivo* is unclear, as, for instance, it is difficult to distinguish MZ cell that travelled to the SG and class-switched *in situ* from classical $CD27^{+}IgM^{-}IgD^{-}$ memory cell. The accumulation of the latter in ectopic lymphoid tissues was, indeed, observed in multiple studies, which could explain the decrease in the circulating $CD27^{+}$ memory B cells.[67,79] Another possible contributor is enhanced differentiation of memory B cells and plasmablasts into plasma cells, accompanied by shedding of the CD27 receptor. Indeed, elevated levels of soluble CD27 were observed in both SS[80] and SLE[81] and associated with higher IgG levels and disease activity.

Plasmablasts, plasma cells, transitional and double-negative B cells

Other B cell subpopulations affected in SS and SLE include transitional, double-negative (DN, $CD19^{+}CD27^{-}IgD^{-}$), plasmablasts and plasma cells. In SLE, the proportion of circulating DN cells is increased.[68] In contrast, in SS the DN percentage is decreased, but only in severe cases. The same was true for plasmablast and plasma cell relative counts: they were increased in a subset of SLE patients[26,76] and, on the contrary, decreased in SS, the latter being considered a consequence of CXCR3 and CXCR4 overexpression.[76] After immune triggering by vaccination, the amount of circulating plasmablasts was increased also in SS compared to healthy donors.[82] Elevation of antibody producing B cell counts might result from enhanced

cytokine impact on differentiation or survival of cells, including autoreactive.[76] An increased proportion of circulating transitional $CD24^{high}CD38^{high}$ cells observed in SLE might arise from similar mechanisms: dysregulation of chemokine receptors or/and diminished negative selection.[76] Interestingly, anti-Ro52 antibodies isolated from a patent with SS displayed low levels of somatic hypermutation, suggesting an origin from immature (naïve) B cells avoiding tolerance.[83]

Age-associated B cells

In 2011, two groups have described a novel B cell population, designated age-associated B cells (ABC).[84,85] As the name suggests, those cells accumulate with age in both mice and humans, and have been identified in spleen, blood and bone marrow.[86] Differentiation of ABC depends on transcription factor T-bet,[85] and they are defined as $CD11c^{+}CD21^{low/-}$ $CD5^{high}CD23^{low/-}CD95^{high}$ in healthy human and naïve mouse with slight variation in phenotype between the studies.[86,87] Interestingly, for the effective activation, ABC require TLR7/9 ligands and IFNγ in addition to BcR ligation.[86] ABC function as producers of IFNγ, IL-10 and TNFα and preferentially IgG2. To date, ABC have been investigated in SS, SLE, MS, infections and common variable immune deficiency.[87,88]

1.5.2 T cells

Decreased numbers of circulating T helper (Th) cells have been described in both SS and SLE patients.[89-91] Further, the balance between Th17 and T_{reg} seems to be shifted towards the former.[92,93] Th17 cells appear to be important contributors to the ectopic lymphoid tissue formation at early stages in SS, as they are attracted by CXCL9 and CXCL10 secreted by activated SG resident cells and amplify and sustain the inflammatory signalling by producing IL-17 and IL-22, that activate B cells and support SG epitheliocytes.[93] Interestingly, a subset of Th17 cells expressing CXCR5 has been identified in juvenile dermatomyositis,[94] SS[95] and RA.[96]

An important finding in the T cell compartment in systemic autoimmunity is also the elevated frequency of circulating T follicular helper (T_{FH}) cells, expressing CXCR5, and high levels of PD-1 and ICOS in a subset of SS and SLE patients with severe clinical course.[76] Also, higher levels of IL-21, a cytokine produced by T_{FH}, and higher ANA titers were observed in these patients.[76,97] Notably, IL-21 is a potent B cell differentiation factor and may also sustain the B cell activity in the patients.[94,98]

T follicular regulatory (T_{FR}) cells, another component of CXCR5-positive T cell pool, are suppressors of GC B cell activity[99-101] and, unlike conventional T_{reg}, require expression of BCL-6.[102] In autoimmune conditions their abundance in GC negatively correlates with autoantibody production,[103] however their circulating counterpart seems to have restricted immune suppressive properties and their prevalence is rather an indirect indication of ongoing inflammation.[104]

1.5.4 Natural killer cells

NK cells are a type of innate lymphoid cells (ILC1) bearing both cytotoxic and cytokine producing functions. A variety of activating and inhibitory NK cell receptors were identified that convey self-non-self discrimination or presence of cytokines. Classically, NK cells with predominantly cytotoxic functions are defined as $CD16^{+}CD56^{low}$, and cytokine producing NK cells are $CD16^{low/-}CD56^{+}$.[105] A third type, $CD16^{-}CD56^{high}$ decidual NK cells, with exclusively cytokine production properties, are vital for the first trimester of pregnancy, when they orchestrate vascularization and placentation by VEGF, CXCL10 and CXCL12 secretion.[106] Both circulating and tissue resident NK cells, e.g. in SG and skin, can produce IFNγ, IL-10 and TNFα, exhibit direct or (auto)antibody mediated cytotoxicity and possess plasticity, and thus may have a dual role in autoimmunity.[107]

In SS patients, Davies *et al.* demonstrated deviations in absolute counts of circulating NK subsets in different clusters of patients, which were also partially treatment-dependent.[91] Similarly, Seror *et al.* observed that a subgroup of SS patients responding to the anti-BAFF monoclonal Belimumab had lower NK cell SG infiltration.[108] The interpretation of these findings seems controversial in terms of the NK cell role (immunosuppressive vs stimulatory), compartmentalization, cause vs consequence, and treatment vs disease subtype/severity.

1.5.3 Plasmacytoid dendritic cells

pDCs are the main type I IFN producing cells. Upon stimulation by nucleic acids or nucleic acid-containing immune complexes cytosolic RIG-I–like receptors (RLRs) or the endosomal TLR7 and TLR9 receptors are activated and large levels of IFNs are produced.[109] pDCs are also effective at antigen presentation and chemokine (CCL3, CCL4, CXCL9, CXCL10) and cytokine (IL-6, IL-12) production, thus potentially contributing to autoimmunity.[110] pDCs were not a focus of this thesis, but are discussed in the results section in relation to our data. Direct consequences of type I IFN production were extensively studied, however pDCs were recently found to be involved in novel pathological pathways in autoimmunity. Zhao *et al.* suggested a model of ectopic GS formation, where pDC-derived IFNα induced CXCL13 production by MΦs, which in turn attracted T_{FH} and CXCR5-positive B cells.[111] Co-culturing B cells with pDC in presence of immune complexes turned out to directly force class-switched memory B cells to acquire DN phenotype, shedding light on the origin of skewed B cells subset composition in SLE.[112]

1.6 Comorbidities in Sjögren's syndrome

An increased risk of B-cell lymphoma has been well documented in Sjögren's syndrome,[4-7] and multiple large epidemiological studies summarized by Pego-Reigosa *et al.* also indicated higher risks of fibromyalgia and infections (primarily candidiasis and tuberculosis) and slightly higher risk of dyslipidaemia, cardiovascular disease and thyroid cancer in SS compared to the general population.[113] There is also a risk for neonatal lupus in the foetus of pregnant women with SS. In my thesis work, I have studied two of the comorbidities of SS; non-Hodgkin lymphoma (NHL) and neonatal lupus erythematosus.

1.6.1 B-cell lymphomas

The most frequent autoimmunity-associated lymphoma types are mucosa-associated lymphoid tissue (MALT) lymphoma and diffuse large B-cell lymphoma (DLBCL).

DLBCL is the most frequent NHL (30 – 40% of all cases) with an aggressive course, and comprising two major molecular subtypes described in 2000 by Alizadeh and colleagues; germinal center B cell-like (GCB) and activated B cell-like (ABC).[114-116] The other DLBCL subtypes, e.g. primary mediastinal B-cell lymphoma, are very rare, and will not be discussed here. Multiple studies of SHM and gene expression signatures indicate that DLBCL cells are derived from mature GC B cells (GCB subtype) or post-germinal B cells (ABC subtype).[5,115]

GCB DLBCL cells resemble normal GC B cells, as they hold the immunohistochemistry hallmarks of ongoing SHM, express CD10, CD38 and transcription factor BCL-6, which inhibits their differentiation and facilitates proliferation. The most specific markers for ABC DLBCL cells is expression of c-MYC, IRF4 and mutations in FLIP and MYD88, which reflect the constantly "activated status" of these cells, and ensures their reduced responsiveness to apoptotic and differentiation stimuli.[5,115,117-119] The overexpression of hallmark molecules (BCL-6, IRF4, c-MYC) in DLBCL often results from translocations, especially to the Ig locus, but can also arise from *in situ* mutations (*MYD88, FLIP*).[5,115] Attempts are currently being made to identify genetic biomarkers of tumour subtype using genomics and transcriptomics: next-generation sequencing,[120] whole exome sequencing, nanostring,[121] and reverse transcriptase multiplex ligation-dependent probe amplification,[122] which can be used for paraffin-embedded biopsies. The prognosis is significantly better in GCB DLBCL compared to the ABC group.[115,117,118]

Mucosal Associated Lymphoid Tissue (MALT) lymphomas are a subset of MZ lymphomas, and are the most common lymphoma subtype in SS.[114] The MZL group contains both somatically mutated and non-mutated subtypes, and can originate from post-GC B cells or, intriguing, from outside GC and even lymphoid tissues in a T-cell independent fashion (extranodal lymphomas)[5,123] MZ lymphomas, including MALT, are thought to derive from MZ B cells. MALT lymphomas display fewer translocations than DLBCL, but more point mutations. However, there are some typical translocations in extranodal MALT, such as *API2-MALT1* and *BCL-10* and *MALT1* translocations to the Ig locus, which result in constitutive NFκB signalling through TRAF2 and TRAF6.5 The point mutations seem to result from the SHM, and include *c-MYC, BCL6, CDK2A/B, PAX5, A20,* and *FAS*.[5,123]

In MALT lymphomas, chronic inflammation and infections play important roles in pathogenesis. Anti-apoptotic signalling through BcR, activation via PRR and co-stimulatory molecules, and the cytokine environment all interact to maintain cellular survival and high proliferation rates. Gastric MALT lymphoma is driven by *Helicobacter pylori* and is regarded as a classic example of infection-related cancer. MZL of other locations are also associated with infections, such as ocular, cutaneous, splenic and small intestine MZLs.[5,123] The common mechanism behind the lymphomagenesis is, again, NFκB signalling and during the first stage, it is BcR or TLR-dependent (and often T cell-independent).[123] In the context of the salivary glands, this first stage is called lymphoepithelial sialadenitis (LESA), and it can be

regarded as a transition step to lymphoma, with the hyperproduction of several clones of antibodies, including autoreactive ones.[5,124]

Phenotypically, MALT lymphoma cells are BCL-6, CD5 and CD10-negative, but bear several MZ B cell markers, such as CD20, CD27, CD21/35, IgM and IgD. However, their phenotype differs slightly between the studies.[5,124]

1.6.2 Lymphomas in autoimmunity

Multiple factors of inflammation might predispose to lymphoma development, and type I IFN exposure is potentially one.[125,126] At the same time, type I IFNs are considered to have direct (anti-proliferative, pro-apoptotic) and indirect (activation of NK cells, cytotoxic T cells and pDC to present oncoantigens) tumour-suppressor effects. Moreover, IFNα is an approved therapeutic agent in melanoma, breast cancer, hairy cell leukemia and CML, although with limited effect and use, partially due to side-effects.[31] Since MALT lymphomas and some DLBCL develop in an environment of the elevated type I IFNs (viral infections, SS, SLE, RA), one could speculate that the IFN tumour-suppressor mechanisms are disrupted in these conditions.[31,127] Moreover, the unresponsiveness to the IFN, logically, should be a prerequisite to a successful lymphoma development. On the other hand, type I IFN induces a variety of effects, that potentially promote oncogenesis (described in more detail in Type I interferon in the SS and SLE section). Ngo and colleagues observed the signature of IL-6, IL-10 and IFNβ signalling in ABC DLBCL cell lines with hyperactive MYD88.[33] IFNβ was also present in the supernatant of the human B-cell lymphoma OCI-Ly3 cell line, which, according to authors, could be an autocrine mechanism, promoting lymphoma survival.

The NFκB pathway is the major regulator of B cell activation and differentiation, with extremely broad outcomes, including survival, proliferation, cytokine production, SHM and CSR.[128] NFκB signalling dysregulation has been reported as one of the key pathogenetic mechanisms of lymphomagenesis of MALT lymphoma in SS and DLBCL in SLE and RA.[5,123-125,129,130] NFκB activation occurs through stimulation of the BcR complex, as well as through co-stimulatory molecules (mainly of TNF receptor superfamily) and TLR.[50,129,131] Except for NFκB and type I IFN, PI3K pathway is also often active in the chronic BcR stimulation,[129] as well as upon the CD40 ligation,[132] which provides additional survival signalling.

A well-studied link between autoimmunity and oncogenesis is provided by the NFκB inhibitor A20 (TNFAIP3). A20 is upregulated upon TLR and TNFα stimulation to create a negative feedback loop for NFκB. Single nucleotide polymorphisms (SNP) and mutations in the A20 locus were found in 60% of SS-associated lymphomas.[123] Other shared susceptibility loci between autoimmune diseases and lymphomas include HLA[133] and, intriguingly, *CXCR5*.[41,134]

1.6.3 TRIM21 and cancer

Overexpression of Trim21 in the lymphoma B cell line A20 leads to activation-induced apoptosis upon stimulation through CD40,[41] indicating the involvement of NFκB signalling.

TRIM21 was shown to negatively regulate NFκB signalling through interactions with TRAF6[49] and IKKb,[50] although it might have targets at other levels that are not yet investigated, e.g. TRAF3 and MAP kinases. Another tumour suppression mechanism of TRIM21 may be ubiquitination of its reported target BCL-2,[48] a central anti-apoptotic molecule, upregulated in many cases of lymphoma. These direct and indirect observations of a possible role for TRIM21 in cancer has led investigators to explore expression of TRIM21 in different forms of malignancies. To date, maintained TRIM21 expression turned out to be a beneficial prognostic factor in hepatocellular carcinoma[135] and breast cancer,[136] but, opposingly, predicted for shorter survival in pancreatic cancer and showed no prognostic value in colorectal cancer.[137]

1.6.4 Congenital heart block

The presence of maternal Ro/SSA (anti-Ro52 and anti-Ro60) and La/SSB (anti-La) autoantibodies during pregnancy is associated with a risk of neonatal lupus erythematosus (NLE) in the child, which includes congenital heart block (CHB), skin manifestations, liver and hematological involvement. IgG is known to be transported across the placenta during pregnancy, and Ro/SSA and La/SSB autoantibodies have been shown to be transferred to the foetus. The autoantibodies are found in > 95% of the cases of *in utero* CHB[138,139], a rare condition that develops in the foetus at gestation week 18 – 24 and refers to a complete third degree atrioventricular block (AVB III), but is sometimes also used to indicate lower degrees of AV block and/or other cardiac manifestations such as endocardial fibroelastosis. It manifests as markedly decreased heart rate, usually less than 70 bpm, versus expected 120 – 160 bpm.[140] The reported mortality is variable, but generally high, 4 – 19%,[140-144], and is usually related to a more generalized cardiac involvement such as dilated cardiomyopathy and endocardial fibroelastosis. Fluorinated steroids administrated to the mothers, cross the placenta and have shown to inhibit the progression of second-degree AV block. Recent publications report that the anti-malaria drug Hydroxychloroquine (HCQ) might reduce the risk of CHB.[145-147]

The studies discussed here, address specifically autoantibody-associated CHB. Anti-Ro52 IgG purified from maternal sera, as well as Ro52 monoclonal antibodies, induced CHB in mouse and rat pups, although the antibodies targeting the human protein did not bind Ro52 in rodents.[139,148] Whether maternal autoantibodies bind their conventional targets in human fetal heart remains one of the unsolved questions in CHB. One possibility is a so-called apoptosis-inflammation hypothesis, which includes translocation of Ro/La to the extracellular space as a part of physiological apoptosis in fetal conduction cells, followed by antibody binding and inflammation.[149] Another theory is cross-reactivity of maternal autoantibodies with non-Ro/La related epitope(s) expressed in the fetal heart. Several alternative autoantigens, as well as autoantibodies,[138,150] have been suggested, including calcium channels,[151] calreticulin,[150] a serotonin receptor 5-HT4[152,153] and several more. Of interest, autoantibodies of M class to calreticulin were detected in anti-Ro/La exposed neonates, and especially in CHB (this study did not include HD),[154] and anti-Ro60 IgM was found in both healthy and, at increased levels, in autoantibody-exposed subjects.[155]

The association of CHB with anti-Ro/La antibodies is well established, but a recurrence rate of only 12 – 20% in following pregnancies despite persisting maternal autoantibodies,[156] suggests that additional factors are required for disease development. These may be immunological, pregnancy-related, genetic or environmental.[157,158]

1.6.5 Neonatal immunity

Except for anti-Ro/SSA and anti-La/SSB-positivity, not many immunological parameters are known to predispose for CHB. Here, I'll focus on cellular and molecular aspects of this condition.

The physiological umbilical cord cell composition and status display striking differences compared to adult peripheral blood. The major cell differences include presence of myeloid, lymphoid and erythroid precursors and stem cells as well as the well-established neutrophilia and relative lymphopenia, that reverse at the fifth day of life.[159] In the B cell compartment, the cells transitional and naïve B cells are of the highest frequencies throughout ontogenesis, while switched memory and MZ B cells are of the lowest frequencies.[160,161] The same logic applies to the T cell compartment: a high prevalence of naïve T cells and decreased numbers of memory cells of both helper and cytotoxic subsets. Low frequencies of T_{reg}, and absence of CXCR5-expressing T_{FH} are common findings.[162] Both absolute and relative numbers of NK cells are increased in the cord blood. Immunological profiling of cord blood, including the humoral (immunoglobulins, cytokines) and cellular (activation markers, MHC expression) is comprehensively reviewed in Basha, Surendran and Pichichero, 2014.[163]

1.6.6 Pregnancy and autoimmunity

Pregnancy is a complex process, involving a variety of immune and non-immune factors and interactions that drastically change over time. Several cell types are involved during a successful pregnancy, the process of implantation requires a low degree of inflammation while excessive inflammation might cause implantation failure or miscarriage. The major findings in blood cell composition in late pregnancy were known before the flow cytometry era and include granulocytois, monocytosis and lymphopenia.[164,165] The expansion of granulocyte and monocyte fractions conforms to the high levels of both G-CSF and M-CSF.[166] Intriguingly, besides high counts, monocytes display an activated phenotype.[165]

Absolute and relative B cell lymphopenia is also a well-established phenomenon associated with gradual oestradiol increase throughout pregnancy.[167] Lima and colleagues reported that this decrease in B cell counts mainly resulted from a decrease in transitional, MZ, DN B cells and plasmablast subpopulations, while naïve B cells counts were intact, and Breg counts were increased.[168] Muzzio *et al*. confirmed the suppression of B cell lymphopoiesis in mice, resulting from reduced BM precursors and decreased BAFF levels.[167]

Several studies, but not all, indicated a general T cell lymphopenia in humans and rats during pregnancy.[166,169] In the second trimester, a decrease in T_{reg} percentages has been observed, while activated $CD25^{high}$ T helper frequencies, on the contrary, were increased.[170] Late T cell patterns of PBMC in pregnancy also include alterations in the Th1/Th2 ratio.[165,166] Both

cytotoxic and cytokine producing activities of NK cells seem to be gradually suppressed throughout pregnancy, as well as frequencies of circulating $CD56^{dim}$ NK cells, while $CD56^{bright}$ NK relative cell counts remain stable.[166]

2 AIMS

SS and SLE are frequent and severe autoimmune diseases of great societal impact. A genetic basis for SS has been suggested, including polymorphisms in the *CXCR5* gene locus, but the association remains functionally unexplained.

Trim21/Ro52 knock out mice display signs of systemic inflammation and splenomegaly, while Trim21 overexpression restricted the growth of a mouse B cell line. Further, maintained TRIM21 expression has been proposed as a good prognostic marker in several types of cancer. Taken together, these observations point to a potential role for TRIM21 in B cell homeostasis.

Finally, Ro/SSA and La/SSB autoantibodies are associated with a passive autoimmune condition, neonatal lupus erythematosus (including congenital heart block), that may affect the foetus of women carrying these autoantibodies. However, the mechanisms driving the inflammation in the exposed foetus are unclear.

The aims of the current thesis were therefore to:

- link genetic variants of the *CXCR5* gene locus to expression patterns and cell compartmentalization in SS;
- investigate the role of the autoantigen TRIM21/Ro52 in B cell homeostasis and function;
- characterize immune activation in newborns of Ro/SSA and La/SSB autoantibody-positive mothers.

3 RESULTS AND DISCUSSION

3.1 Genetic and cellular features of CXCR5 in Sjögren's syndrome

Genetic susceptibility contributes to the development of autoimmune and rheumatic diseases. Among other genetic variants, polymorphisms in a locus on chromosome 11q23.3 were identified as associated with Sjögren's syndrome in a GWAS published by Lessard and colleagues in 2013.[58] The peak of the genetic association signal includes two genes; *DDX6* and *CXCR5*. To understand if the expression of either gene was dependent on cis-genotypes, we first ran an eQTL analysis in an independent cohort of healthy donors (HD) described by Fairfax *et al*.[171] Since the specific SNPs identified by Lessard *et al.* had not been typed by Fairfax, a proxy SNP (rs4938573, LD $r^2 > 0.8$) was used. The analysis demonstrated that the disease risk allele (T) was associated with a significantly lower expression of CXCR5 in $CD19^+$ B cells. In **Papers I and II**, we aimed to connect this genetically based observation with the pathological process in SS.

3.1.1 eQTL effect in *CXCR5* gene locus might be explained by B cell redistribution (Paper I)

CXCR5 is a chemokine receptor expressed on B and T cells required for their homing to follicles in secondary lymphoid organs. We therefore assessed CXCR5 expression profiles on B and T cell populations in a cohort of untreated SS patients. Within the B-cell compartment, we first confirmed previous findings in SS patients, such as lower frequencies of marginal zone (MZ) B cells, accompanied by tendencies towards higher naïve and lower memory B cell prevalence compared to HD. Some of the patients had higher $CD19^+$ B cell frequencies than HD. As described previously, most circulating B cells expressed CXCR5.[172]

However, in the SS patients, the frequencies of CXCR5-positive cells among total B cells were reduced, resulting from lower frequencies of CXCR5 positive memory, MZ and $CD27^-$ IgD^- double-negative (DN) subsets. This observation complemented our eQTL analysis results. Moreover, the cell surface expression levels of CXCR5 followed a similar pattern, with significantly lower median fluorescence intensity (MedFI) in memory and DN B cells. Interestingly, naïve B cell proportions in SS patients tended to be increased, while the difference in their surface CXCR5 expression between HD and SS patients appeared minimal. CXCR5 expression is known to gradually increase throughout B cell differentiation, and reaches a plateau at the follicular/naive B cell stage.[173]

T cell homing to GC appears to differ from that of B cells: T cells require antigen encounter, downregulation of CCR7, and transitional upregulation of CXCR5, which is re-enforced in the GC.[174,175] Indeed, in HD, circulating CXCR5-positive T cells expressed 2 – 3 times lower levels of CXCR5 than B cells and comprised not more than 15% of all T cells. When comparing HD and SS patients, the total T cell population did not reveal any major differences. However, in the CXCR5-positive T-cell subset, decreased frequencies of Th17 cells became apparent. Also, the frequency of T_{FH} cells characterised by CXCR5 positivity

and high expression programmed death protein 1 (PD-1), were increased in four patients in our cohort. This increased frequency of T_{FH} did not correlate with any of the clinical parameters, however the data, including histology, were limited. These findings are particularly interesting given the importance of T_{FH} in autoantibody-positive autoimmune diseases and suspected Th17 cells contribution to SS.[95]

For twelve patients in our cohort, *CXCR5* genotype data were available, and grouping by genotype confirmed the association between the T risk allele and reduced CXCR5 expression on MZ B cells also in our patients. We speculate that taken together, these observations reflect the homing of CXCR5-positive B cells from the circulation to target organs in SS. In support of this idea, the levels of plasma CXCL13 reversely correlated with percentages of circulating CXCR5 positive B cells.

Presence of focal lymphocytic infiltrates in the minor salivary glands is one of the criteria for SS. The foci are dominated by T cells, but also contain B cells, FDC, pDC and macrophages.[126] CXCR5-CCL13 and CXCR4-CCL12 have been suggested as the major chemokine axes involved in the formation of the infiltrates.[176]

To understand if the decrease in frequencies of circulating CXCR5-positive cells could result from homing to the target organ in SS, we assessed expression of CXCR5 in salivary gland tissue biopsies. Indeed, CXCR5 expression was strikingly higher in patients with SS than in sicca patients.

In SS, the infiltrates may become chronically large, replacing substantial portions of glandular tissue, reminiscent of physiological lymphoid tissue organogenesis.[177] In around a quarter of SS cases, structures resemble conventional GC are found, in which FDC are surrounded by proliferating B and T cells.[8,126,178] Approximately half of the patients in our IHC cohort displayed GC.

To summarize the findings in this paper, we observed enrichment of CXCR5-positive cells in the target organ in SS, elevated CXCR13 levels in plasma, and reduced frequencies of circulating $CXCR5^+$ B cells which also had lower cell surface expression of CXCR5. We show that SNP located in the *CXCR5* locus is associated with the decrease in CXCR5 expression on circulating B cells. We postulate that the lower frequency of CXCR5 in circulating B cells most likely reflect their homing to the target organ although disease associated genotypes may also contribute.

3.1.2 CXCR5-positive circulating T cells are a heterogenous population, and T_{FR} cells are of interest in Sjögren's syndrome (Paper II)

Following the publication of **Paper I**, a letter was addressed to the editor by Válter R Fonseca and Luis Graca; "Contribution of $Foxp3^+$ T_{FR} cells to overall human blood $CXCR5^+$ T cells".[179] Given the possibility to respond to the letter, we were intrigued by the suggestion from Fonseca and Graca that the circulating CXCR5-positive T helper cell population is heterogenous with an increase in frequency of a subset of T follicular regulatory (T_{FR}) cells defined as $CD4^+CXCR5^+FoxP3^+$ in SS, and developed our response into a short report.

We first aimed to replicate the finding of VR Fonseca and L Graca in our cohort described in **Paper I**. We first analysed $CXCR5^+FoxP3^+CD25^+$ T cells to identify the whole T_{FR} population. The frequency of $FoxP3^+CD25^+$ T cells among $CXCR5^+CD4^+$ T cells was augmented in SS patients confirming Fonseca´s study. However, the frequency of $CXCR5^+$ T cells in $FoxP3^+CD25^+CD4^+$ T cells was not different in SS patients and HD donors. This discrepancy might result from a dimmer signal from FoxP3, which would impact the $FoxP3^+CD25^+$ gate purity, thus defusing differences in the downstream population frequencies. Hence, using CXCR5 in a first gating step might provide a better resolution of T_{FR} populations.

The ratio between total T_{FH} ($CD4^+CXCR5^+$) and T_{FR} cells was increased in SS patients, confirming previous reports.[180] Further, SS patients had higher frequencies of PD-1-positive T_{FR}, suggesting, on one hand, an active state of these cells, and on the other, the effect of type I IFN, which is known to upregulate PD-1.[181]

T_{FR} were first characterized in 2011 as a subset of T_{reg} cells functioning in lymphoid follicles and possessing hallmark transcription factors FoxP3 and BCL-6.[99-101] A study from Miyara *et al*. revealed that the circulating T_{reg} counterpart can be further sub-phenotyped into three fractions, according to FoxP3 and CD45RA expression.[182] Fraction I cells ($FoxP3^{low}CD45^+$) are considered naïve T_{reg}, Fraction II ($FoxP3^{high}CD45^-$) effector T_{reg}, and fraction III ($FoxP3^{low}CD45^-$) are effector T cells.[182] Applying this phenotyping approach to the CXCR5-positive portion of T_{reg} (i.e. T_{FR}), did not reveal differences within fractions I, II and III between SS patients and HD. We however confirmed previous data showing that CXCR5-positive T_{reg} have an enriched naïve phenotype (Fraction I) suggesting that T_{FR} are bona fide regulatory T cells.

Together with elevated frequency of PD-1 expressing cells, these observations imply the unique properties of T_{FR} as a new subset of circulating CXCR5-positive cells, underlining the importance of their sub-phenotyping in autoimmune diseases. The estimation of T_{FR} frequencies in circulation is a relevant surrogate marker of autoantibody production activity in ectopic lymphoid tissue in RA[183] and SS,[180] however in SLE this correlation is not as strong. [103]

3.2 TRIM21/Ro52 in autoreactive and malignant B cells

TRIM21/Ro52 first caught research attention as an autoantigen for Ro/SSA autoantibody. Ro/SSA and La/SSB were described in 1961 and started to be implemented into clinical practice in 1964.[184] Later, for several autoimmune diseases the association between the autoantibody specificity and clinical features of disease was established.[185] This supported the idea that autoantibodies might affect the functionality of their targets, and not just reflect autoreactivity in general and trigger inflammation. TRIM21 turned out to have a self-standing role in the immune regulation and in basic cellular processes. In **Papers III and IV** we aimed to expand the knowledge about this multifunctional protein.

3.2.1 Trim21 in B cells (Paper III)

Ro52/TRIM21 is a main autoantigen in Sjögren's syndrome and SLE. Interestingly, *Trim21* knock-out mice (*Trim21*$^{-/-}$) develop systemic autoimmunity with B cell-related features including autoantibodies, hypergammaglobulinemia and glomerulonephritis after tissue injury, implying a potential role for Ro52/TRIM21 in the pathogenesis. Autoantibodies directed to Ro52/TRIM21 can inhibit its E3 ligase activity,[41,186,187] and may thus potentially lead to a Ro52/TRIM21 deficient state in patients with Sjögren's syndrome and SLE. To understand how Trim21 deficiency may contribute to B cell disturbances, we analyzed the B cell responses of *Trim21*$^{-/-}$ mice after immunization with T cell-dependent (OVA) and T cell-independent (Ficoll, LPS) antigens.

NP-OVA immunization yielded differences in antigen-specific IgM and IgG levels, with higher levels in *Trim21*$^{-/-}$ mice, as well as higher frequencies of NP-specific B cells (assessed by both flow cytometry and ELISPOT) and plasma cells. IgM and IgG levels were also higher in *Trim21*$^{-/-}$ mice immunized with NP-Ficoll. Both OVA and Ficoll act through the BcR, and one explanation of these data might be that Trim21 regulates signalling downstream of the BcR. Indeed, *Trim21*$^{-/-}$ B cells displayed higher proliferative response *in vitro* upon stimulation by BcR, which was observed by both [3H]-thymidine incorporation and Ki67 staining.

It may seem unexpected that NP-LPS immunization did not reveal differences in anti-NP antibody levels, since Trim21 has been suggested to act downstream of several TLRs.[44,63,188] However, LPS stimulation of *Trim21*$^{-/-}$ B cells *in vitro* has been previously shown to generate similar degree of IgM production as in wild type mice, consistent with our observations.[63]

Flow cytometric analysis of splenocytes *ex vivo* revealed higher frequencies of follicular B cells in samples from NP-OVA-immunized *Trim21*$^{-/-}$ mice. While no difference in CD19^{+} cell frequency has been described in naïve mice earlier,[44] we revisited the issue of B cell subpopulations in naïve *Trim21*$^{-/-}$ mice, and indeed observed an expansion of follicular B cells in both young and aged mice. To understand the differences between follicular B cells derived from *Trim21*$^{-/-}$ and *Trim21*$^{+/+}$ mice, we sorted follicular B cells and subjected them to microarray analysis.

Analysis of the microarray data revealed substantial differences between follicular B cells of *Trim21*$^{-/-}$ - and *Trim21*$^{+/+}$ mice. Gene set enrichment analysis using Gene Ontology (GO) terms revealed the term "Regulation of B cell differentiation" as the most significant result. A shift towards follicular B cells might reflect a subtle immune-related process, for instance, due to presence of limited amounts of non-pathogenic microorganisms. The "Cell Cycle" GO term contains 1636 genes, and, despite significant results of ranked list enrichment test, seems more likely to reflect a high overlap with differentiation-related pathways than a genuine enrichment.

Interestingly, among the differentially expressed genes (DEG), multiple entries were regulated by the sterol regulatory element-binding protein (SREBP) (data not shown). In relation to that, it is interesting to note that patients with SS are known to be at higher risk of

metabolic syndrome.[113] Further, several metabolic disorders reveal underlying pro-inflammatory mechanisms, e.g. macrophage and Th1 and Th17 activation.

A limitation of this study is that is does not distinguish between the intrinsic and extrinsic effects of Trim21 deficiency on B cells. Some of the data described here might thus derive from influences from other cell types on B cell homeostasis and functions.

In sum, we observed enhanced proliferation and antibody production in immunized mice and BcR stimulated cells. Our data from naïve mice are suggestive of Trim21 affecting B cell differentiation and, unexpectedly, sterol metabolism. Thus, Trim21 might play a slightly different role in non-stimulated B cell signalling. The anti-proliferative and anti-differentiational effects of Trim21 might, at least partially, be explained by already described interactions of Trim21 within the NFκB[50,189] pathway or/and ubiquitination of IRFs, though so far unidentified targets may also exist.

3.2.2 TRIM21 in lymphoma (Paper IV)

Anti-proliferative properties of TRIM21 have been demonstrated in several previous studies, including our **Paper III**,[41,135,136] and higher expression level of TRIM21 has proven to be a marker of better prognosis in several types of tumours, but not in pancreatic cancer,[137] where this association was reversed. In our studies of lymphoma, we questioned if maintained expression of TRIM21 is beneficial also for prognosis in diffuse large B-cell lymphoma (DLBCL) and autoimmunity-associated DLBCL. DLBCL is the most common B-cell lymphoma in both RA[190] and SLE.[191]

We show that survival parameters (overall and progression-free survival) positively correlate with TRIM21 expression in the tumour tissue, both in patients with and without autoimmune disease. We also confirm the anti-proliferative effect of TRIM21 in human *ex vivo* PBMC.

In our cohort of patients with autoimmune diseases, most patients were diagnosed with RA, and one third with SLE. The risk of DLBCL in RA is approximately two times higher than that in the general population,[192] and it correlates with RA disease activity.[193] The prognosis is worse than in DLBCL in non-autoimmune patients, most likely due to the higher prevalence of the ABC type of lymphoma. SLE and a subset of RA patients are characterized by an IFN signature, and TRIM21 is an IFN-stimulated gene. One could thus expect higher TRIM21 expression in autoimmunity cohort. Surprisingly, we observed the opposite: 42% of patients fell into the TRIM21 low subgroup (versus 12% and 20% in the other two cohorts), although these data are consistent with the worse prognosis.

In accordance with previous studies,[193] in the cohort of lymphoma patients with autoimmunity, there was a shift towards ABC subtype (67%). IRF4, one of the hallmark molecules for ABC DLBCL,[117] is known to downregulate TRIM21,[43] which could contribute to the IFN-TRIM21 discrepancy. At the same time, there was no correlation between DLBCL subtype and TRIM21 expression in any of the cohorts, indicating the importance of other mechanisms. For instance, lower TRIM21 expression might give the cells a proliferative advantage, thus the amount of those would increase with disease (lymphoma) duration.

Therefore, we concluded that TRIM21 might have an independent prediction value in both autoimmunity-related and self-standing DLBCL.

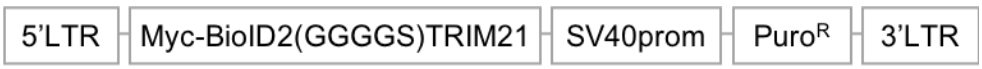
One mechanism of tumour suppression by TRIM21 might be restriction of proliferation, as illustrated by the pokeweed mitogen experiment included in this study, as well as data from **Paper III**, where mouse *Trim21*$^{-/-}$ splenocytes displayed enhanced proliferation upon BcR stimulation. Another potential contributor might be activation-induced apoptosis (AIA). Indeed, exogenous expression of *Trim21* made a mouse lymphoma A20 susceptible to CD40-induced apoptosis.[41] Burington *et al*. investigated the signalling behind AIA in DLBCL and concluded that it was mediated by p53, and that expression of BCL-6 and high degree of DNA damage increased susceptibility to AIA.[132] If this hypothesis holds true, maintained TRIM21 expression could be an indication of administration of anti-CD40 monoclonal antibodies, e.g. Dacetuzumab, to patients with DLBCL.

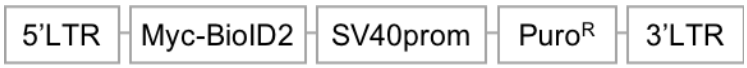
3.2.3 Identifying the TRIM21 interactome using proximity labelling by BioID2

Multiple substrates for TRIM21-mediated ubiquitination have been described. However, a picture of TRIM21 interactions remains fragmentary. We have recently initiated a project to assess the interactome of TRIM21 using proximity labelling with biotin followed by mass spectrometry. I have chosen to include data from the project here, although a full manuscript has not been generated. Two advantages of proximity labelling compared to e.g. immunoprecipitation are the more robust capture and an opportunity to track the interactors within a desired time frame and proximity (reviewed in[194]). BioID2 is a modified biotinylase from *E.coli* (BirA),[195] capable of labelling the adjacent proteins in a non-specific fashion. The technique includes introduction of the protein of interest, fused to BioID2 by a linker sequence, to the model cells. Cells are exposed to biotin, harvested, and the cell lysates are subjected to mass spectrometry for identification and quantification of the biotinylated proteins.

Plasmid generation and A375 transduction

The *TRIM21* cDNA sequence with a N-terminal GGGGS linker was cloned into a pBABE-Myc-BioID2 plasmid (Addgene) by digesting with XhoI and SalI and ligation with T4 ligase. The plasmid was extracted from the gel and propagated in Endura Competent Cells (Lucigen) using electroporation and subsequent ampicillin selection. The plasmid was purified with NucleoBond Xtra Midi kit (Macherey-Nagel). The success of cloning was verified by digestion with XhoI and SalI **(Fig 1A)** and by sequencing. The expression of Myc-BioID2-TRIM21 fusion protein was verified by western blotting of transfected HEK293T cell lysate with the anti-TRIM21 antibody **(Fig 1B)**. Next, HEK293T cells were transfected with pBABE-Myc-BioID2 with and without TRIM21, pUMCV and pMD2.G plasmids (Addgene) and X-tremeGENE 9 (Roche) to generate the retroviral vectors:





The virus-containing supernatants were used to transduce A375 cells. The transduced clones were selected with 1ug/ml Puromycin and expanded. We then exposed the cells to 50uM biotin for 20h before lysis and performed western blot using HRP-conjugated streptavidin **(Fig 1C)**.

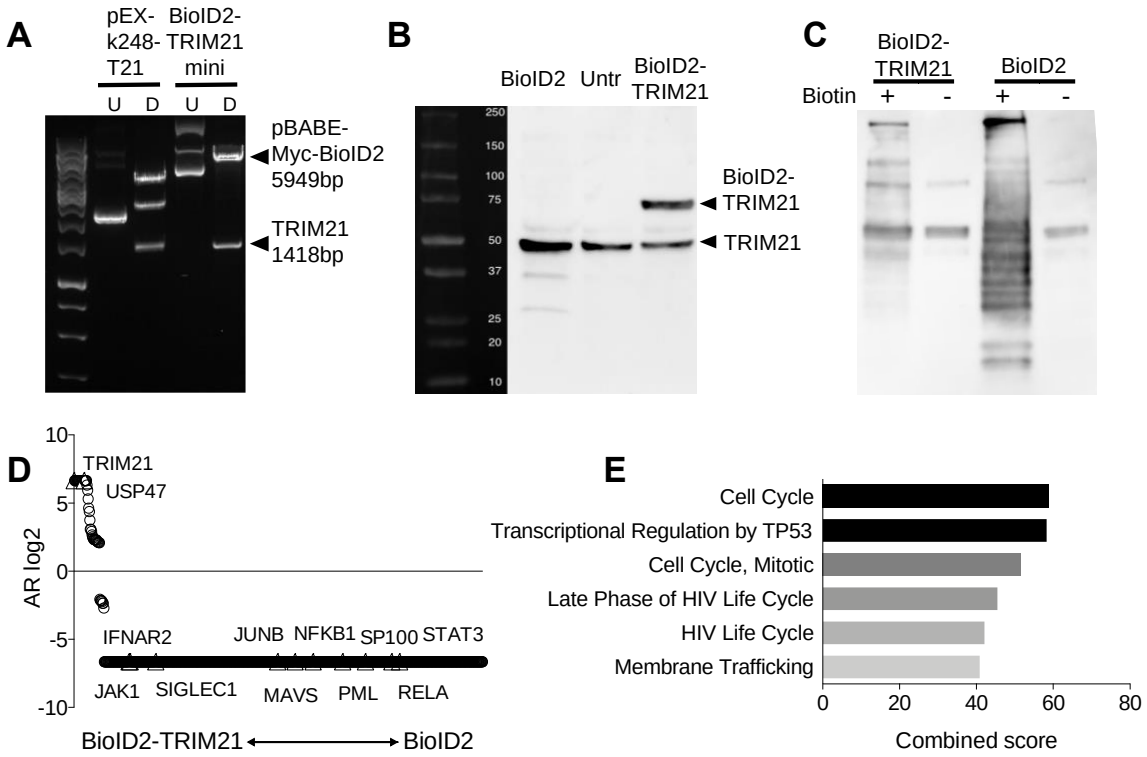


Figure 1. Main steps of TRIM21 proximity labelling experiment (A) *TRIM21* cloning. pEX-k248-T21, source plasmid; U, undigested; D, digested with SalI and XhoI. **(B)** Transitional expression of Myc-BioID2-TRIM21 plasmid in HEK293T cells, mouse anti-human antibody against TRIM21, HRP-conjugated secondary antibody. Untr, untransfected. **(C)** Biotinylation in transduced A375 cells after incubation with 50uM biotin for 20h. **(D)** Abundance ratios of LC-MS hits, n = 730. **(E)** Overrepresentation analysis, by Enrichr tool, Reactome library. All depicted overlaps are significant (p=3.7^10^{-9} and lower); the colour intensity is proportional to significance. Combined scores are resultant of three enrichment analysis methods suggested by Chen EY *et al*;[196] AR, abundance ratio.

Stable isotope labelling of proteins and Mass Spectrometry

Once the BioID2 biotinylating capability was confirmed, the cells were cultured for one week with Stable isotope labelling by amino acids in cell culture (SILAC) medium (ThermoFisher Scientific). A375 cells transduced with Myc-BioID2-TRIM21 were cultured with medium supplemented with stable isotope-containing (heavy) $^{13}C_6$ $^{15}N_2$ L-lysine-2HCl and $^{13}C_6$ $^{15}N_4$ L-arginine-HCl, and control cells were cultured with light (naturally prevalent) amino acids. Cells were then harvested for protein extraction, as described by in[197,198]. The concentrations of proteins in "heavy" and "light" samples were equalized, and the lysates were merged. The purification of biotinylated proteins was performed using streptavidin-coated magnetic beads. The resulting bead suspension contained TRIM21 interactome proteins, labelled with heavy amino acids and a control "non-specific" interactome proteins of BioID2, composed of light

amino acids. Liquid chromatography - mass spectrometry (LC-MS) was performed after on-bead trypsinization.

Data analysis

The set of identified proteins generated using Mascot software was obtained from Proteomics Biomedicum Facility, Karolinska Institutet. Only characterized master protein entries were used for further analysis. We first ran the protein ID list through the Contaminant Repository for Affinity Purification (CRAPome),[197] to remove background contaminants. CRAPome score less than 50 was used as a threshold. The heavy to light protein abundance ratios (AR) were log2 transformed.

TRIM21 interactors

Filtering as described above yielded a list of 900 entries. The entries with log2 AR between -2 and 2 were considered background noise and removed, resulting in 730 hits (**Fig 1D**) for running an overrepresentation analysis using the Enrichr tool,[196,199] Reactome library. The terms with significant overlap with our data are illustrated by **Fig 1E**.

The two Reactome terms with the highest degree of overlap with our dataset were Cell Cycle (R-HSA-1640170) and Transcriptional Regulation by TP53 (R-HSA-3700989), which goes in line with previous findings, including the ones in **Papers III and IV**. The vesicular trafficking-related terms were identified also in anti-Ro/LA-positive mothers from our neonatal lupus study **(Paper V)**. TRIM21 is upregulated in SS and SLE, and our experimental conditions here might mimic that due to additional (exogenous) TRIM21 expression.

Interestingly, several protein hits in our study have been previously described as TRIM21 interactors. Out of 116 unique TRIM21 interactors listed at BioGRID (https://thebiogrid.org/112615/summary/homo-sapiens/trim21.html), 11 (TRIM21, RC3H1, CBL, HAUS2, JUNB, MNAT1, NCOA3, PAWR, SPDL1, ZNF598, SQSTM1) were present in our dataset (n=730). Other interesting entries included IFNAR2, NFκB1, RELA, STAT3, adaptor of cytosolic RNA sensors MAVS, autoantigens PML and SP-100 and many more **(Fig 1D)**.

However, a majority of proteins was only present in the control sample, most likely due to TRIM21-specific ubiquitination and (at least partial) degradation in Myc-BioID2-TRIM21-tranduced ("heavy") cells. Thus, both genuine TRIM21 substrates and non-specifically biotinylated proteins would gain high abundance in the control sample. Unfortunately, our current experiment design does not allow distinguishing between them. To circumvent this issue, we are planning to repeat the experiment using a mutated version of TRIM21. Removing the critical catalytic cysteine from the RING domain will result in abolished ubiquitination capacity.[200] As a result, MS hits with lower abundance in the cells transduced with functional Myc-BioID2-TRIM21 ("heavy") sample should reveal genuine substrates of TRIM21-mediated ubiquitination.

3.3 Congenital heart block

Another comorbidity in SS and SLE, congenital heart block (CHB) is a rare, but dangerous condition, dictating a need for further research in order to create effective therapeutic and preventive management strategies. The peculiar feature of CHB is a location- and time-specific inflammatory process in the foetus, induced by maternal antibodies that do not affect the maternal heart. Despite a sustained effort, the fetal autoantigen in CHB has not been definitively identified, highlighting the complexity of CHB pathogenesis. Thus, **in papers V and VI** we chose to approach the problem from a different perspective and focused on the factors of fetal/neonatal immunity.

3.3.1 Type I IFN in newborns exposed to autoantibodies against Ro and La (Paper V)

Autoantibodies against Ro and La can induce production of type I IFN in PMBC and pDC, which has been suggested as the central mechanism for generation of the IFNα found in patients with SS and SLE, and for the subsequent upregulation of IFN stimulated genes.[201,202] During pregnancy, maternal IgG, including autoantibodies of IgG class is transported across placenta. The foetus of the mothers carrying anti-Ro/La autoantibodies is at risk of neonatal lupus erythematosus (NLE) including a congenital heart block (CHB). The pathogenic mechanism is not well understood, and while many attempts have been made to identify fetal targets for the autoantibodies, the question whether the antibodies may induce an activation of the IFN system also in the foetus has not been addressed. In this study we therefore aimed to characterize type I IFN activation in newborns antenatally exposed to maternal anti-Ro/La autoantibodies.

To address the question if the neonates born to anti-Ro/La positive mothers differ in their gene expression from neonates born to heathy donors (HD) we analysed the gene signatures from PBMC of mother-baby pairs. Gene enrichment analysis revealed Gene Ontology (GO) terms related to type I IFN and anti-viral response in both anti-Ro/La positive mothers and anti-Ro/La exposed neonates when compared to HD mothers and neonates, correspondingly. We next performed a DELFIA assay[203] to measured IFNα levels, and in anti-Ro/La positive subjects (both mothers and their newborns) they were significantly elevated, confirming our microarray findings.

Interestingly, the third set of GO terms differed between anti-Ro/La exposed mothers and their neonates. In the neonates, GO:0034341 Response to interferon gamma was identified (investigated in more detail in **Paper VI**), while in the mothers, vesicle-related process was revealed.

Interesting hits among maternal differentially expressed genes (DEG) include IRF7, a transcription factor downstream of TLR9 and a target for TRIM21-mediated ubiquitination,[43,44] and TNIP1, a susceptibility gene for SS, SLE and RA[58,204,205] and a part of the NFκB pathway. In anti-Ro/La-exposed neonates, TGFB1, a multifunctional and potent cytokine was upregulated.[206]

The presence of type I IFN was further confirmed by calculating an IFN score, a value derived from accumulated normalized expression of selected IFN-regulated genes. IFN scores were significantly higher in both mothers and neonates exposed to autoantibodies and, moreover, highly correlated with each other. Interestingly, the subgroup of neonates of mothers treated with immunomodulatory drugs, such as hydroxychloroquine (HCQ) and azathioprine, had significantly lower IFN score than the non-treated subgroup, while maternal IFN scores were equally high to the non-treated group. HCQ, a drug widely administered during pregnancy to patients with SS, SLE and other autoimmune diseases,[8,207] is a basic lipophilic substance with a capacity to accumulate in lysosomes and endosomes, thus increasing their internal pH.[208] This renders the conditions for TLR triggering suboptimal, and thus interrupts induction of type I IFN production in pDC. HCQ is known to cross placenta, hence, the observed decrease in type I IFN score in treated neonates might be explained by effect of HCQ on neonatal pDC.

Next, we visualized microarray expression data separately for Regulation of type I IFN production and Response to type I IFN Gene Ontology terms and observed an activation of both processes. These data suggested that type I IFN in anti-Ro/La-exposed neonates could originate from their own cells. It has previously been shown that adult immune cells can produce type I IFN after exposure to anti-Ro/La antibodies combined with apoptotic/necrotic material, but whether fetal immune cells also have that ability was not known. We tested this *in vitro* by stimulating neonatal cells with anti-Ro/La positive serum together with necrotic material and confirmed for the first time the capacity of neonatal PBMC to produce IFNα.

Finally, as a confirmation of downstream type I IFN activity at the protein level, the expression of the lectin Siglec-1 (sialoadhesin, CD169) on monocytes in both anti-Ro/La exposed mothers and neonates was strikingly higher than that of healthy donors. Siglec-1 was previously suggested as a convenient flow cytometry marker to monitor maternal type I IFN status throughout pregnancy.[209]

While IgG transfer is dependent on FcRn,[210] a vast diversity of substances is delivered through placenta by extracellular vesicles. Earlier studies suggest the IFN does not cross term placenta.[211] However, placenta-derived extracellular vesicles were recently shown to contain multiple chemokines, growth factors and cytokines, including IFNα, IFNβ, IFNγ and TGFα.[212] Considering the mode of action of HCQ, one might hypothesize that, except for a direct effect on neonatal IFNα production through TLR, HCQ could alter maternally-derived cytokine influx by modifying vesicular transport. Indeed, chloroquine, belonging to the same family of drugs as HCQ, was shown to block exosome internalization in microglia.[213] It is tempting to speculate that a vesicle-related process occurring in anti-Ro/La-positive mothers, as indicated by our microarray data, is related to active transfer of cytokines to the foetus. This could be a conserved mechanism developed to supply the foetus with protective substances if a viral infection is sensed in the mother. The origin of type I IFN in the foetus remains to be fully established, and transfer from the mother and production in the foetus are not mutually exclusive possibilities.

Here we show that neonates exposed to maternal Ro and La autoantibodies in utero, alike their mothers, have elevated levels of type I IFN, accompanied by type I IFN signature at mRNA and protein level. Our data indicate that neonatal cells are capable and active as IFNα producers, suggesting a probable fetal origin of the cytokine.

3.3.2 Cord blood cell profiling in newborns at risk of CHB (Paper VI)

The association of anti-Ro/La autoantibodies with neonatal lupus and congenital heart block (CHB) is well known, and the serology of the mothers and babies has been extensively investigated.[140,214] The immune cell profile in peripheral blood of non-pregnant women with anti-Ro/La autoantibodies has also been described, however that of pregnant women with anti-Ro/La autoantibodies is less well known. Similarly, the cord blood cell characteristics of newborns under risk of CHB have not been studied. In **Paper VI**, we therefore described the circulating immune cells in anti-Ro/La positive mothers and their newborns.

We first screened maternal and neonatal major blood cell populations. In the mothers, we observed deviations in B and T cell frequencies, as previously described for patients with systemic autoimmunity. In the anti-Ro/La-exposed neonates, T and B lymphocyte prevalence did not differ from those of healthy donor (HD) neonates, however, higher frequencies of NK/NKT cells were observed. In anti-Ro/La-exposed newborns of mothers receiving immunomodulatory treatment, this difference was no longer present.

To confirm our cellular findings in anti-Ro/La-exposed newborns, we performed gene enrichment analysis of microarray data from the same cohort. Indeed, two libraries from "Cell types" category of Enrichr Tool[196,199] yielded significant overlap between our DEG set and NK-cell-related terms. Interestingly, in both libraries, the second most significant overlap was with plasmacytoid dendritic cell terms. This finding goes in line with the elevated levels of IFNα from **Paper V**.

We next tested if the levels of type II IFN were also elevated as this is the main cytokine produced by NK cells. Although the difference did not reach significance, some of the anti-Ro/La-exposed neonates had high levels of IFNγ, while in all HD, but one, the IFNγ was virtually absent. Moreover, NK cell frequencies and INFγ concentrations correlated **(Fig 2)**, suggesting that neonatal NK cells might be the source of the cytokine.

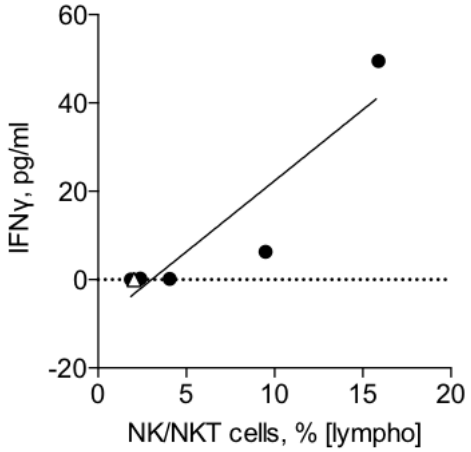


Figure 2. Correlation between NK/NKT cell frequencies and IFNγ levels in anti-Ro/La-exposed and healthy newborns. Spearman's rank correlation coefficient. Data from neonates from families 4 and 9 (HD) and anti-Ro/La-exposed subjects from families 11, 12, 18 and 23. Data from a treated subject (family 23) is illustrated as an open triangle. $r = 0.89$, $p < 0.03$.

The presence of IFNγ was further illustrated by an IFNγ score, which was calculated the same way as described for the type I IFN score,[215] but using genes preferentially regulated by type II IFN. Finally, to attempt to dissect type I and type II signatures, we applied a modular analysis suggested by Chiche *et al*. All three modules exhibited significant HGT results, however, the p values gradually increased from module M1.2 (representing type I IFN signature) to M5.12 (representing type II IFN signature). The unsupervised hierarchical clustering followed the same trend. This was not surprising, considering not all anti-Ro/La exposed individuals had detectable IFNγ in the plasma. Finally, we observed upregulation of HLA-DR on monocytes of anti-Ro/La exposed newborns, reflecting the presence of IFN (predominantly IFNγ). With these indications of a role of NK cells in pathologies following anti-Ro/SSA exposure, we sought to understand if they could contribute to the cardiac inflammation in CHB. Indeed, even a short-term stimulation of cardiomyocytes with type I IFN yielded upregulation of chemoattractants and activating ligands for NK cells.

Our findings are suggestive of IFNγ and NK cells involvement in NLE, however, the data may be interpreted ambiguously. A subset of SS and SLE patients have increased IFNγ levels, but there is no consensus on its clinical meaning, association with disease activity or increased risk of lymphoma.[38,216,217] NK cells have been shown to infiltrate adult heart tissues in multiple cardiac inflammatory diseases and even initiate the inflammatory process in viral myocarditis (by secretion of all three types of IFN and IL-12), however, their functions vary from directly cytotoxic to immunosuppressive and often are combined within the same pathology.[218] Besides, the properties of neonatal NK cells might differ from those of the adult. Rival *et al*. describe a mouse model of autoimmune ovarian disease in the neonate induced by maternal zona pellucida 3 autoantibody, which was mediated by neonatal, but not maternal, NK cells. The difference was explained by a lack of expression of inhibitory receptor Ly49 on neonatal NK cells.[219] In a mass cytometry study, Strauss-Albee *et al*. revealed no major differences between adult and cord blood NK cells, yet IFNγ and TNFα production, as well as ADCC and target apoptosis induction were significantly reduced in neonatal cells.[220]

Taken together, our findings indicate involvement of the innate branch of immunity in anti-Ro/La exposure-related pathologies, potentially mediated by an interplay of pDC and NK cells.

4 CONCLUDING REMARKS AND FUTURE RESEARCH QUESTIONS

This thesis addressed cellular and molecular factors in the pathogenesis of systemic autoimmunity and comorbidities.

The three main themes of our studies reveal:

- A decrease of CXCR5 expression in circulating B cells in patients with Sjögren's syndrome. This might be explained by the homing of B cells with high cell surface expression of CXCR5 to the autoimmune target organs exocrine glands. Genetic polymorphisms of *CXCR5* may also contribute to differential B cell trafficking. The frequency of the T_{FR} cell population is altered in SS, and might be useful as marker of inflammation activity.

- TRIM21/Ro52, an autoantigen in SS and SLE, affects B cell antibody production, proliferation, and, presumably, differentiation decisions. Higher TRIM21 expression in tumour cells is associated with better prognosis in diffuse large B-cell lymphoma.

- Cord blood cells from neonates exposed to maternal anti-Ro/La autoantibodies display signs of type I and type II IFN activity and are capable of IFNα production. NK cells and pDC supposedly contribute to the immune activation in these subjects. Both elevated type I IFN scores and NK cell frequencies in the neonates are affected by maternal immunomodulatory treatment.

The future questions appearing most relevant from a scientific and clinical point of view are:

- What are the mechanistic effects of the SS-associated *CXCR5* gene locus SNP?

- Is TRIM21 involved in activation-induced apoptosis in B-cell lymphoma? Can it be used as a predictor of sensitivity to therapy with anti-CD40 monoclonal antibodies?

- What are the roles of NK cells and IFNγ in cardiac inflammation in congenital heart block? Which yet unknown ways does HCQ impact immune activation in anti-Ro/La exposure in utero?

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