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THE TRIM21/RO52 E3 LIGASE AND ITS ANTIBODIES IN AUTOIMMUNE DISEASE

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Stockholm 2010

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Published by Karolinska Institutet.
Printed by Larserics Digital Print AB, Box 20082, SE-161 02 Bromma, Sweden.

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ISBN 978-91-7457-146-2

ABSTRACT

Patients with the systemic autoimmune diseases systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) often have autoantibodies against Ro/SSA (composed of the TRIM21/Ro52 and Ro60 antigens) and La/SSB. The biological function of the TRIM21/Ro52 protein itself has remained unknown until its recent description as a tripartite-motif (TRIM) family member and an E3 ligase involved in ubiquitination. Congenital heart block (CHB) is a passively acquired autoimmune condition that may develop in fetuses of anti-Ro/La positive women following transfer of maternal autoantibodies across the placenta and disruption of the fetal atrioventricular (AV) conduction system. Although anti-Ro, and especially anti-TRIM21/Ro52 antibodies, have been associated with development of CHB, the risk for CHB in an anti-Ro positive pregnancy is only 1-2%. In addition, a recurrence rate of 12-20% despite persistence of maternal antibodies indicates that additional factors are required for the establishment of heart block.

The aims of this thesis were 1) to contribute to the elucidation of the biological function of the TRIM21/Ro52 protein, especially regarding its role in autoimmunity, and 2) to characterize the involvement of anti-TRIM21/Ro52 antibodies in CHB pathogenesis and identify risk factors other than maternal autoantibodies for the development of heart block.

Using in vitro and in vivo studies, TRIM21/Ro52 was shown to be an IFN-inducible protein expressed in immune cells, mainly localized in the cell cytoplasm but able to translocate into the nucleus upon inflammatory stimuli such as IFNα. Ubiquitination assays demonstrated that TRIM21/Ro52 is a RING-dependent E3 ligase that can interact with different E2s both in the cytoplasm and in the nucleus, and that its E3 enzymatic activity is inhibited by anti-RING antibodies present in serum of patients with SLE or SS. Importantly, TRIM21/Ro52 was shown to ubiquitinate several interferon regulatory factors (IRFs), which are transcription factors activated downstream of TLR/IFN signaling, and disruption of the *Trim21* locus in vivo led to increased production of pro-inflammatory cytokines and development of systemic autoimmunity following tissue injury/infection.

Anti-TRIM21/Ro52 antibodies recognizing the p200 part (amino-acids 200-239) of the protein were specifically implicated in the pathogenesis of CHB by demonstrating that they induced AV block in rodents following transfer during gestation, while antibodies targeting other domains of TRIM21/Ro52 did not. Using a rat immunization model of heart block, maternal MHC genes were shown to regulate the generation of pathogenic anti-TRIM21/Ro52 antibodies, while a different MHC haplotype was linked to susceptibility to disease in the offspring. In addition, maternal age and seasonal timing of pregnancy were identified as risk factors for the development of CHB in anti-Ro/La antibody positive pregnancies in a Swedish cohort of families with individuals affected with CHB.

In summary, the TRIM21/Ro52 autoantigen is a negative regulator of IFN/TLR responses via ubiquitination of several IRFs and as such may play an important role in the pathogenesis of SLE and SS. Anti-TRIM21/Ro52 p200 antibodies can initiate development of fetal heart block and factors such as fetal genetic susceptibility, maternal age and seasonal timing of pregnancy may promote the establishment of CHB.

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I. Strandberg L*, Ambrosi A*, Espinosa A, Ottosson L, Eloranta ML, Zhou W, Elfving Å, Greenfield E, Kuchroo VK, Wahren-Herlenius M. **Interferon-α induces up-regulation and nuclear translocation of the Ro52 autoantigen as detected by a panel of novel Ro52-specific monoclonal antibodies.** J Clin Immunol. 2008 May;28(3):220-31.

II. Espinosa A*, Hennig J*, Ambrosi A, Sandberg M, Sheng Y, Arrowsmith CH, Nyberg F, Sunnerhagen M, Wahren-Herlenius M. **Anti-Ro52 autoantibodies from patients with Sjögren's syndrome inhibit the E3 ligase activity of Ro52 by blocking the E3:E2 interface between Ro52 and UBE2E1.** Submitted.

III. Espinosa A*, Dardalhon V*, Brauner S*, Ambrosi A, Higgs R, Quintana FJ, Sjöstrand M, Eloranta ML, Gabhann JN, Winqvist O, Sundelin B, Jefferies CA, Rozell B, Kuchroo VK, Wahren-Herlenius M. **Loss of the lupus autoantigen Ro52/Trim21 induces tissue inflammation and systemic autoimmunity by disregulating the IL-23-Th17 pathway.** J Exp Med. 2009 Aug 3;206(8):1661-71.

IV. Espinosa A, Brauner S, Ambrosi A, Kuchroo VK, Wahren-Herlenius M. **Response to comment on "Gene disruption study reveals a nonredundant role for TRIM21/Ro52 in NF-κB-dependent cytokine expression in fibroblasts".** J Immunol 2009 183: 7620-7621.

V. Ambrosi A*, Dzikaite V*, Park J, Strandberg L, Kuchroo VK, Herlenius E, Wahren-Herlenius M. **Congenital heart block is induced by lupus-associated Ro52 autoantibodies specific for amino acid 200-239.** Manuscript.

VI. Strandberg L, Ambrosi A, Jagodic M, Dzikaite V, Janson P, Khademi M, Salomonsson S, Ottosson L, Klauninger R, Åden U, Sonesson SE, Sunnerhagen M, de Graaf K, Kuchroo VK, Achour A, Winqvist O, Olsson T, Wahren-Herlenius M. **Maternal MHC regulates generation of pathogenic antibodies and fetal MHC-encoded genes determine susceptibility in congenital heart block.** J Immunol. 2010 Sep 15;185(6):3574-82.

VII. Ambrosi A*, Salomonsson S*, Eliasson H, Zeffer E, Dzikaite V, Bergman G, Fernlund E, Theander E, Rydberg A, Skogh T, Wållberg-Jonsson S, Öhman A, Lundström U, Mellander M, Winqvist O, Fored M, Ekbom A, Alfredsson L, Källberg H, Olsson T, Gadler F, Jonzon A, Kockum I, Sonesson SE, Wahren-Herlenius M. **Development of heart block in SSA/SSB autoantibody-positive pregnancies is associated with maternal age and display a season-of-birth pattern.** Submitted.

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LIST OF ABBREVIATIONS

5-HT_4	5-hydroxytryptamine
aa	amino acid
APC	antigen presenting cell
AV	atrioventricular
BCR	B cell receptor
CD	cluster of differentiation
CHB	congenital heart block
DC	dendritic cell
DNA	deoxyribonucleic acid
ECG	electrocardiogram
GFP	green fluorescent protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon-regulatory factor
ISRE	interferon stimulated response element
LPS	lipopolysaccharide
MHC	major histocompatibility complex
(m)RNA	(messenger) ribonucleic acid
NF-κB	nuclear factor κB
NLE	neonatal lupus erythematosus
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
PBMC	peripheral blood mononuclear cell
PRR	pattern-recognition receptor
RBL	RING/B-box linker
RIG-I	retinoic acid-inducible gene I
RING	really interesting new gene
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
TCR	T cell receptor
TGFβ	transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRIM	tripartite motif

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INTRODUCTION

The subject of this thesis is the E3 ligase TRIM21/Ro52 and its antibodies, and their respective role in autoimmune disease. Ro52 was first described as an autoantigen in rheumatic diseases but in recent years its function as an E3 ubiquitin ligase and its involvement in the regulation of immune responses has gained increasing attention. Anti-Ro52 antibodies are particularly associated with the passive autoimmune condition congenital heart block (CHB), which may develop in fetuses of women with anti-Ro antibodies. This introduction therefore begins with a description of the immune system and autoimmunity, before focusing on the Ro52 protein, with a brief overview of TRIM proteins and the ubiquitin system. Finally, CHB is presented in details, with emphasis on past and current findings on CHB pathogenesis and the role of anti-Ro52 antibodies.

Immunity and autoimmune diseases

The immune system is a wonderful and intricate collection of cells and molecules giving an organism the benefit of immunity, a state where the individual is protected against infections. One of its remarkable characteristics is its ability to distinguish between self and dangerous non-self antigens: while able to recognize and elicit a protective response against a great variety of invading pathogens (dangerous non-self), the immune system does not react against each individual's own (self) antigens. However, mechanisms maintaining this state of unresponsiveness to self antigens (also called immunological tolerance) may fail, leading to autoimmune reactions, in which the immune system attacks the individual's own cells and tissues.

Innate and adaptive immunity. The immune system is traditionally divided into the innate and the adaptive (or acquired) immune system. Innate immunity constitutes the first line of defense of an organism and relies on a set of germ line-encoded pathogen recognition receptors to respond early and rapidly to microbial infections. In contrast, adaptive immune responses develop more slowly and mediate the later, more effective and more specific, phase of defense against an infection. As their name suggests, innate immunity is present from birth while adaptive or acquired immunity, which requires creation of new, unique antigen receptors through somatic recombination (Hozumi and Tonegawa, 1976), needs time to develop and mature to a full functional state.

Cells and receptors of the innate immune system. The principal components of the innate immune system are the epithelia, which constitute a physical and chemical

barrier against microbes, plasma proteins, such as the proteins of the complement system, and cells in the circulation and tissues. Neutrophils and monocytes/macrophages are the two main types of phagocytes. They circulate in the blood and are recruited to sites of infections where they can ingest and kill microbes. Dendritic cells (DCs) are also phagocytic cells and play an important role in the initiation of the adaptive immune response by displaying peptide antigens from the ingested microbes and activating T lymphocytes. Another class of innate immune cells are the natural killer cells, which are able to recognize and kill infected or tumor cells.

The receptors of the innate immune system recognize structures common to various types of microbes but absent from mammalian cells, such as lipopolysaccharide (LPS), a bacterial cell wall component, viral double-stranded ribonucleic acid (RNA), or unmethylated CpG oligonucleotides, common in microbial deoxyribonucleic acid (DNA) but rare in mammalian DNA (Akira et al., 2006). Such receptors are called pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) are transmembrane proteins that constitute one of the two classes of PRRs. The Toll protein, first discovered for its role in dorso-ventral patterning in *Drosophila* embryos (Hashimoto et al., 1988), was later shown to be crucial for protecting the fly against fungal infections (Lemaitre et al., 1996). Subsequently TLRs were described as important receptors of the innate immune system in mammals (Medzhitov et al., 1997) and to date 10 and 12 TLRs and their ligands have been described in humans and mice, respectively. The other class of PRRs are the cytosolic PRRs, which recognize microbial structures present in the cytoplasm of infected cells and include the retinoic acid-inducible gene I (RIG-I) family members and nucleotide-binding oligomerization domain (NOD) proteins (Meylan et al., 2006). Stimulation of cells through these PRRs leads to activation of two main families of transcription factors, nuclear factor κB (NF-κB) and interferon (IFN)-regulatory factors (IRFs), and to the secretion of various cytokines that will contribute to the early response to infections by activating macrophages (IFNγ) and natural killer cells (interleukin (IL)-12) or by inhibiting viral replication and spread of the infection (type I IFN).

In addition to their role in the early defense against infections, innate immune cells play an important role in the initiation of the adaptive immune response by providing costimulatory signals necessary for the activation and differentiation of T lymphocytes into effector cells. Depending on the nature of the pathogen and the cell type, distinct PRRs may be engaged, resulting in secretion of different cytokines that will further shape the ensuing adaptive response to be most effective against that particular pathogen. In a reciprocal way, the adaptive immune system often uses mechanisms of innate immunity to fight infections, e.g. activated T lymphocytes may secrete IFNγ that will stimulate macrophages to more efficiently engulf and kill microbes.

T and B lymphocyte development. T and B cells represent the adaptive arm of the immune system. In contrast to innate immune cells, which are all capable of expressing the same PRRs, lymphocytes produce antigen receptors that are clonally distributed, meaning that, as it was predicted more than 50 years ago (Burnet, 1957), each T and B cell clone expresses a single type of antigen receptor with a unique specificity. Incomplete allelic exclusion at the T cell receptor (TCR) α chain locus has however been described and results in the generation of T cells with a dual specificity. Although this phenomenon is likely to be rare, it might have implications in the development of autoimmunity, with T cells bearing an autoreactive TCR being potentially activated via recognition of an environmental antigen by their second non-autoreactive TCR (Heath and Miller, 1993; Padovan et al., 1993; Zal et al., 1996).

B cells are able to recognize several classes of molecules, e.g. proteins, carbohydrates and lipids, via their B-cell receptor (BCR) whereas T cells can only recognize peptide antigens presented on class I or II major histocompatibility complex (MHC) via their T cell receptor (Zinkernagel and Doherty, 1974). Class I-associated peptides are produced from cytosolic antigens by the proteasome (Michalek et al., 1993) and presented by MHC I, which is expressed by all nucleated cells, while class II-associated peptides are derived from internalized extracellular antigens processed in lysosomes (Waldron et al., 1974; Ziegler and Unanue, 1981) and are presented by MHC II, which is expressed by antigen-presenting cells (APCs). Professional APCs are also able to present on class I MHC molecules intracellular antigens derived from another infected cell that they have ingested, a process called cross-presentation (Bevan, 1976).

As all immune cells, T and B cells are produced in the bone marrow, but while B cells also mature in the bone marrow, T cell maturation takes place in the thymus. During maturation, B and T cells undergo positive and negative selection, a process by which only useful cells, with the potential to recognize foreign antigens, survive while useless and potentially harmful, self-reactive, cells die (Hayakawa et al., 1999; von Boehmer et al., 1989). T cells that receive a positive selection signal via MHC I will develop into cluster of differentiation 8 ($CD8^+$) T cells while those that receive a positive signal via MHC II will become $CD4^+$ T cells (Teh et al., 1988). As a consequence, $CD8^+$ T cells recognize peptides produced from cytosolic proteins and bound to MHC I on the surface of infected/tumor cells or APCs that have phagocytosed such cells, while $CD4^+$ T cells recognize MHC II-bound peptides derived from extracellular microbes and presented by APCs.

Lymphocyte activation. Activation of T cells is initiated by recognition of their specific antigen bound to MHC on APCs. However TCR engagement is not sufficient to complete T cell activation and a second costimulatory signal, provided by APCs, is needed (Bretscher and Cohn, 1970; Lafferty and Cunningham, 1975). APCs that have ingested

microbes and/or been exposed to cytokines produced in the course of the innate immune response upregulate surface molecules that will act as costimulators for T cell activation, e.g. B7-1 and B7-2 that interact with CD28 on T cells. While naïve T cells can only be activated by mature DCs, previously activated T cells can also be reactivated by macrophages and B cells (Inaba and Steinman, 1984).

Within 1 or 2 days after activation, T cells start to proliferate, resulting in the expansion of antigen-specific clones. $CD8^+$ T cells differentiate into cytotoxic T lymphocytes, able to kill infected or tumor cells expressing the antigen, while $CD4^+$ T cells differentiate into so-called T helper (Th) cells that respond to their specific antigen by expressing cytokines and surface molecules to activate phagocytes and B cells. Depending on the cytokine milieu at the time of activation, helper T cells may differentiate into different subsets producing distinct sets of cytokines that will tune the immune system to respond in the most appropriate way to the current threat. At least 4 different lineages have been described: Th1, Th2 (Mosmann et al., 1986), Th17 (Harrington et al., 2005; Park et al., 2005) and regulatory T cells (Chen et al., 2003; Sakaguchi et al., 1995).

B cell responses are initiated through engagement of the BCR to its specific antigen. In the case of a protein antigen, B cells will require further activation by T helper cells in peripheral lymphoid organs to proliferate, undergo affinity maturation (Eisen and Siskind, 1964) - a process by which BCRs with higher affinity for the inducing antigen are generated through somatic hypermutation (Kim et al., 1981) -, class-switch (Wang et al., 1970) and differentiate into antibody-producing plasma cells. Non-protein antigens, such as lipids or polysaccharides, are able to elicit B cell responses without T cell help through cross-linking of many BCRs on a single cell (Dintzis et al., 1983; Feldmann and Easten, 1971). Such antigens are called T-independent antigens and induce antibody responses characterized by less heavy chain class switching and affinity maturation than T-dependent humoral responses.

Tolerance. As mentioned earlier, the normal immune system does not react to the individual's own antigens. This state of immunological tolerance is established when developing lymphocytes encounter self antigens in the central lymphoid organs (central tolerance) or, in the case of self antigens that are not present in the thymus or bone marrow, when mature lymphocytes encounter these antigens in the peripheral tissues (peripheral tolerance).

Central tolerance is achieved through deletion of T/B cells that interact strongly with self antigens. In this process, also called negative selection, lymphocytes recognizing self antigens with high affinity receive signals that trigger apoptosis and the cells die before completing maturation (Goodnow et al., 1989; Kappler et al., 1987). Alternatively, self-reactive B cells can undergo receptor editing to produce a new BCR that will no longer

be specific for the self antigen (Radic et al., 1993; Tiegs et al., 1993) and some self-reactive $CD4^+$ T cells may develop into regulatory T cells (Jordan et al., 2001).

Peripheral tolerance can be maintained via three different mechanisms: anergy, deletion and immune suppression. Anergy is defined by functional inactivation of lymphocytes and occurs when T or B cells recognize their specific antigen but do not receive costimulatory signals from APCs, or T cell help, respectively (Nossal and Pike, 1980; Quill and Schwartz, 1987). Alternatively, self-reactive lymphocytes may be deleted via a process called activation-induced cell death, which likely results from a lack of costimulatory signals and/or survival stimuli, or expression of death receptors and their ligands, e.g. Fas and FasL (Russell et al., 1991; Watanabe-Fukunaga et al., 1992). Finally, immune suppression by regulatory T cells (formed in the thymus or in the periphery) is an important component of peripheral tolerance (Sakaguchi et al., 1995); self-reactive regulatory T cells may suppress the activation and responses of nearby cells, specific for the same antigen but also for others, via production of cytokines such as IL-10 and TGF-β and/or direct cell-cell interactions. B regulatory cells, producing IL-10, have also been described (Katz et al., 1974; Mizoguchi and Bhan, 2006).

From autoimmunity to autoimmune disease. Autoimmunity is defined as the loss of tolerance to self antigens and the establishment of an immune response towards these antigens. Following infection or injury, transient autoimmune episodes may occur and induce further damage, however these can eventually be resolved, with or without treatment (e.g. reactive arthritis). It has also been suggested that transient autoimmune responses are a necessary part of the healing process after tissue trauma (Kipnis et al., 2002).

In contrast to these transient events, an autoimmune disease is defined by the presence of a chronic autoimmune response that does not resolve. It is characterized by the presence of auto- (self-) reactive T and B lymphocytes and production of autoantibodies. Examples of autoimmune diseases where T cells are the main effectors of the pathogenic immune response include rheumatoid arthritis, multiple sclerosis and type I insulin-dependent diabetes mellitus. While autoreactive $CD8^+$ cytotoxic T lymphocytes may directly kill host's cells (e.g. pancreatic beta cells in type I diabetes), $CD4^+$ T helper cells may contribute to tissue injury by secreting pro-inflammatory cytokines that will recruit and activate innate immune cells such as macrophages and neutrophils, which will in turn induce tissue damage via cell-mediated responses and release of proteases and reactive oxygen species. By contrast, diseases such as myasthenia gravis and Grave's disease are the result of a direct pathogenic effect of autoantibodies, directed to the acetylcholine receptor and inducing muscle weakness and paralysis in the former, recognizing the thyroid-stimulating hormone receptor and inducing hyperthyroidism in the latter.

Despite the characteristic presence of autoreactive cells and antibodies in autoimmune diseases, it remains difficult to define a disease as genuinely and etiologically autoimmune. It is possible in animal models to recapitulate features of autoimmune diseases by transferring autoreactive cells/antibodies, e.g. induction of experimental autoimmune encephalomyelitis in the mouse/rat by transfer of T cells specific for myelin protein (Ortiz-Ortiz et al., 1976), induction of arthritis by transfer of serum from K/BxN mice (Korganow et al., 1999). However, if the limited genetic and environmental variations inherent to the use of experimental animals make it possible to prove the direct etiologic role of specific autoreactive cells in induction of disease, the opposite large variation in genetic backgrounds and environmental exposures in humans leaves the etiology of autoimmune diseases largely unknown to date. Circumstantial evidence such as the detection of autoantibodies well before clinical onset of disease (Arbuckle et al., 2003), or reports of allogeneic bone marrow transplantations transferring disease, support the idea that a certain disease is indeed genuinely autoimmune, however the understanding of how and why such autoimmune responses develop remains a challenge.

Genetic and environmental factors. The observation that some autoimmune diseases run in families and that the risk for a particular disease is higher in the twin sibling of an affected individual than in the general population led to the early realization of the existence of a genetic component in the etiology of autoimmune diseases. As revealed by the recent genome wide association studies and the study of congenic mouse or rat strains with different susceptibilities to spontaneous or induced autoimmune disease, most inflammatory autoimmune diseases are associated with multiple susceptibility loci. While the MHC genes show the strongest association, many non-MHC genes contribute to the risk of developing a certain disease, e.g. the tyrosine phosphatase PTPN22 (involved in lymphocyte activation signaling) has been associated in humans with several autoimmune diseases (Begovich et al., 2004; Bottini et al., 2004; Kyogoku et al., 2004), the IRF5 transcription factor with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) (Graham et al., 2006; Miceli-Richard et al., 2007) and the cytosolic PRR NOD-2 with Crohn's disease (Hugot et al., 2001; Ogura et al., 2001).

Although twin studies have revealed the genetic component of autoimmune diseases, they have also shown that the concordance rates between identical twins are far from 100%, suggesting that environmental factors must interact with genetic predispositions to cause autoimmune disease. The role of infection in the development of autoimmunity as such an environmental trigger has been a subject of discussion for a long time. It was initially proposed that infections may induce autoimmunity by molecular mimicry, a mechanism whereby cross-reaction between microbial and self antigens initiates the autoimmune response (Fujinami and Oldstone, 1985). Although

this phenomenon has been identified as the cause of rheumatic fever, its actual relevance in the pathogenesis of most autoimmune diseases remains unknown. An alternative hypothesis is that infections may trigger autoimmunity by damaging tissues and thus releasing antigens otherwise hidden from the immune system, and, by simultaneously activating innate immune cells, provide the secondary signals necessary to the activation of autoreactive lymphocytes, eventually leading to break of tolerance (Miller et al., 1997; Rosen et al., 1995). However it is not clear how such a response would be perpetuated and develops into an autoimmune disease. It is possible that the autoimmune response itself would induce further damage, and that the continuous release of endogenous danger signals from the tissue would lead to a chronic autoimmune reaction. Another possibility is that recurrent infections may feed the original autoimmune response, leading progressively to disease.

Environmental triggers other than infections have also come to light in the last decade. Development of rheumatoid arthritis has been associated with smoking in combination with a certain MHC haplotype (Padyukov et al., 2004). Arthritis can be induced in rodents following exposure to mineral oil (Kleinau et al., 1991) and interestingly, an increased risk for rheumatoid arthritis has been observed in individuals exposed to hydraulic oils (Klareskog et al., 2002). Recently, a season-of-birth pattern has been linked to the risk of developing multiple sclerosis in populations from Northern countries (Willer et al., 2005).

Systemic lupus erythematosus and Sjögren's syndrome. SLE and SS are both autoimmune inflammatory rheumatic diseases characterized by B cell hyperactivity and presence of autoantibodies. While SS most commonly affects exocrine glands, with lymphocytic infiltrates and progressive tissue destruction (Jonsson et al., 2000), SLE is a more severe disease involving many organs and characterized by the deposition of immune complexes, particularly in the kidneys, joints, skin and blood vessels, leading to glomerulonephritis, arthritis, skin rash and vasculitis (Klippel, 1997). Both diseases are more prevalent among women than men, with a ratio of 9:1 (female:male) and SLE predominantly affects women in their child-bearing years (Petri, 2002). Although the mean age at onset for SS is between 45 to 55 years of age, this disease affects a broad range of individuals, including children, and it has been suggested that young versus elderly SS patients may represent two distinct subgroups, with the autoantibodies being more prevalent in early-onset SS (Haga and Jonsson, 1999).

Both SLE and SS are associated with the presence of anti-nuclear antibodies (ANA), rheumatoid factor, anti-Ro/SSA (Ro52 and Ro60) and anti-La/SSB antibodies (Griesmacher and Peichl, 2001). Among ANA anti-double stranded DNA antibodies are most specific for SLE, while anti-Ro/La antibodies are more common in SS patients. Both diseases are characterized by the presence of an "IFN signature", i.e. increased

expression of IFN-regulated genes (Bennett et al., 2003; Gottenberg et al., 2006), and an increased risk of developing B cell lymphoma (Theander et al., 2006).

The Ro52/TRIM21 autoantigen and E3 ligase

Autoantibodies to Ro/SSA have been known to be present in patients with rheumatic diseases, in particular SLE and SS, since the late 60's (Alspaugh and Tan, 1975; Clark et al., 1969) and have been used as a diagnostic tool, although the molecular identity of the Ro/SSA autoantigen was unknown. In the 80's, Ro/SSA was found to consist of two different proteins ; Wolin et al first identified Ro60 as part of the complex (Wolin and Steitz, 1984) and Ben-Chetrit et al later described a 52-kilo Dalton (kD) protein comprised in the Ro/SSA autoantigen, which was therefore named Ro52 (Ben-Chetrit et al., 1988). The complementary DNA of human Ro52 was subsequently cloned in the early 90's (Chan et al., 1991; Chan et al., 1990; Itoh et al., 1991), the human Ro52 gene was mapped to chromosome 11 (Frank et al., 1993) and Ro52 was shown to belong to the tripartite-motif (TRIM) protein family (Reymond et al., 2001). Trim21 is the official gene symbol for Ro52 and the protein is therefore also called TRIM21. The term Ro/SSA 52 kD may be used as well, especially in clinical settings.

TRIM proteins. The TRIM family is composed of proteins sharing a conserved N-terminal structure made of one Really Interesting New Gene (RING) domain, one or two B-box domains and one coiled-coil region (Borden et al., 1993; Reddy et al., 1992; Reymond et al., 2001). TRIM proteins possess an E3 ligase activity associated with their RING domain, which has been shown to mediate protein-protein interactions, especially the binding of E2 enzymes to E3 ligases (Lorick et al., 1999; Meroni and Diez-Roux, 2005). While the conserved N-terminal motif in TRIM family members is suggestive of an effector function, the variability in the C-terminal domain may be responsible for different subcellular localizations, cell type-specific expressions and biological functions of individual TRIM proteins (Short and Cox, 2006). The most common C-terminal sequences in TRIMs are the PRY and SPRY domains, found either alone or in combination to form the PRYSPRY (also called B30.2) domain. Structural analysis of the B30.2 revealed that it forms a putative interaction site allowing the TRIM proteins to bind different targets, with sequence polymorphisms in the B30.2 of different TRIMs conferring specificity to the binding and therefore contributing to the variety of the biological functions of TRIM family members (Grutter et al., 2006).

The observation that many TRIMs are upregulated by IFNs suggests a possible role in innate immune responses (Rajsbaum et al., 2008). In fact, several TRIM proteins have

been shown to restrict infections by retroviruses, e.g. TRIM5 (Stremlau et al., 2004), TRIM22 (Barr et al., 2008), TRIM25 (Gack et al., 2007) and TRIM28 (Wolf and Goff, 2007). However, the importance of TRIMs in innate immune defense does not seem to be limited to antiviral activity, but to extend to the regulation of IFN responses and PRR signaling pathways. Although this aspect of TRIM protein function is only beginning to be investigated, several TRIMs have already been implicated in the control of NF-κB and IRF transcription factors activation pathways. TRIM25, in addition to its ability to inhibit replication of HIV, has thus been shown to promote RIG-I-induced signaling and subsequent type I IFN production and NF-κB activity (Gack et al., 2007). Conversely, TRIM30α and TRIM27 have been described as negative regulators of TLR signaling, via inhibition of NF-κB and IRF responses (Shi et al., 2008; Zha et al., 2006).

The ubiquitin system. Ubiquitination is a post-translational modification mechanism used by eukaryotic cells to control diverse biological processes such as protein degradation, trafficking or activation. It is a complex three-step enzymatic reaction resulting in the covalent attachment of one or more ubiquitin moieties to the target protein, **Fig 1** (Ciechanover et al., 1980; Ciehanover et al., 1978; Hershko and Heller, 1985; Hershko et al., 1983).

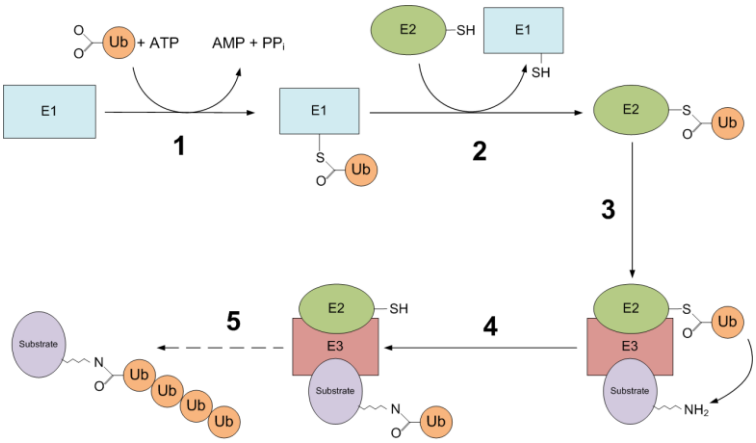


Figure 1. Post-translational modification with ubiquitin. A ubiquitin molecule is first activated and bound by the E1 enzyme **(1),** transferred to an E2 enzyme **(2)** and finally attached to the substrate with the help of an E3 ligase **(3-4)**. In the case of a RING E3 ligase, ubiquitin is directly transferred from the E2 to the substrate, with the E3 acting as a scaffold protein (depicted here). Several ubiquitin moieties can be sequentially attached to form a polyubiquitin chain by repeating step 4 **(5)**.

The ubiquitin molecule is first bound and activated by a ubiquitin activating enzyme (E1) through an ATP-dependent mechanism where the C-terminal glycine residue (G76) of ubiquitin forms a thioester bond with a catalytic cysteine residue in the active site of the E1. The activated ubiquitin molecule is then transferred to a cysteine residue of a ubiquitin conjugating enzyme (E2). In a third and final step, a ubiquitin ligase (E3) mediates the transfer of ubiquitin from the E2 to a lysine residue of the target protein, either by binding ubiquitin covalently before transferring it to the target (homologous E6AP carboxyl terminus -HECT- E3 ligases) or by acting as a scaffold protein to bring together the E2 and target protein (RING E3 ligases) (Freemont et al., 1991; Huibregtse et al., 1995; Lovering et al., 1993).

Proteins can be modified by ubiquitination in three different ways: monoubiquitination, where a single ubiquitin molecule is attached to the target protein; multiubiquitination, where several single ubiquitin moieties are covalently bound to separate lysine residues on the target protein; and polyubiquitination, where a chain of ubiquitin molecules is added to a single lysine residue on the target. Polyubiquitin chains are thought to be built by sequential attachment of ubiquitin moieties through their own lysine residues (Chen and Pickart, 1990), but it has also been suggested that addition of pre-formed ubiquitin chains might occur (Li et al., 2007). As ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), several different types of ubiquitin chains are possible, K48 and K63 chains being the most commonly described (Kirkpatrick et al., 2005).

The ubiquitination process has long been associated with non-lysosomal protein degradation, starting with the work of Ciechanover and colleagues in the late 70's, and K48 ubiquitin chains have been shown to tag proteins for degradation in the 26S proteasome (Chau et al., 1989; Hershko and Heller, 1985). However, ubiquitination has since been linked to several other cellular events, such as activation of transcription factors (Kaiser et al., 2000) or internalization and degradation of plasma membrane proteins (Hicke, 1997). Ubiquitination, like other post-translational modifications, needs to be tightly regulated and carried out in a target-specific manner. While the process is reversible, through the activity of deubiquitinating enzymes (Amerik and Hochstrasser, 2004), the fate of ubiquitin-modified proteins is determined via the recognition of specific patterns of ubiquitination by different ubiquitin-binding domains contained in ubiquitin-binding proteins (Hicke et al., 2005). The specificity of the ubiquitination process itself is maintained thanks to a large number of E3 ligases (>400) that ensure the transfer of ubiquitin moieties to specific targets, while fewer E2s (about 50) and only one E1 are required.

E3 ligases in immune responses. Ubiquitination is a post-translational modification involved in many different biological processes, including immune responses.

Interestingly, the use of transgenic mice in recent years has brought forward the involvement of many E3 ligases in the regulation of immune cell activation, through ubiquitination and subsequent degradation or activation of diverse transcription factors, signaling molecules or cell surface receptors. Mice lacking the RING E3 ligase Casitas B-lineage lymphoma b (Cbl-b) show dysregulated T cell signaling, leading to impaired induction of T cell tolerance and autoimmunity (Bachmaier et al., 2000; Chiang et al., 2000). Similarly, deletion of the E3 ligase GRAIL leads to increased T cell responses and impaired anergy induction in absence of costimulation (Nurieva et al., 2010). Deletion of the E3 ligase Itch also leads to severe immune dysregulation and the enzyme has been shown to mediate the ubiquitination and degradation of the activator protein 1 (AP-1) subunit c-Jun, resulting in the downregulation of many AP-1 dependent cytokine genes (Fang et al., 2002; Perry et al., 1998). Besides regulating lymphocyte activation, E3 ligases have also been implicated in the control of TLR-induced responses and the growing evidence about the involvement of TRIM proteins in such a process has already been mentioned. In addition, the adaptor protein and E3 ligase TRAF6 has been shown to mediate IκB (inhibitor of NF-κB) kinase activation via formation of K63-polyubiquitin chains (Deng et al., 2000) and to be involved in IRF7 activation and IFNα production following TLR stimulation (Kawai et al., 2004).

TRIM21/Ro52. Ro52 is a member of the TRIM family of proteins, and as many other TRIMs has been shown to exhibit E3 ligase activity (Espinosa et al., 2006; Wada and Kamitani, 2006a, b). The TRIM21/Ro52 gene is located on chromosome 11 in human, 7 in mouse, and consists of seven (human) or eight (mouse) exons with a conserved exon/intron structure spanning approximately 8 kilobases (**Fig 2**). In both the human and mouse genomes, the TRIM21 gene is part of a large TRIM gene cluster containing TRIM2, 6, 22 an 34 in addition to TRIM21.

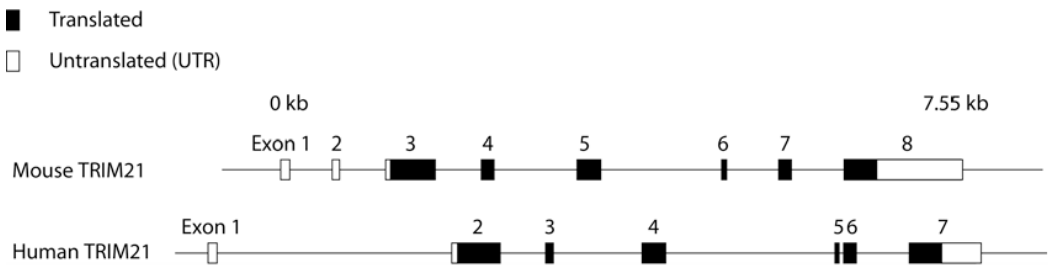


Figure 2. The exon/intron structure of the TRIM21/Ro52 gene in mouse and human.

The Ro52 protein comprises 475 amino acids in human (470 in mouse), with the TRIM motif (RING, B-box and coiled-coil domains) making about half of the protein on the N-terminal part, **Fig 3** (Ottosson et al., 2006). The coiled-coil domain is actually made of two predicted coiled-coil regions (CC-1 and CC-2), with CC-2 containing a putative leucine zipper motif. The C-terminal domain of Ro52 is denoted B30.2 or PRYSPRY and spans amino acids 252-470 in human (268-465 in mouse).

An alternatively spliced Ro52 mRNA transcript, where exon 4 is deleted, has been described and detected in a variety of tissues, including fetal heart and salivary glands (Chan et al., 1995). The significance of this alternative transcript is however unclear, as the existence of the corresponding Ro52 isoform, denoted Ro52-β, has never been demonstrated at the protein level.

Ro52 expression was originally suggested to be ubiquitous from in situ hybridization experiments (Reymond et al., 2001), however this has not been confirmed at the protein level.

MOUSE (aa 1-470)

Domain:	Sequence (aa):	Exon:
RING	(20-58)	3
B-box	(96-127)	3
Coiled-coil 1	(128-166)	3-4
Coiled-coil 2	(186-245)	5
B30.2	(252-470)	7-8

HUMAN (aa 1-475)

Domain:	Sequence (aa):	Exon:
RING	(16-54)	2
B-box	(92-123)	2
Coiled-coil 1	(125-166)	2-3
Coiled-coil 2	(179-235)	4
B30.2	(268-465)	6-7

Figure 3. The domain structure of TRIM21/Ro52. The RING, B-box and coiled-coil domains constitute the TRIM motif. The C-terminal of TRIM21/Ro52 comprises a B30.2 (or PRYSPRY) domain. Coiled-coil 2 contains a putative leucine zipper motif.

Ro52 function. Overexpression studies in cell lines have implicated Ro52 in the regulation of IL-2 and IL-12 production as well as in the regulation of B cell proliferation and sensitivity to activation-induced cell death (Espinosa et al., 2006; Ishii et al., 2003; Kong et al., 2007). However the mechanisms underlying these observations have remained unclear. Ro52 E3 ligase activity was first demonstrated in 2006 by two independent research groups (Espinosa et al., 2006; Wada and Kamitani, 2006a). Since Ro52's ubiquitination substrate was unknown, its enzymatic activity was first shown through autoubiquitination and proved to be dependent on the presence of the RING

domain (Espinosa et al., 2006). Several putative substrates have since been described for Ro52, including the immunoglobulin (Ig) G heavy chain (Rhodes and Trowsdale, 2007; Takahata et al., 2008; Yang et al., 1999; Yang et al., 2000), the cell cycle inhibitor p27 (Sabile et al., 2006), and IRF transcription factors (Higgs et al., 2008; Kong et al., 2007).

Although the interaction between human Ro52 and human IgG heavy chain is well characterized, its relevance in physiological conditions remains unclear. Ro52 binds the constant part of the IgG heavy chain through its B30.2 domain with a very high affinity (estimated at K_D=3,7.10^{-9}) at a site overlapping the binding site of several other proteins, including *Staphylococcus aureus* protein A and *Streptococcus* spp. protein G, and it has been suggested that Ro52 may play a role in innate immunity by regulating IgG functions (James et al., 2007; Rhodes and Trowsdale, 2007; Yang et al., 1999). However, experimental data are still needed to clarify the consequences of Ro52 binding to IgG.

Sabile et al have shown that Ro52 interacts with Skp2, a component of the SCF^{skp2} multisubunit complex involved in the degradation of many regulatory proteins, including the cyclin-dependent kinase inhibitor p27 (Sabile et al., 2006). In addition, the same authors demonstrate that knock-down of Ro52 in a cell line leads to accumulation of p27 and impaired progression to the S phase of the cell cycle. However direct p27-Ro52 interaction as well as ubiquitination of p27 by Ro52 remains to be demonstrated. The ubiquitination and degradation of the substrate protein bound by Skp2 is known to be mediated by the interaction of the SCF complex with the Cul1-Rbx1/Roc1 catalytic complex, where Rbx1/Roc1 is responsible for RING E3 ligase activity (Willems et al., 2004). It is worth noting that Sabile and colleagues observed an interaction between Ro52 and Cul1 similar to that of Cul1-Rbx1/Roc1, suggesting that several E3 ligases may be involved in Skp2-mediated degradation of different substrates, thereby conferring specificity to the process. It is therefore possible that Ro52 plays a role in the degradation of p27 and the control of cell proliferation under certain conditions (e.g. following mitogenic stimuli) and/or in specific cell types.

While the studies on Ro52 presented in this thesis were ongoing, two research groups reported Ro52-mediated ubiquitination of IRF8 and IRF3 (Higgs et al., 2008; Kong et al., 2007). In both cases, the interaction between IRF and Ro52 was dependent on the B30.2 domain of Ro52. Higgs et al showed that deletion of the Ro52 RING domain as well as knock-down of Ro52 led to increased IRF3 transcriptional activity and protein levels, indicating that Ro52 may negatively regulate IFN production by ubiquitinating IRF3 for proteasomal degradation. In contrast, Kong et al suggested that Ro52 may contribute to the stabilization of IRF8 following TLR stimulation after observing an enhanced production of IL-12p40, a target gene of IRF8, in a Ro52-transduced macrophage cell line. Given the fact that Ro52 mRNA expression is

upregulated by IFNs (Der et al., 1998; Rhodes et al., 2002; Zimmerer et al., 2007), influenza virus (Geiss et al., 2002) and LPS (Thomas et al., 2006), it is tempting to hypothesize a role for Ro52 in the regulation of IFN responses. IRF proteins therefore represent the most attractive targets of Ro52-mediated ubiquitination proposed to date.

Anti-Ro52 antibodies. Ro52 was first identified as part of the autoantigen complex Ro/SSA and although recent advances have uncovered a potential role for Ro52 in the regulation of immune responses through its E3 ligase activity, it remains unknown whether the anti-Ro52 autoantibodies present in patients with SLE or SS play a role in the pathogenesis of these autoimmune diseases. One stumbling block in conceiving a pathogenic function of anti-Ro52 antibodies is the fact that the protein is intracellular and thereby inaccessible to circulating IgGs. Since the late 70's, several research groups have shown that antibodies can penetrate living cells (Alarcon-Segovia et al., 1978; Zack et al., 1996), but these data were only obtained from in vitro studies and it remains controversial whether cytoplasm entry by antibodies is an in vitro artifact or can also happen in vivo. The expression of Ro52 on the surface of apoptotic keratinocytes and ductal epithelial cells has been described (Lawley et al., 2000; Ohlsson et al., 2002; Saegusa et al., 2002), and although this would expose the otherwise intracellular antigen to the circulating autoantibodies, it remains unclear whether antibodies would then exert a pathogenic effect by binding to their target and inhibiting its enzymatic activity.

Congenital heart block

CHB is a passively acquired autoimmune condition that may develop in fetuses of women with anti-Ro/SSA (Ro52 and Ro60) and anti-La/SSB antibodies. In contrast to SLE and SS, where a pathogenic role for anti-Ro52 antibodies remains to be determined, maternal anti-Ro52 antibodies have been directly incriminated in the development of CHB, although the molecular mechanisms are still being unraveled.

Neonatal lupus erythematosus. Neonatal lupus erythematosus (NLE) is a syndrome that may develop in infants of mothers with SLE, SS, or even asymptomatic mothers. The most common manifestations are skin rash, present at birth or precipitated by exposure to UV light, and CHB, diagnosed in utero or shortly after birth, but other features such as hepatitis and cytopenias may occur. All of the NLE symptoms except

CHB are transient and will resolve as maternal autoantibodies are cleared from the child's circulation at approximately 6 to 8 months of age (Lee, 2009).

Skin rash and CHB rarely occur simultaneously, with the overlap of the two symptoms making up only about 10% of the NLE cases (Lee, 1990). In addition, skin manifestations are transient, while complete CHB is irreversible. It is therefore possible to speculate that the maternal diagnosis and/or autoantibody profile as well as the pathogenic mechanisms involved in skin versus cardiac manifestations of NLE are different. Although the association between the presence of maternal autoantibodies to the Ro/SSA and La/SSB antigens and development of NLE in the child was described a long time ago, the mechanisms involved in NLE, and especially the factors leading to development of skin rash rather than cardiac manifestations in some children, while others do not develop any NLE symptoms at all, remain unexplained.

Congenital heart block. CHB is a rare disease, affecting 1 in 15,000 to 20,000 births in the general population (Michaelsson and Engle, 1972). Its association with the presence of maternal autoantibodies to Ro/SSA and/or La/SSB is however well established, and the risk of complete CHB is 1-2% in an anti-Ro/SSA positive pregnancy (Brucato et al., 2001; Buyon et al., 2001) and rises to 12-20% in mothers with a previously affected infant (Buyon et al., 1998; Julkunen and Eronen, 2001; Llanos et al., 2009b). Atrioventricular (AV) block is the hallmark manifestation of CHB and can lead to a life-long pacemaker dependence or even death, most often occurring in utero or perinatally (Buyon et al., 1998; Eronen et al., 2000; Waltuck and Buyon, 1994). Other cardiac disturbances, such as sinus bradycardia (Brucato et al., 2000; Brucato et al., 2001; Cimaz et al., 1997), prolongation of the QT interval (Cimaz et al., 2000; Gerosa et al., 2007), endocardial fibro-elastosis and dilated cardiomyopathy (Moak et al., 2001; Nield et al., 2002a; Nield et al., 2002b) have also been reported. CHB is usually detected between 18 to 24 weeks of pregnancy by standard fetal echocardiography techniques.

AV block is defined as a block or delay in the signal conduction at the AV node and can be divided in three degrees of severity. First-degree AV block is characterized by a prolonged AV conduction time, which can be visualized on an electrocardiogram (ECG) by a lengthened PR interval (Jaeggi and Nii, 2005). This interval is measured from the beginning of the P wave (depolarization of the atria) to the onset of the QRS complex (depolarization of the ventricles), **Fig 4**. During fetal life, AV time intervals can be estimated by following atrial and ventricular depolarization through their mechanical (m-mode, tissue Doppler) or hemodynamic (pulse-wave Doppler) consequences. In the case of a second-degree AV block, some of the atrial impulses are not conducted to the ventricles. A third-degree AV block corresponds to a complete block of all signal conduction through the AV node and is irreversible in CHB.

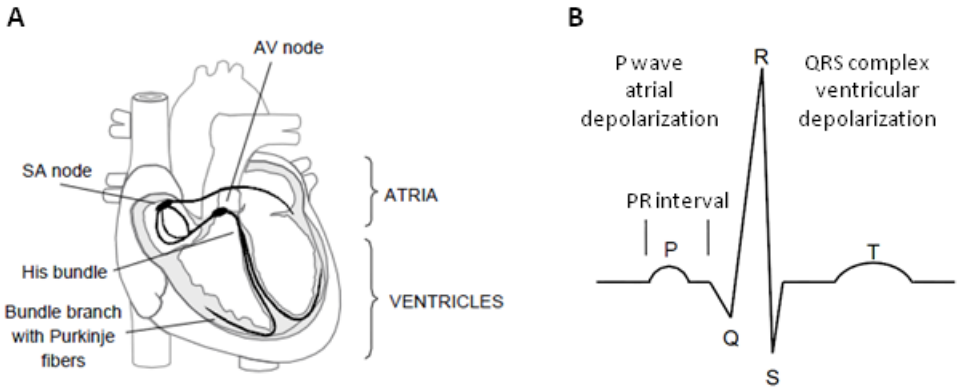


Figure 4. Conduction of electric impulses through the heart. (A) Electrical impulses are generated in the sino-atrial (SA) node and propagated from the atria to the ventricles via the atrioventricular (AV) node, before being conducted through the bundle of His to the bundle branches and the Purkinje fibers, and finally through the entire myocardium. **(B)** Electrocardiogram showing the P wave and the QRS complex, which correspond to the atrial and ventricular depolarization, respectively. The PR interval is measured from the beginning of the P wave to the beginning of the QRS complex.

Maternal autoantibodies and CHB. Since the early 80's, several studies have demonstrated a correlation between the presence of anti-Ro/SSA and -La/SSB antibodies in maternal serum and development of NLE in infants (Scott et al., 1983; Taylor et al., 1988). Although the association found between CHB and antibodies to the three autoantigens Ro52, Ro60 and La varies across the different studies, most of the data indicate that anti-Ro, and especially anti-Ro52 antibodies, are present in a high proportion of mothers of children with CHB (Eronen et al., 2004; Fritsch et al., 2006; Julkunen et al., 1993; Salomonsson et al., 2002). This finding was further confirmed recently in a population-based study in Sweden, where the serum of 96% (77/80) and 61% of autoantibody-positive women who had a child with heart block displayed reactivity towards the Ro52 and Ro60 proteins, respectively (Zeffer et al., 2010). As anti-Ro60 antibodies are most often found with anti-Ro52 antibodies, it is however difficult to assess their individual contribution to the development of CHB. In addition, most studies still rely on clinical assays that do not distinguish between Ro52 and Ro60 to investigate the presence of anti-Ro antibodies in maternal sera. In contrast to anti-Ro antibodies, the association of anti-La antibodies to CHB is still a matter of debate. While Silverman et al found the level of anti-La antibodies to be higher in mothers whose children developed cutaneous NLE rather than CHB (Silverman et al., 1995), a study by Gordon et al suggested that, on the contrary, the presence of anti-La antibodies increased the risk for heart block (Gordon et al., 2004). However, a recent study seems

to confirm the importance of maternal anti-Ro antibodies in development of cardiac NLE and shows an association of anti-La antibodies with a more benign prognosis and development of cutaneous lupus rather than CHB (Jaeggi et al., 2010). The current consensus is that antibodies to Ro60 and La may contribute to the inflammatory reaction that leads to AV block but that CHB may also develop in their absence.

Despite the almost universal presence of anti-Ro antibodies in mothers of children with CHB, the risk of fetal heart block in an anti-Ro positive pregnancy is only 1-2%. Attempts at finding other antibodies involved in CHB have yielded a few candidates, however these studies are few and most of the time too small to demonstrate a reliable association between the presence of antibodies and fetal outcome. Antibodies to calreticulin, a protein involved in calcium storage, have thus been found more frequently in mothers of children with heart block than in mothers of healthy children and have been suggested to contribute to initiation of the disease (Orth et al., 1996). Antibodies to the M1 muscarinic acetylcholine receptor have also been associated with the development of CHB and were shown to bind their target in the neonatal myocardium and to interfere with its function (Bacman et al., 1994; Borda and Sterin-Borda, 2001). The presence of these antibodies has however not been investigated in other and larger cohorts and the relevance of these findings in heart block pathogenesis remains uncertain. Antibodies to a cleavage product of α-fodrin have also been suggested as a serological marker for CHB (Miyagawa et al., 1998), however these antibodies are often found in patients with Sjögren's syndrome (Locht et al., 2008), a condition in which anti-Ro antibodies are also vastly prevalent, raising the possibility that the association of anti-α-fodrin antibodies with CHB may only reflect the close relation between CHB and anti-Ro52 antibodies. Llanos and colleagues recently evaluated the reactivity of sera from mothers of children with CHB to the α-enolase protein, following a report of cross-reactivity of specific anti-Ro52 antibodies to this protein (Ambrosi et al., 2007), however only a small proportion of CHB sera were positive, indicating that these antibodies may only represent a subset of mothers at risk (Llanos et al., 2009a). Similarly, reactivity to the α_{1D} calcium channel subunit was recently found in sera from mothers of CHB children, however such reactivity was limited to about 14% of all CHB mothers tested (Karnabi et al., 2010).

In all, anti-Ro52 antibodies seem to remain to date the maternal autoantibodies that correlate the most to CHB, despite the low penetrance of the condition in anti-Ro positive pregnancies. It is possible that not only the presence, but also the levels, of maternal anti-Ro52 antibodies are of importance in predicting fetal outcome, as is suggested by the recent findings of Jaeggi and colleagues where cardiac conduction disturbances were associated with moderate to high levels of anti-Ro antibodies but not to low levels (Jaeggi et al., 2010).

Fine specificity of anti-Ro52 antibodies associated with CHB. The specificity of the immune response to the Ro52 protein has been the subject of many studies, both because it is a major autoantigen in SS and because of its potential role in the development of fetal heart block. Dominant epitopes have been described in the central part of the Ro52 protein, in the region predicted to contain a leucine zipper (Blange et al., 1994; Kato et al., 1995), but anti-Ro antibody positive sera have also been shown to react against the N-terminal zinc-finger domain of Ro52 (Pourmand and Pettersson, 1998).

Sera from anti-Ro52 antibody positive mothers of children with or without heart block have been screened in an attempt to determine a specificity profile within the pool of anti-Ro52 antibodies associated with the development of cardiac conduction disturbances. Fritsch et al found a higher frequency of antibodies to Ro52 peptides comprising amino acids (aa) 107-122 and 277-292 in mothers of children with CHB compared to mothers of healthy children, however this was only true for mothers who had SLE and not for asymptomatic or SS mothers (Fritsch et al., 2006). Elevated levels of antibodies towards additional Ro52 peptides (aa 1-13 and aa 365-382) were also noted during the risk period for CHB (18-30 weeks of pregnancy).

Epitope mapping using overlapping peptides covering the immunodominant central region of Ro52 revealed a significant association between maternal antibodies to aa 200-239 of Ro52 (denoted p200) and the risk of CHB (Salomonsson et al., 2002; Strandberg et al., 2008). Higher anti-Ro52/p200 antibody levels have also been correlated to longer AV time intervals in a prospective study of anti-Ro52 positive women during susceptibility weeks 18-24 of pregnancy (Salomonsson et al., 2005).

Clues to CHB pathogenesis from experimental models. Two major particularities of CHB make it an autoimmune condition difficult to study in humans and for which an adequate animal model is therefore very desirable: it is very rare and occurs in utero, at about mid-pregnancy. Over the past 15 years several groups have performed in vitro and in vivo studies, generating direct evidence of a pathogenic role of maternal anti-Ro/La, and especially anti-Ro52 antibodies, in CHB.

Presence of anti-Ro antibodies in the cardiac tissue of fetuses dying of CHB, together with deposition of complement, fibrosis and calcification, was demonstrated over 20 years ago by several groups (Clancy et al., 2004; Lee et al., 1987; Litsey et al., 1985) and provided the first link between maternal antibodies and pathogenesis of heart block by placing the antibodies at the site of injury. Of note, antibodies, complement as well as signs fibrosis and calcification were observed not only at the AV node but also in the entire myocardium, suggesting a potential involvement in other cardiac manifestations of CHB that have been described, such as sinus bradycardia, cardiomyopathy and QTc prolongation.

In vitro studies performed on rat or human hearts isolated with the Langendorff technique demonstrated a direct pathogenic role of antibodies from mothers of children with CHB, as perfusion of hearts with maternal IgG containing anti-Ro/La antibodies or with affinity-purified anti-Ro52 antibodies induced bradycardia and complete AV block within 15 minutes (Boutjdir et al., 1997; Boutjdir et al., 1998). Subsequent reperfusion of the heart with a wash solution allowed only partial recovery. Similar results were obtained by different research groups using Langendorff-perfused rabbit hearts exposed to anti-Ro/La IgG, with different degrees of AV block and sinus bradycardia observed (Garcia et al., 1994; Hamilton et al., 1998; Restivo et al., 2001; Viana et al., 1998).

Evidence for a pathogenic role of anti-Ro/La antibodies in vivo has been gathered from animal models based on passive transfer of antibodies during gestation or active immunization of females before mating. Transfer of anti-Ro/La antibodies purified from two mothers of children with CHB into pregnant female BALB/c mice induced first-degree AV block in 47 to 90% of the offspring, depending on the day of gestation at which the injection was performed (Mazel et al., 1999). Sinus bradycardia was also observed, albeit in a somewhat smaller proportion of pups. While this study interestingly showed a time dependency of AV block development following passive transfer of maternal antibodies, which corresponds to the human situation where AV block is usually not detected later than the 24th week of pregnancy, no second- or third-degree AV block was observed and the exact specificity of AV-block inducing antibodies remained unclear.

Active models of heart block, where female rats or mice are immunized with a particular antigen before gestation, allows for a more controlled investigation of the ability of specific antibodies to induce AV block in the offspring (**Table 1**). Several studies have shown that immunization of mice and rabbits with the Ro52, Ro60 or La proteins leads to development of heart block in the offspring, however to somewhat low frequencies (Boutjdir et al., 1997; Miranda-Carus et al., 1998; Suzuki et al., 2005; Xiao et al., 2001b). Immunization with the Ro52β protein also induced AV block in mice, however it is difficult to assess the relevance of this finding as the endogenous Ro52β protein has never been detected in humans nor rodents, despite a report of Ro52β mRNA expression in the fetal human heart (Chan et al., 1995). It may be worth noting that Xiao et al reported a large number of neonatal deaths following immunization of female rabbits with Ro52, which might have been related to higher-degree blocks, however no histological evaluation of neonatal hearts was performed to support this hypothesis.

In an attempt to narrow down the specificity of the anti-Ro52 antibodies inducing heart block and/or identify a cross-reactive target, three different Ro52 peptides have been used in immunization models (**Table 1**). Salomonsson et al demonstrated that

first-degree AV block developed in 19% of pups born to rat females immunized with the Ro52 p200 peptide (aa 200-239), which had been shown to associate with a higher risk for CHB in humans (Salomonsson et al., 2005). The other two Ro52 peptides used (aa 365-382 and aa 366-379) were selected on the basis that antibodies to these peptides cross-reacted with the serotoninergic 5-hydroxytryptamine (5-HT_4) receptor (Eftekhari et al., 2000). However, while pups from mice immunized with 5-HT_4 peptides showed signs of bradycardia, AV block as well as skin rash and neuromotor problems, pups born to females immunized with the Ro52 peptides did not develop any cardiac abnormality (Eftekhari et al., 2001).

Antigen	Species (strain)	AVB (%)	AVB II/III (%)	Reference
Ro52	Mouse (BALB/c)	25	10	Boutjdir 1997
Ro52	Mouse (BALB/c)	9	3,6	Miranda-Carus 1998
Ro52	Rabbit (New Zealand)	9	0,7	Xiao 2001
Ro52β	Mouse (BALB/c)	12	6	Miranda-Carus 1998
Ro52 (mouse)	Mouse (BALB/c)	9	0	Miranda-Carus 1998
Ro52 (aa 365-382)	Mouse (BALB/c)	0	0	Eftekhari 2001
Ro52 (aa 366-379)	Mouse (BALB/c)	0	0	Eftekhari 2001
Ro52/p200 (aa 200-239)	Rat (DA)	19	0	Salomonsson 2005
Ro60	Mouse (BALB/c)	19	0	Miranda-Carus 1998
Ro60 *	Mouse (C3H/HEJ)	14	0	Suzuki 2005
La	Mouse (BALB/c)	7	0	Miranda-Carus 1998
La *	Mouse (C3H/HEJ)	7	0	Suzuki 2005

Table 1. A comparison of the success of immunization models of heart block. AVB was determined by ECG recorded on pups at birth. * The authors reported reactivity to Ro and La in animals immunized with La or Ro60, respectively.

Altogether, these data confirm the importance of the maternal anti-Ro/La, and especially anti-Ro52, antibodies in the pathogenesis of CHB, however the precise specificity of the heart-block inducing antibodies, as well as their target, remain a matter of debate. In addition, the immunization models used in the past 15 years have never yielded more than 25% first-degree AV block in pups exposed to maternal anti-Ro antibodies, and very few cases of second- or third-degree AV block have been observed, indicating that, as is the case in humans where the penetrance of the disease is relatively low in anti-Ro pregnancies (1-2%), other factors besides maternal antibodies are needed for the full establishment of CHB.

Targets of maternal antibodies in the fetal heart. Despite the well-established association of maternal anti-Ro antibodies with CHB, the pathogenesis of the disease remains unclear. The major problem in the elucidation of the molecular mechanisms involved, and one that has divided CHB researchers for years, is the fact that the major targets of maternal autoantibodies associated with fetal heart block (Ro52, Ro60 and La) are intracellular proteins and therefore not within the reach of the targeting antibodies. From there, two schools of thought, not mutually exclusive and each supported by experimental data, have emerged: the "apoptosis" and the "cross-reactivity" hypotheses.

According to the "apoptosis hypothesis", anti-Ro and anti- La antibodies gain access to their respective target when it is exposed on the surface of apoptotic cells. The presence of Ro60 and La has indeed been described on apoptotic cardiac myocytes (Miranda et al., 1998). Ro52 has also been detected on the surface of apoptotic but not live cardiac cells in one study, although only one out of the five anti-Ro52 monoclonal antibodies tested bound apoptotic cells, and to a lesser extent than did anti-Ro60 and anti-La antibodies (Clancy et al., 2006). The apoptosis hypothesis postulates that physiological apoptosis taking place during heart development leads to brief exposure of the Ro and La proteins on apoptotic cells, allowing antibodies to bind their cognate antigen. This may divert the removal of apoptotic debris from a normal non-inflammatory pathway towards their engulfment by macrophages through opsonization. Subsequent activation of the phagocytic cells will in turn lead to the production of pro-inflammatory and pro-fibrotic cytokines, recruitment of leukocytes and complement components and establishment of an inflammatory reaction that will eventually irreversibly damage the targeted tissue (Clancy et al., 2004; Miranda-Carus et al., 2000).

The "apoptosis hypothesis" does not however explain the electrophysiological effects of maternal anti-Ro/La autoantibodies on Langendorff-perfused hearts or calcium channel currents, or the specificity of the reaction in targeting the AV node, which remains the major and most common site of disturbances in CHB. In addition, transient first-degree AV block has been reported in as many as 30% of fetuses of mothers with anti-Ro52 antibodies (Sonesson et al., 2004), indicating that maternal autoantibodies may have a direct effect on the conduction system without inducing a self-amplifying cardiac inflammation that eventually leads to complete AV block, as is implied in the "apoptosis hypothesis". The "cross-reactivity hypothesis" therefore suggests that maternal anti-Ro/La antibodies bind to and interfere with the function of cardiac molecules involved in the control of the electric signal generation and conduction. Calcium channels have been the focus of several studies, given their central role in AV conduction but also in sinus node electrogenesis. Boutjdir and colleagues have shown that IgG purified from mothers of children with CHB inhibit L-type and T-

type calcium currents in ventricular myocytes as well as in sino-atrial node cells and exogenous expression systems (Boutjdir et al., 1998; Qu et al., 2005; Qu et al., 2001; Xiao et al., 2001a; Xiao et al., 2001b). The same research group also provided experimental data supporting a possible cross-reactivity of maternal anti-Ro/La antibodies with the α_{1C} and α_{1D} calcium channel subunits has been provided by the same group (Qu et al., 2005; Qu et al., 2001). Boutjdir et al additionally argue for a chronic effect of maternal autoantibodies on the fetal heart through binding of antibodies to calcium channels, subsequent internalization and degradation, which leads to inefficient signal conduction but also to insufficient excitation-contraction coupling and reduction of cardiac contractile function (Xiao et al., 2001b). Fetal cardiomyocytes indeed rely heavily on calcium channels for calcium delivery to the contractile proteins as the sarcoplasmic reticulum, which plays a major role in calcium storage and release in the adult heart, is sparse and less functional in the fetus. Cross-reactivity of maternal anti-Ro52 antibodies with the serotoninergic 5-HT_4 receptor has also been suggested to be involved in the development of AV block. Eftekhari et al showed that antibodies to the Ro52 peptide 365-382 recognized residues 165-185 of the cardiac 5-HT_4 receptor and that affinity-purified anti-5-HT_4 antibodies could antagonize the serotonin-induced calcium channel activation in atrial cells (Eftekhari et al., 2000). However mouse pups born to females immunized with Ro52 peptides that had been selected on the basis of recognition by anti-5-HT_4 antibodies did not develop any sign of AV block (Eftekhari et al., 2001). In a later study by the same authors, only 16% of the sera from mothers of children with CHB were shown to be positive for anti-5-HT_4 antibodies, indicating that cross-reactivity to the serotoninergic 5-HT_4 receptor, if indeed involved in the development of CHB, may only represent a small subset of cases (Kamel et al., 2005).

In support of the “cross-reactivity hypothesis”, monoclonal antibodies recognizing specifically the p200 part of the Ro52 protein (aa 200-239) have been shown to bind to the surface of rat neonatal cardiomyocytes in vitro and disturb calcium homeostasis, leading to apoptosis (Salomonsson et al., 2005), suggesting that maternal anti-Ro antibodies may indeed bind to another molecule than their cognate antigen on heart cells and induce apoptosis, which would then be accompanied by exposure of the intracellular Ro proteins and allow for the establishment and amplification of an inflammatory reaction as described in the “apoptosis hypothesis”.

Genetic factors in CHB. As mentioned before, reported recurrence rates of 12-20% despite persistence of maternal autoantibodies indicate that other factors, such as genetic susceptibility, are needed for the establishment of heart block. An association between the HLA haplotype B8/DR3 in women and the risk of giving birth to a child with heart block was described in the late 90’s (Siren et al., 1999b), however it is likely that

this observation only reflects the association of this particular haplotype with the presence of anti-Ro/La antibodies (Gottenberg et al., 2003). It has been suggested that the susceptibility of a child for heart block was related to a HLA haplotype different from the mother's (Siren et al., 1999a), however large studies allowing for the reliable identification of genetic associations have been lacking, mainly due to the rarity of the disease. Only recently the report of a genome-wide association study performed on individuals with CHB born to anti-Ro/La positive mothers demonstrated a significant association with polymorphisms in the HLA region and at the location 21q22 (Clancy et al., 2010). While these data need to be replicated in another cohort, one should be careful in their interpretation, especially regarding the observed HLA association. As the study was performed by comparing CHB cases to healthy controls from the general population, it is possible that the HLA association may only reflect the HLA bias present in the mothers, who may have SLE or SS or, even if asymptomatic, have autoantibodies to the Ro/La autoantigens.

In one study using a candidate gene approach, two known polymorphisms of the genes encoding the pro-inflammatory and pro-fibrotic cytokines TNFα and TGFβ were investigated. The TGFβ polymorphism assessed was found significantly more frequently in children with CHB while the TNFα polymorphism was found at an increased frequency in both children with CHB or skin rash, compared to healthy controls (Clancy et al., 2003). These findings have however not yet been replicated in a large group of CHB cases.

While the use of genome wide association studies may generate clues regarding the genetic susceptibility to heart block and the molecular mechanisms involved, the rarity of CHB will make it difficult to replicate any findings. In addition, studies of genetic influences in humans may not be powerful enough to identify rare variants associated with the condition, and animal models may therefore be useful to investigate potential genetic influences.

AIMS

The relevance of the Ro/SSA autoantigen and anti-Ro/SSA antibodies in rheumatology practice has been recognized since the late 60's, but it was not until the mid-90's that the autoantigen complex was broken down into its two components, Ro52 and Ro60. From then, studies on the individual Ro52 protein started to emerge, mostly focusing on its role as an autoantigen in SS and SLE. It was not until recent years that interest in the biological function of Ro52 arose, first driven by its identification as a TRIM protein with E3 ligase activity. As for anti-Ro52 antibodies, once they could be detected separately from anti-Ro60 antibodies, their specific association with congenital heart block was further delineated and experimental work started to provide clues as to their pathogenic role in CHB and the potential mechanisms involved.

The aims of this thesis were dual:

- The first part aimed at contributing to a better understanding of the biological function of TRIM21/Ro52 by investigating its expression and cellular localization, characterizing its E3 ligase activity and interaction partners, and most importantly evaluating its role in vivo by using a mouse strain with a disrupted TRIM21/Ro52 gene.
- The second part focused on characterizing the pathogenicity of anti-TRIM21/Ro52 antibodies in an animal model of CHB, but also aimed at identifying risk factors other than maternal autoantibodies for the development of heart block by using an animal model to dissect the genetic influence and a population-based study to uncover other maternal or fetal factors associated with CHB.

RESULTS AND DISCUSSION

Part I: The TRIM21/Ro52 E3 ligase

When the work included in this thesis began, little was known about the Ro52 protein besides its being an autoantigen frequently targeted in SS and SLE and a TRIM protein for which E3 ligase activity had just been demonstrated. In order to generate clues as to Ro52 biological function, we investigated its expression, localization and enzymatic activity as an E3 ligase. In addition, the study of a mouse strain in which the Trim21/Ro52 gene is disrupted by genetic targeting allowed us to advance further in the understanding of its function in vivo.

TRIM21/Ro52 is mainly expressed in immune cells and is induced by type I and type II IFNs *(Papers I and III)*

Analyzing the expression of a protein temporally, spatially and quantitatively can provide useful information as to its function. Ro52 expression was suggested to be ubiquitous from in situ hybridization experiments (Reymond et al., 2001), however this observation has not been corroborated by experimental data, especially at the protein level. We used Ro52-deficient mice, generated by the insertion of an internal ribosome entry site (IRES) -green fluorescent protein (GFP) reporter cassette into the Ro52 locus, to track the spatial expression of Ro52 in vivo in physiological conditions by detection of GFP (**Paper III**). Ro52 was found predominantly expressed in lymphoid organs, with little or no expression in non-immune tissues such as liver, heart, kidney, pancreas and skin. These findings are consistent with microarray analyses showing the expression of Ro52 mRNA mainly in immune cells in both mice and humans (Wu et al., 2009) (http://biogps.gnf.org). The highest expression of Ro52 was observed in $CD3^+$ and $CD11b^+GR\text{-}1^+$ cells. Some expression was also observed in endothelial cells, which may account for Ro52 being previously reported to be expressed in a large variety of tissues based on in situ hybridization experiments (Reymond et al 2001). Alternatively, it is possible that Ro52 is indeed expressed at low levels in all tissues and that its expression is further induced to detectable protein levels in certain conditions.

Many TRIM proteins are induced by IFNα, IFNγ, endotoxins or viruses. In particular, Ro52 induction at the mRNA level has been reported following IFNα, IFNγ, influenza virus A and LPS stimulation (Der et al., 1998; Geiss et al., 2002; Rhodes et al., 2002; Thomas et al., 2006; Zimmerer et al., 2007). To verify that Ro52 expression was indeed induced by IFNα and viruses at both mRNA and protein levels, cell lines and human

peripheral blood mononuclear cells (PBMCs) were stimulated in vitro with IFNα or inactivated herpes simplex virus, and Ro52 expression was detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and by western-blot. Ro52 mRNA expression was induced by IFNα in both human epithelial cell (HeLa) and B cell (Raji, Daudi) lines, as well as in human PBMCs, and peaked at 3-6h, while upregulation of the Ro52 protein was observed after 24h of IFNα stimulation by immunoblotting and immunofluorescence using anti-Ro52 monoclonal antibodies (**Fig 5A and 5B**, **Paper I**). Ro52 mRNA levels were also increased following exposure of PBMCs to inactivated herpes simplex virus (**Paper I**). Using GFP as a reporter, we also found that Ro52 expression was induced in murine bone marrow cells after 24h exposure to IFNγ and, to a lesser extent, IFNα and IFNβ, whereas exposure to TGFβ or TNFα had no effect (**Fig 5C**, **Paper III**).

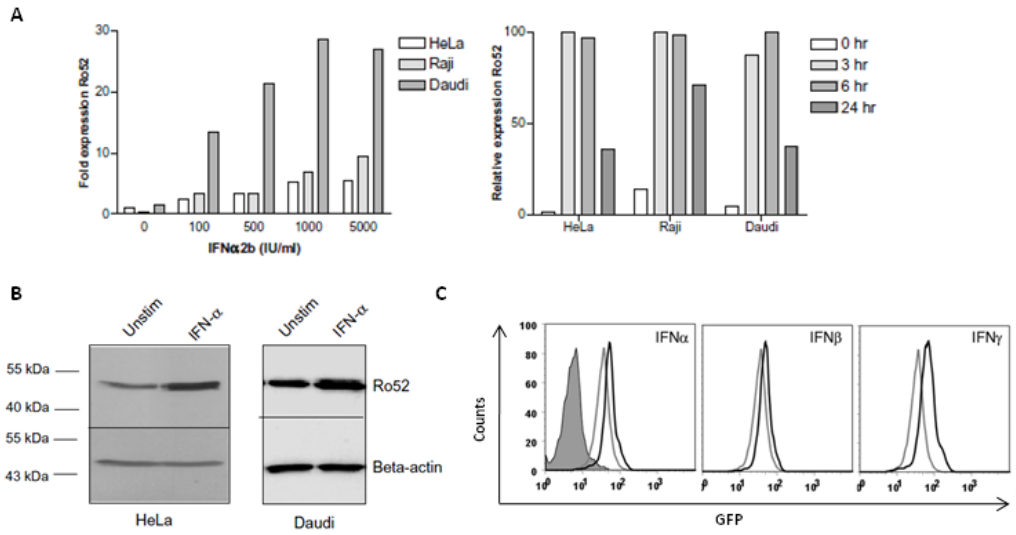


Figure 5. Ro52 expression is increased after IFN stimulation. (A) Exposure of cell lines to IFNα2b at different concentrations increased Ro52 expression from 5- to 30-fold in HeLa and Daudi cells (*left*) and the expression of Ro52 peaked at 3-6h after exposure to IFNα2b at 1000 IU/ml (*right*). **(B)** The increased expression of Ro52 at the protein level following IFNα exposure was confirmed in HeLa and Daudi cells by immunoblotting. **(C)** Both type I and type II IFN induced expression of Ro52 as evaluated by GFP expression in bone marrow cells from Ro52$^{+/-(IRES-GFP)}$ mice cultured for 24h with medium (gray line) or medium + IFN (black line). Filled histogram represents cells from Ro52$^{+/+}$ mice.

The relatively rapid induction of Ro52 mRNA upon IFNα stimulation suggests a direct effect on Ro52 promoter downstream of IFN signaling. Although putative interferon-stimulated response elements (ISRE) can be identified in the promoter region of the

Ro52 gene by bioinformatics approaches, the existence of such an ISRE site has not yet been investigated experimentally. It is also possible that gamma activation sites (GAS) are present in the Ro52 promoter, accounting for the increased transcriptional activity upon type II IFN stimulation, although this effect may also depend on binding of IRF1 to the ISRE site, as IRF1 is activated by STAT1 downstream of IFNγ signaling.

TRIM21/Ro52 is primarily located in the cytoplasm and translocates to the nucleus upon IFNα stimulation *(Paper I)*

The cellular localization of Ro52 has been the subject of several studies, but most of them focused on the Ro/SSA autoantigen complex and reported its expression on apoptotic blebs of keratinocytes and ductal epithelial cells, where it would be accessible to circulating autoantibodies (Lawley et al., 2000; Ohlsson et al., 2002; Saegusa et al., 2002). Our aim was to investigate the intracellular localization of the Ro52 protein in intact cells, and more particularly its distribution between the nuclear and cytoplasmic compartments, as such previous studies have led to conflicting data (Espinosa et al., 2006; Kong et al., 2007; Ohlsson et al., 2002; Pourmand et al., 1998). Using anti-Ro52 monoclonal antibodies targeting different parts of the protein, we observed Ro52 expression at low levels predominantly in the cytoplasm of HeLa cells, with a slight speckled nuclear staining. These data are consistent with the results obtained with recombinant Ro52 fused to GFP and expressed in HeLa cells (Espinosa et al., 2006). As already mentioned, Ro52 expression was considerably increased after 24h exposure to IFNα, but was still mainly cytoplasmic, whereas by 48h Ro52 had translocated to the nucleus in most cells. It is worth noting that nuclear Ro52 was detected with antibodies targeting either the C-terminal or the coiled-coil domain of the protein, indicating that Ro52 moved to the nucleus without undergoing extensive modification or deletion of any of these domains.

In a more recent study using Ro52 deletion constructs fused to GFP, Espinosa et al showed that, while full-length Ro52 was predominantly located in the cytoplasm, deletion of aa 203-248 (part of the coiled-coil domain) led to accumulation of the protein in the nucleus (Espinosa et al., 2008). Interestingly, additional deletion of the C-terminal residues 381-470 abrogated the translocation of the construct in the nucleus, indicating that an intact C-terminal domain is necessary for Ro52 entrance into the nucleus. As a TRIM protein, Ro52 possesses a highly conserved N-terminal RING domain mediating E3 ligase activity, and it is thought that the less conserved C-terminal domains of the TRIM proteins confer specificity to the ubiquitination process by allowing the interaction with specific substrates. It is therefore tempting to speculate that the C-terminal part of Ro52 is required for its interaction with its substrate and subsequent translocation to and/or accumulation in the nucleus.

We have shown that the nuclear localization of Ro52 occurs following exposure to IFNα, and a similar observation has been made when cells are exposed to nitric oxide (NO) (Espinosa et al., 2008). Both IFNα and NO are inflammatory mediators, suggesting that Ro52 may translocate to the nucleus in inflammatory conditions to exert its function by interacting with a nuclear substrate, possibly a transcription factor. Ro52 has indeed been detected at high levels in the nucleus of inflamed skin cells from patients with cutaneous lupus erythematosus (Oke et al., 2009), providing physiological relevance to our in vitro findings. IFNα and NO both induce cell cycle arrest, and it is therefore also possible that Ro52 is involved in the regulation of cell proliferation after its translocation to the nucleus.

TRIM21/Ro52 is an E3 ligase (P*aper II)*

When the work on paper II started, Ro52 had just been described as an E3 ligase by two independent groups (Espinosa et al., 2006; Wada and Kamitani, 2006a) and our aim was to further characterize Ro52 activity in the ubiquitination process. As the identity of its substrate remained unknown, an autoubiquitination assay was used. Autoubiquitination is a process in which E3 ligases modify themselves with polyubiquitin chains in the absence of a substrate. Even though it may only be an experimental artifact of no physiological relevance, it is nevertheless useful to demonstrate the presence of an E3 ligase enzymatic activity.

Using an in vitro ubiquitination assay (Lorick et al., 1999), where all three enzymes required for the ubiquitination process (**Fig 1**) are mixed in the presence of ATP, we showed that Ro52 E3 ligase activity was dependent on the presence of an intact RING domain, as either deletion of the RING or mutation of cysteine residues critical for RING folding abolished autoubiquitination of Ro52 (**Fig 6A**).

Using ubiquitin mutants where all lysine residues are mutated except K48 or K63, we also demonstrated that Ro52 was able to build both K48 and K63 chains (**Fig 6B**). Recent studies have reported Ro52-mediated ubiquitination of IRFs, however whether it leads to their degradation or to an alteration of their function remains unclear, as one group reported Ro52-mediated ubiquitination and subsequent degradation of IRF3 and IRF7 (Higgs et al., 2010; Higgs et al., 2008) and another described Ro52-mediated ubiquitination and stabilization of IRF8 (Kong et al., 2007) while also reporting an interaction of Ro52 with the adaptor protein p62 and IRF8 that led to degradation of IRF8 in the proteasome (Kim and Ozato, 2009). Although this may seem confusing, it is possible that Ro52 may catalyze different types of polyubiquitination depending on the substrate, the cell type, the presence of an interacting partner and/or the environment, e.g. in inflammatory or physiological conditions.

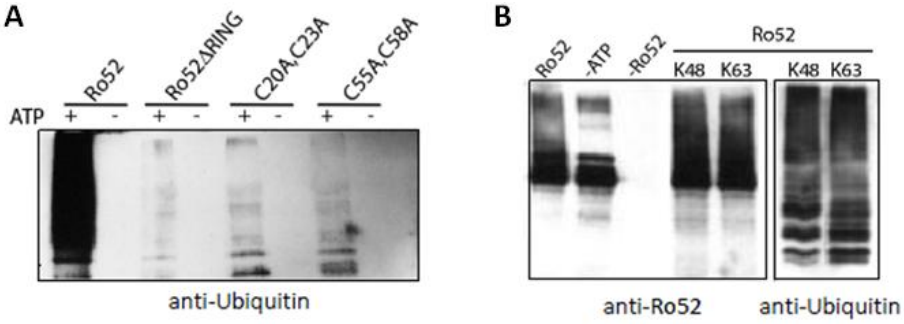


Figure 6. Ro52 is a RING-dependent E3 ligase that can mediate formation of K48 and K63 polyubiquitin chains. (A) Deletion of the RING domain or mutations in cysteine residues critical for RING folding abrogate Ro52 autoubiquitination in an in vitro assay. **(B)** Use of ubiquitin mutants in which all lysine residues are mutated except K48 or K63 shows that Ro52 is able to build both K48 and K63 polyubiquitin chains.

An E2 screen performed using the in vitro autoubiquitination assay revealed that Ro52 E3 ligase activity was supported by both class I (UBE2D1-4) and class III (UBE2E1-2) E2 enzymes. Of particular interest, class I E2s are mainly cytoplasmic while class III E2s charged with ubiquitin are found in the nucleus (Espinosa et al., 2008; Plafker et al., 2004), indicating that Ro52 E3 ligase activity is supported in both cellular compartments. Considering our previous observation that Ro52 is localized in the cell cytoplasm under physiological conditions but translocates to the nucleus in an inflammatory milieu, it is possible that Ro52 exerts different functions in normal versus inflammatory conditions by binding to and ubiquitinating different substrates in each cellular compartment.

Anti-RING autoantibodies inhibit TRIM21/Ro52 E3 ligase activity *(Paper II)*

Anti-Ro52 antibodies are frequently found in the serum of patients with SS or SLE. Although dominant epitopes have been described in the central coiled-coil domain (Blange et al., 1994; Kato et al., 1995), antibodies to the RING domain can also be detected (Pourmand and Pettersson, 1998). In order to investigate whether antibodies from patients could inhibit Ro52 E3 ligase activity we screened patients' sera for reactivity to the Zn^{2+}-binding region (comprising both the RING and the B-box domains) and used a RING-positive serum in an in vitro autoubiquitination assay. Antibodies to the Zn^{2+}-binding region were found in 57% and 55% of anti-Ro52 positive patients with SS and SLE, respectively, with most of the reactivity generated by antibodies binding the RING domain. Addition of serum or Ig fraction containing anti-RING antibodies inhibited

Ro52 autoubiquitination in vitro, while serum containing antibodies only to the coiled-coil domain and a Ro52-specific monoclonal antibody targeting the RING/B-box linker region (RBL) did not.

Although there is no doubt that anti-RING antibodies can inhibit Ro52 autoubiquitination in vitro, the relevance of this finding in vivo is unknown. Several research groups have shown that antibodies can enter living cells and even bind their target and inhibit enzymatic activity (Alarcon-Segovia et al., 1978; Koren et al., 1995; Koscec et al., 1997; Reichlin et al., 1994; Zack et al., 1996), however these studies were based on in vitro systems and the penetration of cells by antibodies remains therefore controversial, as it may only represent an in vitro artifact. The transport of antibodies across biological cell membranes via neonatal fragment crystallizable receptor (FcRn) is well known (Dickinson et al., 1999; Rodewald, 1973; Spiekermann et al., 2002). After internalization and binding to FcRn, IgG is recycled to the cell membrane instead of being directed to the lysosomal compartment for degradation (Lencer and Blumberg, 2005). However it is unknown whether such a mechanism can also lead to the release of IgG into the cell cytoplasm. It is possible that the high concentration of antibodies in the in vitro system may saturate the process of degradation and/or membrane recycling and cause the IgG to be released in the cytoplasm. This hypothesis is of particular interest in the context of SS, since patients often have hypergammaglobulinemia and high autoantibody titers (Hansen et al., 2005). It would be interesting to try and evaluate the capacity of antibodies to penetrate living cells in vivo by using transgenic mice expressing a tagged IgG (e.g. fused to GFP), allowing the tracking of the antibodies without artifacts arising from in vitro manipulation.

Despite the uncertainty of its physiological relevance, the inhibition of Ro52 E3 ligase activity by anti-RING antibodies proved a useful tool in further characterizing the interaction between Ro52 and its E2 partner. Nuclear magnetic resonance and limited proteolysis techniques were employed to map the Ro52 regions involved in the E2:E3 interaction and antibody binding. UBE2E1 was shown to interact with the Ro52 RING-RBL domain in four locations, the L1 and L2 loops, residues 39-42 which are part of a helix structure, and residues 74-78 located in the RBL region. Interestingly, anti-RING antibodies binding to the same Ro52 construct involved amino acids residues located in the L1 and L2 loops, indicating that these antibodies may inhibit Ro52 E3 ligase activity by sterically blocking its interaction with E2. Antibody binding, although preventing Ro52:E2 interaction, did not significantly alter the helix region comprising residues 39-42, suggesting that these residues may not be directly involved in E2 binding but, as part of a structural scaffold on which the L1 and L2 loops are anchored, may still undergo slight changes upon E2:E3 complex formation.

TRIM21/Ro52 ubiquitinates IRFs and negatively regulates the production of pro-inflammatory cytokines *(Paper III and IV)*

To study the biological function of Ro52 in vivo we generated Ro52-deficient mice by inserting an IRES-GFP cassette into the Ro52 locus. Initial observations of the expression of Ro52 predominantly in immune cells as well as the fact that Ro52 expression could be induced by IFNs led to the hypothesis that Ro52 may have a role in the immune system. Ro52$^{-/-}$ mice however developed and aged normally and no abnormality was detected either in the structure of lymphoid organs or in the different immune cell populations, suggesting that Ro52 did not play an important role, if any, in the development of the immune system or in the maintenance of immune homeostasis in naïve mice.

Mice are routinely ear-tagged with metal clips for identification purposes and, while this technique does not induce more than transient swelling and erythema in wild-type mice, we observed that Ro52$^{-/-}$ mice developed severe and progressive dermatitis, originating from the tagged ear. Analysis of the inflamed tissue showed epidermal hyperplasia and inflammatory infiltrates consisting primarily of neutrophils. Interestingly, mice that had developed dermatitis showed signs of systemic autoimmunity, with hypergammaglobulinemia, presence of anti-nuclear antibodies and kidney pathology. Analysis of cells from spleen and lymph nodes of sick Ro52$^{-/-}$ mice revealed hyperproliferation as well as production of pro-inflammatory cytokines such as IL-6 and IL-12/IL-23p40 and Th17 cytokines (IL-17, IL-21 and IL-22), **Fig 7A**. These features were recapitulated in a model of contact hypersensitivity, with Ro52$^{-/-}$ mice showing increased tissue inflammation and production of pro-inflammatory cytokines as compared to Ro52$^{+/+}$ littermates.

Since ear-tagged Ro52$^{-/-}$ mice showed uncontrolled tissue inflammation that led to a systemically increased immune response with high levels of inflammatory cytokines while un-manipulated untagged Ro52$^{-/-}$ mice aged normally, we hypothesized that Ro52 function may be in the regulation of immune responses. We therefore stimulated splenocytes from naïve mice with TLR agonists and observed that Ro52$^{-/-}$ cells indeed produced higher amounts of IL-6, IL-12/IL-23p40, TNF and type I IFN than Ro52$^{+/+}$ cells (**Fig 7B**). In addition, the expression of IL-23/p19 was greater in Ro52$^{-/-}$ than in Ro52$^{+/+}$ bone marrow-derived macrophages following exposure to CpG/IFNγ (**Fig 7C)**. TLR stimulation leads to the activation of two main families of transcription factors regulating cytokine production, NF-κB and IRFs. Recent reports had described Ro52-mediated ubiquitination of IRF8 and IRF3, and we confirmed these data using an overexpression system-based ubiquitination assay. Transfection of 293T cells with Ro52, ubiquitin and different IRFs showed that Ro52 could ubiquitinate not only IRF3 and IRF8, but also IRF5 (**Fig 7D**), which are transcription factors involved in the induction of

IL-6, IL-12/23p40, TNF and type I IFN (Giese et al., 1997; Sakaguchi et al., 2003; Sato et al., 2000; Takaoka et al., 2005). Using a luciferase reporter assay, we additionally showed that Ro52 negatively regulated the transcriptional activity of IRF3 and IRF5 downstream of TLR signaling.

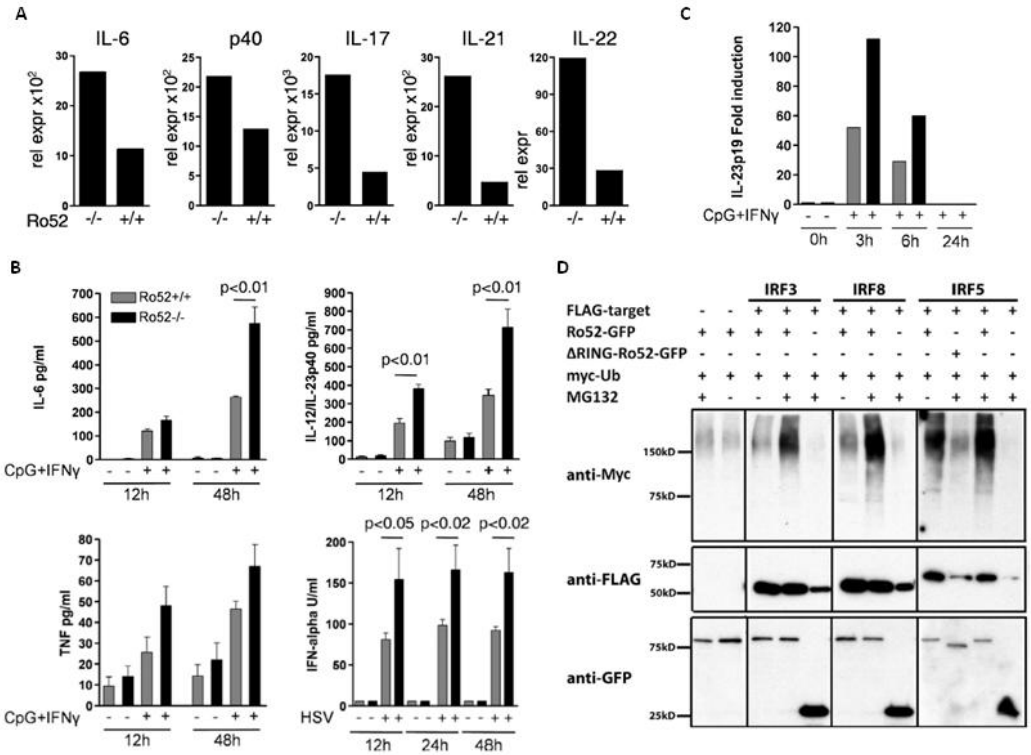


Figure 7. Ro52 downregulates the production of pro-inflammatory cytokines following TLR stimulation via ubiquitination of IRFs. (A) Cytokine expression in lymph node cells from Ro52$^{-/-}$ mice with dermatitis and Ro52$^{+/+}$ mice. **(B)** Cytokine levels in supernatants of Ro52$^{+/+}$ and Ro52$^{-/-}$ splenocytes stimulated with CpG/IFNγ or inactivated herpes simplex virus (HSV). **(C)** IL-23p19 expression in Ro52$^{+/+}$ (grey bars) and Ro52$^{-/-}$ (black bars) bone marrow-derived macrophages upon stimulation with CpG/IFNγ. **(D)** Ro52-mediated polyubiquitination of IRF3/5/8 in transfected 293T cells.

Altogether our data show that Ro52 is an E3 ligase expressed mainly in immune cells and induced by IFNs, and that Ro52 ubiquitinates several IRFs and downregulates the expression of pro-inflammatory cytokines, thereby providing a negative feedback for IFN-mediated immune responses (**Paper III**). Similar findings were reported by Yoshimi and colleagues who investigated Ro52 function in another Ro52-deficient strain (Yoshimi et al., 2009). While they show that Ro52 expression is induced by IFNs, they

also point out that neither IL-1β nor TNF, two cytokines activating NF-κB pathways, induce Ro52. As mentioned earlier, a putative ISRE site is present in the Ro52 promoter region, and it is possible that Ro52 expression itself is regulated by one or several IRFs. Interestingly, while Yoshimi and colleagues also observed an increased production of pro-inflammatory cytokines by Ro52$^{-/-}$ embryonic fibroblasts (EFs) compared to Ro52$^{+/+}$ EFs, they focused on the NF-κB pathway, showing by a luciferase reporter assay that absence of Ro52 in transfected EFs led to increased NF-κB transcriptional activity. Before both studies on Ro52-deficient mice were published, Ro52 had been described to ubiquitinate IRF3 and IRF8, in one case leading to degradation of IRF3 and subsequent downregulation of IFNβ production (Higgs et al., 2008), and in the other leading to stabilization of IRF8 and enhanced IL-12p40 cytokine production in IFNγ/TLR-stimulated macrophages (Kong et al., 2007). Since then, we have added IRF5 to the list of IRFs ubiquitinated by Ro52 and a recent report shows Ro52-mediated ubiquitination of IRF7, leading to its degradation and decreased IFNα production (Higgs et al., 2010). Altogether, these data tend to support a role for Ro52 as a negative regulator of IFN responses via ubiquitination of IRFs, however it is fully possible that Ro52 function may differ depending on the degree of a cell's activation, the cell type and the activating stimulus.

TRIM proteins are thought to be involved in innate immune defense mechanisms, and several TRIMs have been shown to be restriction factors for viruses (Barr et al., 2008; Gack et al., 2007; Stremlau et al., 2004; Wolf and Goff, 2007) or to be involved in the regulation of IFN responses and PRR signaling pathways (Gack et al., 2007; Shi et al., 2008; Zha et al., 2006). As many TRIMs, Ro52 is upregulated by IFNs and it is tempting to speculate on a role for Ro52 in innate immunity. It may be worth mentioning that cultures from lesions of mice that had developed dermatitis following ear tagging revealed the presence of *Staphylococcus aureus*, raising the possibility of a persistent local infection in these mice. One may further speculate that, if Ro52 is indeed important in the generation of functional innate immune responses, Ro52-deficient mice may be unable to clear a bacterial infection, leading to constant production of pro-inflammatory cytokines, including IL-17, which may in turn feed the development of systemic autoimmunity. Indeed, IL-17 is known to play an important role in tissue inflammation and elevated levels of IL-17 have been detected in several autoimmune diseases (Aarvak et al., 1999; Matusevicius et al., 1999; Teunissen et al., 1998), while IL-17 deficient animals develop attenuated collagen-induced arthritis and experimental autoimmune encephalomyelitis (Ishigame et al., 2009; Nakae et al., 2003). In addition, IL-17 has been suggested to drive autoimmune responses and production of autoantibodies by promoting the formation of spontaneous germinal centers (Hsu et al., 2008).

The idea that TRIM proteins have evolved as part of an innate immune defense mechanism and that members of this family may have redundant functions is interesting when comparing the phenotypes of the two Ro52-deficient mice reported by Espinosa and Yoshimi. While our Ro52-deficient mice developed a severe dermatitis and systemic autoimmunity upon triggering (tissue injury following ear-tagging and/or concomitant bacterial infection), Yoshimi et al did not observe such a severe phenotype. As mentioned before, both groups reported that Ro52 was mainly expressed in immune cells and that Ro52$^{-/-}$ cells showed an enhanced production of pro-inflammatory cytokines upon TLR stimulation. However, Yoshimi and colleagues reported an increased expression of other TRIM genes in the absence of Ro52 (*Trim12*, *Trim25*, *Trim30* and *Trim34*) (Yoshimi et al., 2009), which we did not see, suggesting that the stronger phenotype of our Ro52-deficient mice may be due to lack of compensatory mechanisms present in the other Ro52-deficient strain. Interestingly, we detected an increased expression of the remaining 5' exons from the targeted *Trim21* allele, indicating that lack of a functional TRIM21/Ro52 protein leads to increased expression of *Trim21* (**Paper IV**). The difference in the targeting strategies may therefore account for the difference in phenotypes, as the complete absence of TRIM21/Ro52 may lead to compensatory expression of other TRIMs. Additionally, the remaining 5' exons of the TRIM21/Ro52 targeted locus of our Ro52-deficient mice may lead to the production of a truncated protein that might act as a dominant negative mutant, leading to a stronger phenotype than complete deletion of the whole protein.

Summary Part I

Taken together, these studies show that Ro52 is an IFN-inducible protein expressed in immune cells, and is localized in the cell cytoplasm but translocates to the nucleus upon inflammatory stimuli such as IFNα. We also demonstrate that Ro52 is a TRIM protein with E3 ligase activity that can interact with different E2s both in the cytoplasm and the nucleus, and that its E3 enzymatic activity is inhibited by anti-RING antibodies. Finally, our data show that Ro52 can ubiquitinate several IRF transcription factors and that disruption of the *Trim21* locus in vivo leads to increased production of pro-inflammatory cytokines and development of systemic autoimmunity.

Part II: Anti-TRIM21/Ro52 antibodies and congenital heart block

Anti-Ro52 antibodies are frequently found in rheumatic patients with SS or SLE but they may also be present in asymptomatic individuals. In fact, many women giving birth to a child with CHB are only tested positive for the presence of anti-Ro52 antibodies after the child's diagnosis is made, and may or may not develop a rheumatic disease later in life. In contrast to SS and SLE, where the involvement of anti-Ro52 antibodies in the disease pathogenesis is unclear, CHB is described as an acquired autoimmune condition where the maternal antibodies directly affect the fetal heart. The molecular mechanisms underlying the initiation and establishment of CHB however remain unknown, and the relatively low incidence of CHB in anti-Ro positive pregnancies (1-2%) as well as a recurrence rate of 12-20% despite persisting maternal antibodies raise the question of what other factors influence the development of CHB. To address these issues, we investigated the specificity of anti-Ro52 antibodies inducing heart block as well as the genetic contribution to the establishment of CHB using animal models, and we assessed the influence of factors such as maternal age, fetal gender and season of birth on the development of CHB in a population-based study.

Anti-Ro52 antibodies specific for amino acids 200-239, but not antibodies specific for other parts of the protein, induce heart block in vivo *(Paper V)*

Immunization of mice and rats with the Ro52 protein or the p200 peptide (aa 200-239) leads to AV block in the offspring (**Table 1**), however whether only p200-specific antibodies carry the pathogenic potential of the maternal anti-Ro52 antibody pool or whether antibodies targeting other epitopes of the Ro52 protein also induce heart block remains to be determined. To address this question, anti-Ro52 monoclonal antibodies targeting different parts of the protein were generated and transferred into rats during gestation. Development of AV block was assessed in the offspring by recording ECGs on newborn pups and measuring the PR interval, which reflects the efficiency of signal conduction at the AV node. First-degree AV block was only observed in pups exposed to anti-Ro52 p200 antibodies during gestation (**Fig 8A**). Pups born to females that had received antibodies targeting the N-terminal or C-terminal domains of Ro52 had PR intervals comparable to those of pups born to females receiving only vehicle. These data suggest that, while non-p200 anti-Ro52 antibodies may participate to the inflammatory reaction in the fetal heart, anti-p200 antibodies are the main initiators of the disease. This idea is supported by the fact that, when added to cultures of rat neonatal cardiomyocytes, only p200-specific antibodies induced deregulation of calcium

oscillations and progressive intracellular calcium accumulation, indicating a direct effect on cardiomyocyte function and/or survival as calcium overload eventually leads to cell death.

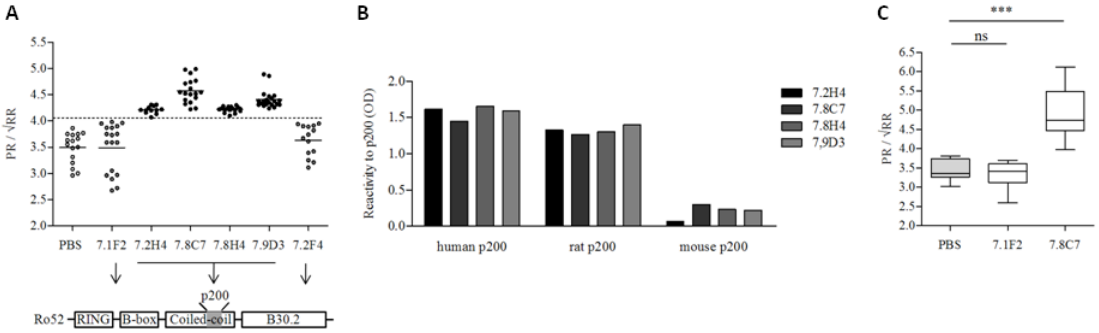


Figure 8. Anti-Ro52 p200 antibodies induce first-degree AV block in rodents when transferred during gestation. (A) Pups exposed to monoclonal anti-Ro52 antibodies specific for p200 (aa 200-239) develop AV block while pups exposed to antibodies recognizing other domains of Ro52 do not. Hatched line represents the threshold for first-degree AV block (defined as PR≥ (mean PR)$_{control}$ + 2SD). **(B)** Comparison of the reactivity of anti-p200 monoclonal antibodies (raised against human Ro52) to human, rat and mouse p200 peptides in ELISA. **(C)** Anti-Ro52 p200 antibodies induce AV block in mice despite low reactivity to mouse p200.

The monoclonal antibodies used in our transfer model were generated against the human Ro52 protein (**Paper I**), and despite high homology between human, rat and mouse p200 peptides, anti-Ro52 p200 antibodies displayed a much lower reactivity to the mouse peptide in ELISA as compared to the human and rat peptides (**Fig 8B**). This prompted us to investigate whether the p200-specific antibodies that induced heart block in rats would also do so in mice. We observed that first-degree AV block developed in virtually all mouse pups exposed to anti-p200 antibodies, despite the poor reactivity of the antibodies towards the mouse Ro52 p200 peptide, indicating the strong pathogenic potential of these antibodies (**Fig 8C**). While it is possible that a higher amount of antibodies in the mouse fetal circulation compensated for their lower affinity to the mouse peptide, one may also hypothesize that the anti-Ro52 p200 antibodies cross-react with another molecule in the fetal heart that displays a higher homology between the rat and mouse species.

Cross-reactivity of heart block-inducing anti-Ro52 antibodies with another cardiac protein is an attractive hypothesis considering that the effects of anti-Ro52 antibodies on intracellular calcium concentration and calcium channels currents reported by us

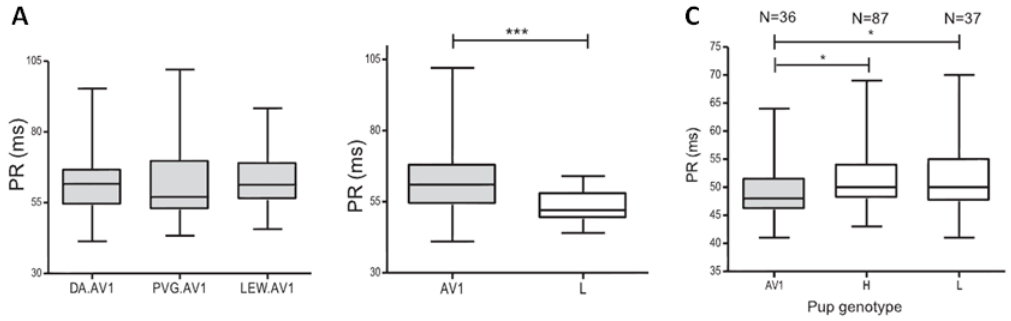
and others (**Paper V**)(Boutjdir et al., 1998; Qu et al., 2005; Qu et al., 2001; Salomonsson et al., 2005; Xiao et al., 2001a; Xiao et al., 2001b) cannot be explained to date by the sole binding of the antibodies to Ro52. In addition, Ro52 is an intracellular protein, and while the presence of Ro60 and La has been described on apoptotic cardiac myocytes (Miranda et al., 1998), surface expression of Ro52 remains unclear. Clancy and colleagues reported the presence of Ro52 on the surface of apoptotic but not live cardiac cells in one study, however only one out of the five anti-Ro52 monoclonal antibodies tested bound apoptotic cells, and to a lesser extent than did anti-Ro60 and anti-La antibodies (Clancy et al., 2006). Our data suggest that the effects of anti-Ro52 antibodies on calcium oscillations of cardiomyocytes may rather stem from cross-reactivity to another molecule on live cells, and that antibody binding to this membrane protein may, by interfering with its function, eventually induce cell death.

Despite a 90-100% incidence of first-degree AV block in our transfer model, we were unsuccessful at inducing complete heart block. These results are consistent with previous attempts at inducing heart block in animals, using either immunization of transfer models, where second- or third-degree AV blocks have only been marginally reported (**Table 1**). It is possible that advanced AV blocks do occur during gestation and lead to fetal death, with physiological resorption of fetal tissue preventing detection of these cases at birth (Suzuki et al., 2005). In addition, we may miss stillborn pups because the maternal mouse frequently consumes the stillborn offspring immediately after birth. Alternatively, one may speculate that, similar to the situation in humans, other factors besides the presence of maternal antibodies determine the fetal outcome. In particular, it is highly possible that different genetic backgrounds will influence the susceptibility to CHB as it does to other animal models of autoimmune diseases (Weissert et al., 1998; Vingsbo-Lundberg et al., 1998). Considering that attempts at inducing AV block have been made mainly in only three different mouse strains and one rat strain (**Table 1**), one may speculate that higher-degree AV block may indeed be possible to achieve, if only finding the "right" susceptible genetic background.

Maternal MHC regulates the generation of pathogenic anti-Ro52 antibodies while fetal MHC modulates susceptibility in an animal model of CHB *(Paper VI)*

To investigate the influence of MHC and non-MHC genes on the development of CHB, we used an immunization-based animal model of heart block, where female rats are immunized with the Ro52 protein, mated, and incidence of heart block in the offspring is assessed by ECG at birth. In a preliminary experiment, we compared the development of heart block in four different rat strains (DA, PVG, LEW and LEW.AV1). Two of them

shared the same non-MHC genes while differing in their MHC haplotype (LEW, which has the $RT1^{l}$ haplotype, and the congenic strain LEW.AV1, generated by introgressing the DA $RT1^{av1}$ haplotype into the LEW strain). Three of them shared the same MHC haplotype $RT1^{av1}$ while differing in their non-MHC genes (DA, PVG and LEW.AV1). We observed that first-degree AV block developed in about 45% of DA, PVG and LEW.AV1 rats but in only 10% of LEW pups, indicating that non-MHC genes did not significantly change the susceptibility to heart block in the three strains investigated while MHC genes did (**Fig 9A and 9B**).



B

Strain	DA.AV1	PVG.AV1	LEW.AV1	LEW.L
First-degree AV block (%)	45	44	47	10

Figure 9. Maternal and fetal MHC genes influence development of CHB in rats. Female rats were immunized with Ro52, mated and presence of AV block in pups was assessed by measuring the PR interval on ECG at birth. **(A)** There was no significant difference between the PR intervals of pups from three strains with the same MHC haplotype (AV1) but different non-MHC genes (DA, PVG, LEW), *left*, whereas pups from strains with an AV1 MHC haplotype (DA, PVG, LEW.AV1) had significantly longer PR intervals than pups from a strain with the L haplotype (LEW), *right*. **(B)** Percentage of first-degree AV block in the four strains investigated. **(C)** An F2 cross between LEW.AV1 and LEW.L revealed that pups with the MHC L haplotype and heterozygous (H) AV1/L pups had longer PR intervals than pups with the AV1 haplotype, indicating an influence of fetal MHC in disease susceptibility (all pups were born to heterozygous AV1/L F1 females).

The lower frequency of heart block in the LEW pups compared to the LEW.AV1 animals may be due to either a lack of generation of pathogenic anti-Ro52 antibody specificities in the $RT1^{l}$ mothers and/or fetal resistance to disease in $RT1^{l}$ pups. To distinguish between a maternal or fetal effect of MHC, we performed an F2 cross between the LEW and LEW.AV1 strains. The F1 females (all $RT1^{av1/l}$) were immunized

with Ro52 and then mated with F1 males ($RT1^{av1/l}$) to generate pups with either the homozygous genotypes $RT1^{av1/av1}$ or $RT1^{l/l}$ or the heterozygous genotype $RT1^{av1/l}$. To our surprise, analysis of the PR interval in these pups revealed that pups of the $RT1^{av1/l}$ or $RT1^{l/l}$ genotypes had significantly longer PR interval than homozygous $RT1^{av1/av1}$ pups (**Fig 9C**). From this follows that the fetal MHC $RT1^{l}$ haplotype confers a higher susceptibility to heart block and that the results from the first study on DA, PVG, LEW and LEW.AV1 were due to a lack of generation of pathogenic anti-Ro52 antibodies in $RT1^{l}$ mothers and not to a fetal resistance to disease of $RT1^{l}$ pups.

Since the presence of antibodies to the p200 peptide of Ro52 is important in the induction of CHB and that the fine specificity of these antibodies differed between $RT1^{l}$ and $RT1^{av1}$ animals, we investigated the mechanisms underlying the generation of different antibody specificities. We identified the rat MHC class II molecule RT1.B as the main contributor in the generation of p200-specific T cell lines and compared the RT1.B α and β chains for both AV1 and L. This analysis revealed important structural and electrostatic differences in the peptide-binding clefts of the RT1.B AV1 and L molecules, suggesting that different peptides will be preferentially presented by these two MHC class II molecules. TCRBV spectratyping of the AV1 and L p200-specific T cell lines showed different TCRBV usage, indicating that they probably recognize slightly different epitopes within the p200 peptide, which will then be reflected in the antibody response generated.

Altogether our data show that allelic variation within the MHC locus determines both the maternal ability to generate pathogenic antibodies and the fetal susceptibility to the effect of these antibodies, and that these traits are linked to different haplotypes in the mother and the fetus. This is similar to the human situation where the MHC haplotype associated with maternal production of anti-Ro52 antibodies (B8, DR3) is rarely found in children with heart block (Siren et al., 1999a; Siren et al., 1999b). While we have shown that allelic differences in MHC class II molecules in the mother affect the repertoire of peptides presented and the specificity of antibodies generated, both classical and non-classical MHC genes may affect the fetal susceptibility to disease. Among the non-classical set, genes encoding complement components and TNFα are attractive candidates as both complement and TNF are implicated in the inflammatory reaction taking place in the fetal heart, as observed by histological and in vitro analyses (Clancy et al., 2004; Lee et al., 1987; Miranda-Carus et al., 2000).

Maternal age and season of birth influence the development of heart block in anti-Ro/La positive pregnancies *(Paper VII)*

The occurrence of congenital heart block in the general population is 1 in 15,000-20,000 pregnancies (Michaelsson and Engle, 1972), which makes it particularly difficult to investigate potential risk factors associated with the disease. In an effort to address this issue, we identified patients diagnosed with heart block before 15 years of age in the Swedish population through the use of national and local patient registers and through a network of pediatric and adult cardiologists and rheumatologists at the six university hospitals in Sweden. Patients with cardiac structural abnormalities as well as patients with post-operative or infection-induced block were excluded, and families for whom a) a blood sample from the mother and b) information on date of birth for the mother, the patient with heart block and all siblings were available were included in our study.

Out of a total of 145 families, we found that in 80 (55%) the mother carried antibodies to Ro and/or La, while in the other 65 the mother was seronegative for these antibodies. These proportions are similar to what Villain et al observed in a cohort of 111 children with heart block, where about 50% of cases were associated with the presence of maternal autoantibodies. Non-autoantibody associated heart block has been suggested to represent a distinct entity characterized by diagnosis of heart block later in childhood and better prognosis, indicating operation of different pathogenic mechanisms. We therefore investigated potential risk factors for heart block in the autoantibody-positive and -negative groups separately.

We observed a recurrence rate of 12.1% in pregnancies immediately following the birth of a child with heart block in the group of autoantibody-positive women, which is lower than what has recently been reported in large cohorts in two independent studies (Julkunen and Eronen, 2001; Llanos et al., 2009b). This discrepancy may be due to differences in patients' ethnic origin but also in patients' recruitment. While recurrent heart block cases may be overrepresented in studies by Llanos and Julkunen, where the cohorts were formed either by specifically seeking families with cases of neonatal lupus or based on registers from tertiary referral centers, one should also consider the possibility that differences in the age range of the mothers at the birth of their first child with heart block may lead to different rates of recurrence between the studies. Indeed, a previous study from our group suggested that maternal age may influence the outcome in anti-Ro52 antibody positive pregnancies (Skog et al., 2008), and one may speculate that our cohort, in which about half of the cases are born before 1990, may comprise fewer recurrent cases because of a higher number of women having their children at a younger age. The mean maternal age at the birth of the first child has indeed increased dramatically in the last few decades (from 24 to 29 years of age between 1970 and 2009 in the Swedish population, information obtained from the

governmental agency Statistics Sweden). Unfortunately, the small number of families with recurrent cases in our cohort did not allow us to analyze a potential association between heart block recurrence and maternal age.

Investigating the influence of the age of the mother on the development of heart block in individual pregnancies revealed that the risk of fetal heart block increased significantly with increasing maternal age in autoantibody-positive women, but not in autoantibody-negative women (**Table 2**). Since the age of the mother is inherently linked to parity, the influence of both factors on pregnancy outcome was analyzed using a logistic regression model. We observed that only maternal age had a significant effect (p = 0.01), while parity did not significantly affect pregnancy outcome (p = 0.35). We did not find any evidence of an interaction between maternal age and parity regarding increased odds ratio for the risk of heart block either (p = 0.21). Older maternal age is known to associate with pregnancy complications in the general population, however its specific influence on the development of autoantibody-associated heart block from a functional point of view remains to be determined. It is possible that the increased risk for fetal heart block with increased maternal age may reflect the influence of other factors associated with age. Hypothyroidism is one such potential factor and of particular interest as anti-Ro positive women with hypothyroidism have been shown to be at higher risk of having a child with complete congenital heart block than women with autoantibodies only (Spence et al., 2006). Alternatively the observed effect of maternal age may be due to the actual appearance of the anti-Ro/La antibodies in the mothers over time. Since most women in our cohort were tested for presence of these antibodies after giving birth to a child with heart block, we could however not investigate this hypothesis further.

	Anti-Ro/La positive		**Anti-Ro/La negative**	
Age	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***
≤24	1.0 (reference)	reference	1.0 (reference)	reference
25-29	1.6 (0.7-3.6)	0.22	0.9 (0.4-1.9)	0.77
30-34	2.3 (1.0-5.5)	0.05	1.7 (0.6-4.9)	0.30
≥35	4.2 (1.4-11.9)	0.01	0.9 (0.2-4.4)	0.90

Table 2. Odds ratio (OR) of fetal heart block in relation to maternal age. OR were calculated by logistic regression with adjustment for parity for antibody-positive or antibody-negative pregnancies.

Recent evidence showing that season of birth may influence the development of autoimmune disease later in life prompted us to investigate whether this was a potential risk factor in heart block. CHB in anti-Ro/La antibodies positive pregnancies is usually detected between weeks 18-24 of pregnancy, indicating that the pathogenic mechanisms leading to conduction defects in the fetal heart are initiated before/during this period of pregnancy. We hypothesized that events during the winter season in Sweden, such as low sun exposure and low vitamin D levels as well as increased infection rate, might affect the outcome of pregnancy if occurring during the 18-24 week window period, which corresponds in turn to a birth in the summer. Analysis of the number of births during the summer (June-August) and the rest of the year revealed a significant difference between affected children and healthy siblings in the Ro/La-positive pregnancy group with births of children affected by heart block representing 58.5% of all births in the summer and only 39% of all births during the rest of the year (p = 0.015). This increased proportion of affected births during the summer was not observed in the group of Ro/La-negative pregnancies (**Fig 10**).

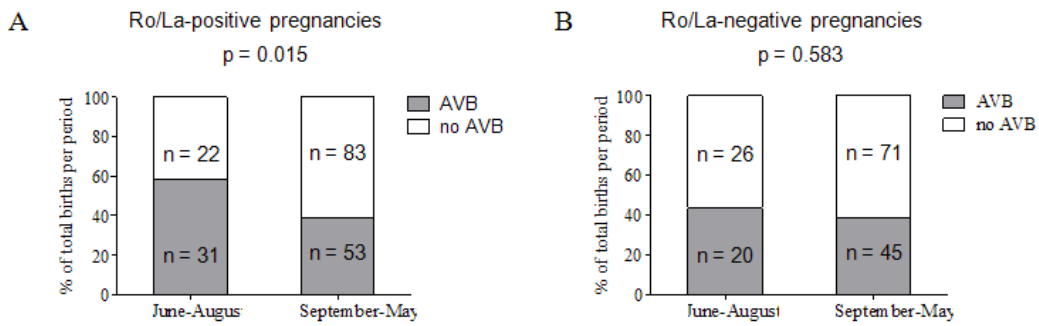


Figure 10. Development of autoantibody-associated heart block shows a season-of-birth pattern, with an increased proportion of affected births during the summer. Proportions of affected (grey fields) and healthy (white fields) births during summer and the rest of the year represented as percentage of the total number of births during the respective periods for anti-Ro/La positive **(A)** or negative **(B)** pregnancies. The number (n) of births in each group is indicated.

While we observed an inverse correlation between vitamin D levels in Swedish women over the year and the number of CHB cases, it remains unclear whether this corresponds to any functional consequence or simply reflects the association of CHB pathogenesis with the winter season in general. It is indeed quite possible that other events, such as infections, may play a role. Of interest, recent studies have shown that,

in addition to damage stemming from a direct intrauterine fetal infection, maternal CMV infection may impair placental function and lead to hypoxia-like conditions and contribute to fetal damage (Gabrielli et al., 2009; Maidji et al., 2010). Although the majority of women of childbearing age are seropositive to CMV, secondary infections may occur and might induce transient placental dysfunction. This may be especially relevant in the context of CHB as hypoxia has been suggested to amplify the harmful effects of anti-Ro/La antibodies in the fetal heart (Clancy et al., 2007).

Summary Part II

We have shown that transfer of monoclonal anti-Ro52 antibodies in rats and mice during gestation can induce AV block in the offspring and that this pathogenic effect is limited to antibodies specific for aa 200-239 of the Ro52 protein. Using an immunization model of heart block, we have also shown that maternal MHC genes regulate the generation of pathogenic anti-Ro52 antibodies while a different MHC haplotype determines susceptibility to disease in the offspring. Finally, we have identified maternal age and seasonal timing of pregnancy as risk factors for the development of CHB in anti-Ro/La antibody positive pregnancies in humans.

HYPOTHESIS

TRIM21/Ro52 in autoimmune disease: good or bad?

Dysregulated immune responses, in particular exaggerated/chronic activation of IFN pathways, have been implicated in the development of autoimmune diseases. Patients with SLE or SS display an increased expression of type I IFN and IFN-stimulated genes ("IFN signature"), and genetic studies have revealed an association between IRF5 polymorphisms and SLE/SS (Baechler et al., 2004; Borchers et al., 2003; Graham et al., 2006; Nordmark et al., 2009; Sigurdsson et al., 2005). In parallel, the importance of IL-17 in autoimmune diseases has been shown in animal models and humans, and recent studies have also highlighted a role for the IL-17 and Th17 pathway in SLE and SS (Crispin et al., 2008; Doreau et al., 2009; Nguyen et al., 2008; Sakai et al., 2008; Wong et al., 2008).

The TRIM21/Ro52 protein has long been known as an autoantigen targeted in SLE and SS, and as such has been thought to contribute to disease through the formation and deposition of immune complexes, and subsequent activation of inflammatory pathways. The findings presented in this thesis offer a new insight into the potential role of TRIM21/Ro52 in the pathogenesis of SLE and SS. Our data indeed suggest that TRIM21/Ro52, which is induced by IFNs, is an important negative regulator of IFN/TLR responses via ubiquitination of IRF transcription factors and subsequent downregulation of pro-inflammatory cytokine production. In addition, we show that the tissue inflammation and systemic autoimmunity that develop in Ro52-deficient mice following injury/infection is dependent on the IL-23/Th17 axis, as it is completely abrogated in Ro52$^{-/-}$p19$^{-/-}$ mice. This observation further stresses the importance of TRIM21/Ro52 in controlling IFN responses and preventing the establishment of an inflammatory loop, which may eventually lead to autoimmunity via exaggerated activation of the IL-17 pathway (**Fig 11**). Polymorphisms in the TRIM21/Ro52 gene have been linked to the development of SLE and SS (Frank et al., 1993; Nakken et al., 2001), and one may speculate that the protein encoded by these variants displays an impaired function, resulting in a decreased negative feedback control of IFN responses. In addition, considering the apparent ability of TRIM21/Ro52 to ubiquitinate several IRFs, the exaggerated IL-17 response in our Ro52-deficient mice, the elevated levels of IL-17 in SLE patient sera and SS patient salivary glands, and the essential role of IRF4 in the differentiation of Th17 cells (Brustle et al., 2007), it will be interesting to investigate further a potential role for TRIM21/Ro52 in the regulation of Th17 responses.

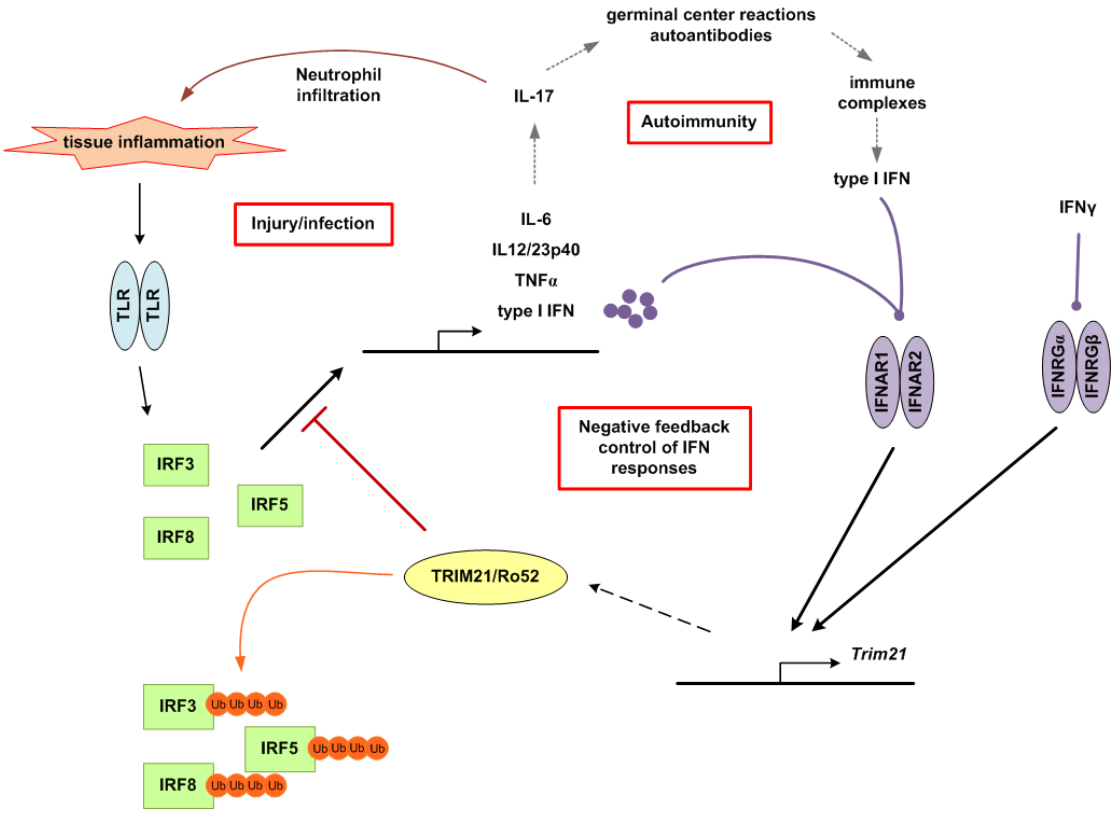


Figure 11. A model for the role of TRIM21/Ro52 in the regulation of IFN responses and development of autoimmunity. TRIM21/Ro52, an E3 ligase induced by IFNs, downregulates the production of pro-inflammatory cytokines following TLR stimulation via ubiquitination of IRF transcription factors. TRIM21/Ro52-deficient mice develop tissue inflammation and systemic autoimmunity following tissue injury and/or infection, suggesting that TRIM21/Ro52 plays an important role in the establishment of autoimmunity via the control of TLR/IFN responses.

If we think that TRIM21/Ro52, as a TRIM protein, may be part of an ancient mechanism of innate immune defense against viruses, it may be surprising to find that it negatively regulates IFN/TLR responses. However, other TRIM proteins have also recently been described as negative regulators of TLR signaling, via inhibition of NF-κB (TRIM30α) and IRF (TRIM27) responses (Shi et al., 2008; Zha et al., 2006). It is possible that some TRIM proteins have evolved to maintain a beneficial balance between protection against infection and detrimental immune responses by being upregulated upon infection and providing a feedback control of responses triggered by the pathogens. Alternatively, one may consider that TRIM21/Ro52 may exert different effects depending on the cell type and/or the activation status of the cell. Our finding that TRIM21/Ro52 is expressed at a higher level in $CD3^+$ cells than in $CD19^+$ or $CD11b^+$ cells at a steady-state level

supports the idea of a cell type-specific function. In any case, it will be highly interesting to investigate the ability of Ro52-deficient mice to respond to and fight off bacterial/viral infections.

Anti-TRIM21/Ro52 antibodies in CHB: how guilty are they?

Two main hypotheses have been proposed to date to explain the pathogenesis of CHB in fetuses of women with anti-Ro/La autoantibodies. The first one postulates that the maternal autoantibodies bind directly to the Ro52, Ro60 and La proteins when they are exposed on the surface of apoptotic cardiocytes during fetal heart development, while the second one argues for the cross-reactivity of maternal antibodies towards a cardiac cell surface molecule that is involved in the electrical activity of the heart, directly interfering with conduction of the signal at the AV node.

We have shown that anti-Ro52 antibodies recognizing the p200 stretch of the protein but not anti-Ro52 antibodies directed at other parts of the protein can induce AV block in rats when transferred during gestation. In addition, anti-Ro52 p200 antibodies induce AV block in mouse pups despite poor recognition of the mouse Ro52 p200 peptide. While these findings support the "cross-reactivity" hypothesis rather than a general recognition of the Ro52 protein by maternal antibodies, the two mechanisms are not exclusive. We propose a model where anti-Ro52 antibodies cross-react to another membrane protein on live cardiac cells, interfering with its function and leading to the dysregulation of calcium oscillations and eventually cell death. Ro52, Ro60 and La may then be released from the dying cell and/or exposed on apoptotic blebs, and binding of the maternal anti-Ro/La antibodies to their cognate antigen may promote the clearance of apoptotic material via a pro-inflammatory pathway, as described in (Clancy et al., 2004; Miranda-Carus et al., 2000). In such a scenario, one may speculate that factors such as fetal genetic predisposition and/or environmental events will promote the evolution of a first-degree AV block, initiated in the "cross-reactive" phase and potentially transient, to a more severe degree of heart block, in the "inflammatory" phase of the disease (**Fig 12**).

Of note, we did not detect expression of GFP in the heart of our Ro52-deficient mice (where a GFP reporter cassette was inserted in the Ro52 locus), indicating that Ro52 is not expressed in the adult heart in normal conditions. Looking at mouse embryos at different times of development using this reporter mouse strain will generate valuable information regarding fetal cardiac expression of Ro52. In addition, transfer of pathogenic anti-Ro52 p200 antibodies in Ro52-deficient mice will allow us to assess the importance of the presence of the Ro52 protein itself in the pathogenesis of CHB.

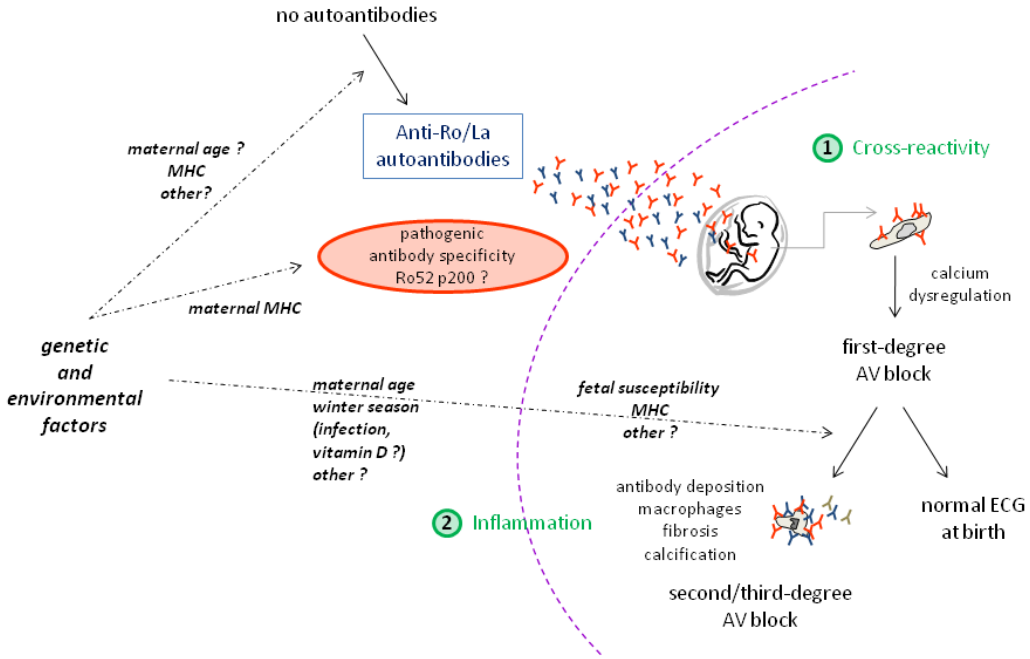


Figure 12. A two-step model of the pathogenesis of antibody-associated CHB. In a first phase, maternal anti-Ro52 antibodies bind to a cross-reactive epitope on fetal cardiomyocytes, leading to calcium dysregulation and first-degree AV block (1). In a second phase, the influence of fetal genetic susceptibility and environmental factors leads to a self-amplifying inflammatory reaction, resulting in irreversible damage in the fetal heart and complete CHB (2). The influence of maternal MHC genes on the generation of pathogenic antibodies is also depicted.

CONCLUSION

The findings presented in this thesis demonstrate that the TRIM21/Ro52 autoantigen is a RING-dependent E3 ligase mainly expressed in immune cells and upregulated upon IFN stimulation. Interestingly, while TRIM21/Ro52 E3 ligase activity is supported by E2 enzymes present in both nuclear and cytoplasmic compartments, we show that TRIM21/Ro52 is predominantly localized in the cell cytoplasm but translocates to the nucleus upon IFNα stimulation, raising the possibility that TRIM21/Ro52 may exert a different function in inflammatory conditions. The study of a mouse strain in which the TRIM21/Ro52 locus is disrupted reveals a key role for TRIM21/Ro52 as a negative regulator of IFN/TLR responses via ubiquitination of several IRFs and downregulation of the production of pro-inflammatory cytokines, suggesting that TRIM21/Ro52 may be involved in the development of the systemic autoimmune diseases SLE and SS, besides being an autoantigen in both conditions.

Investigation of the pathogenicity of anti-TRIM21/Ro52 antibodies in CHB shows that monoclonal antibodies specifically recognizing amino-acids 200-239 but not antibodies targeting other domains of the protein can induce AV block in an animal model, supporting the hypothesis that maternal anti-TRIM21/Ro52 p200 autoantibodies are important in the initiation of the disease, possibly by cross-reacting with another cardiac molecule. Furthermore, we demonstrate the influence of MHC genes on CHB development in an animal model, with the generation of maternal pathogenic antibodies and the fetal susceptibility to disease being associated with two different haplotypes. Finally, we identify maternal age and seasonal timing of pregnancy as novel risk factors for CHB in anti-Ro/La positive pregnancies in a Swedish population-based cohort. These data will be of importance in improving the counseling of autoantibody-positive women contemplating a pregnancy.

In all, the results presented in this thesis provide new insights into the role of the TRIM21/Ro52 E3 ligase and its antibodies in the development of the autoimmune diseases SLE, SS and CHB, as well as into the influence of genetic and environmental factors on CHB development.

ACKNOWLEDGEMENTS

My years as a PhD student have been rich in learning experiences and I would like to thank all the people who have contributed, in some way or other, to make these years so interesting, especially:

My supervisor, *Marie Wahren-Herlenius,* it has been a privilege to be a PhD student in your lab and I would like to thank you for supporting and guiding me throughout these years, while giving me so much freedom, for your energy and broad knowledge, and for providing us all with the best of opportunities to learn and do great science. Also, thank you for being ever so enthusiastic about every little piece of data, even when I was coming to you with a somewhat more sober view of things!

My co-supervisor, *Alexander Espinosa,* you were still a PhD student when I started, and having a bench and a desk close to yours has been invaluable. Throughout our discussions, from cloning trouble-shooting and mysterious Western-blot bands to mouse immunology and broad scientific issues, I have learnt a lot and I can only add one thing, that I hope someday to play piano as well as you (although I fear I will always be lagging a few years behind ☺)!

My co-supervisor, *Vijay Kuchroo,* thank you for giving me the opportunity to work in your lab, evolving in such an atmosphere of great scientific knowledge and determination has made my year in Boston immensely rewarding and I came back to Stockholm passionate about mouse immunology and T cells!

Head of the Rheumatology Unit, *Lars Klareskog,* thank you for making the lab such a great place for research, where basic and clinical immunology constantly meet.

The MWH lab: *Nånnis,* for being such a great lab mate, for all our chats, my first Swedish Midsummer, your enthusiasm and for unknowingly contributing to the improvement of my "mice handling skills" by needing someone at your side to anesthetize them in the Boston lab! *Linn,* for taking the time to teach me immunizations and ECG recording even though you were finishing your PhD, for all the nice Friday afternoon talks while immunizing and for trying to convince Erwan that Toronto was a great place to move to! *Vilija,* for always having so much energy and enthusiasm, for sharing your knowledge in all clinical matter, good luck with your thesis writing, it's now your turn! *Vijole,* I do not think I can thank you enough for sparing me the stress of the first step of a cardiomyocyte prep whenever you could, and thanks for thinking of

bringing me chocolate when I was writing on a recent late Friday evening … *Wei,* for supervising my very first ubiquitination assays when I started and for company in the lab during weekends! *Åse,* for taking such good care of me when I first arrived and for always being ready to help out (not to mention often having chocolate in your drawer). *Maria,* your lab skills (and no doubt Alex's influence!) have left you no choice but to be the PhD student perpetuating the molecular biology tradition of the MWH lab! *Lasse,* thanks for always being around to help (and even though you are no longer a member of the MWH lab, I still consider you as such!). New and previous members of the MWH group, *Therese, Sabrina, Joanna, Marika, Elizabeth, Amanda, Monica E, Robert K*.

The Boston lab: *Valérie,* for sharing your great lab skills and scientific knowledge with me, for a very special karaoke session in the lab one evening while I was finishing my umpteenth Western-blot, and for taking care of me during my first stay in Boston. *Jenna*, for sharing books, for your company both in the lab and in the mouse room at LND, and for taking over the Ro52 breeding ; *Anneli*, for being so helpful during my one month at HIM and for our talks about the "after-PhD" life ; *Nasim*, for all the evening rides whenever I had missed the last shuttle, the coffee-and-cookie breaks that would cheer me up. *Nicole,* for great company at the FASEB conference (in the middle of nowhere in Vermont) and for always providing answers to my questions. *Amit,* for advice on my project; *Rucha,* for help with immunizations; *George,* for keeping cool no matter what; *Nora,* for help with experiments on the Ro52 mice during my first stay; *Ava,* for making each of my trips so much easier! All past and present members of the Kuchroo lab who have kindly helped me whenever I came to them with a question.

All the co-authors, especially *Janosch,* for your deep interest in science and nice talks whenever you were visiting our lab; *Sven-Erik Sonesson,* for your statistical skills, your clinical expertise and your kindness; *Stina,* for spending so much time making the Swedish heart block cohort a reality !

The past and present members of the Rheumatology and Neuroimmunology Units at CMM, Karolinska Institutet, Solna.

★

My friends, *Lâm* and *Lisbeth*, for countless dinners, BSGs, and fikas, for reminding me that there is a life outside the lab and for your friendship; *Carole* and *Isabelle,* we meet only too rarely, but every time it's like all those years since our time at INSA-Lyon had never passed, thank you for your friendship over time and distance! *Heather,* for sharing interest and enthusiasm about research, for a winter of ice-skating sessions that

invariably ended prematurely in favor of fika and for long talks about anything and everything whenever we are both in Stockholm!

My family, pour m'avoir toujours soutenue dans mes choix, même lorsque je suis partie de plus en plus loin!

Erwan, for always listening, whether on skype or sitting next to me, whether understanding what I am talking about or not ☺, for making me laugh with silly things whenever I need it, and for nurturing my determination (even if unintentionally) by reminding me from time to time of how much I love science and research (see, I told you I would not use the word "support")!

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