From the Rheumatology Unit, Department of Medicine
Karolinska Institutet, Stockholm, Sweden

AUTOANTIBODIES IN CONGENITAL HEART BLOCK

Robert Klauninger

Karolinska Institutet

Stockholm 2009

Published by Karolinska Institutet.

ISBN 978-91-7409-700-9

Printed by
REPROPRINT AB
Stockholm 2009
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

ABSTRACT

Neonatal lupus erythematosus (NLE) affects neonates of Ro/SSA and La/SSB positive women. NLE is associated with several clinical manifestations including skin rash, cytopenia and congenital heart block (CHB). CHB is the most severe manifestation which can develop into a lethal atrioventricular (AV) block. There is a strong association between maternal Ro/SSA and La/SSB antibodies and development of CHB, but a recurrence rate of 20% in following pregnancies despite persisting maternal autoantibodies indicates that also other factors contribute to development of CHB.

The aim of this thesis was to investigate transfer of autoantigen-specific immunoglobulins of different isotypes from the mother to the child, and the significance of the postnatal maternal Ro52-, p200-, Ro60- and La antibody levels in infants during their first year. Further, we aimed to identify factors other than autoantibodies influencing the fetal outcome in Ro52 positive pregnancy using experimental models. These studies were focused on investigating fetal susceptibility.

Sera from Ro/SSA and La/SSB positive women and their children were analyzed by ELISA using purified, recombinant antigens and synthetic peptides. Ro52, Ro60 and La IgG antibodies all transferred from the mothers to their fetus *in utero* and were present in the infant at birth, but had decreased significantly in the infants at 4-5 weeks of age. Little or no Ro/La specific IgM or IgA was present in the infants, and NLE developed independently of breastfeeding. We conclude that Ro/La autoantibodies are predominantly transferred to the child via the placenta, and that there appears to be no reason for a Ro/SSA positive mother to refrain from breast feeding.

Using MHC congenic rat strains we show that heart block develops in rat pups of three strains carrying MHC haplotype $RT1^{av1}$ (DA, PVG.AV1 and LEW.AV1) after maternal Ro52 immunization, while pups from LEW rats ($RT1^{l}$) rarely developed block. Different anti-Ro52 antibody fine-specificities were generated in $RT1^{av1}$ versus $RT1^{l}$ animals. Maternal and fetal influence was determined in an F2 cross between LEW.AV1 and LEW strains, which revealed higher susceptibility in $RT1^{l}$ than $RT1^{av1}$ pups once pathogenic Ro52-antibodies were present. This was further confirmed in that $RT1^{l}$ pups more frequently developed heart block than $RT1^{av1}$ pups after passive transfer of $RT1^{av1}$ anti-Ro52 sera, and by microarray analysis of fetal hearts that revealed increased expression of distinct MHC-encoded genes in $RT1^{l}$ compared to $RT1^{av1}$ pups. Our findings suggest that generation of the pathogenic Ro52 antibodies is restricted by maternal MHC, and that a different set of genes encoded by the fetal MHC locus regulate susceptibility and determine the fetal disease outcome in anti-Ro52 positive pregnancies.

LIST OF PUBLICATIONS

I. *Klauninger R, *Skog A, Horvath L, Winqvist O, Edner A, Bremme K, Sonesson SE, *Wahren-Herlenius M. **Serologic follow-up of children born to mothers with Ro/SSA autoantibodies.** Lupus. 2009;18:792-8.

II. Strandberg L, Ambrosi A, Jagodic M, Dzikaite V, Janson P, Khademi M, Salomonsson S, Ottosson L, Klauninger R, Ådén U, Sonesson SE, Sunnerhagen M, de Graaf KL, 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.** Submitted manuscript.

*Indicates authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ANA	anti-nuclear antibodies
AVB	atrioventricular block
CFA	complete Freund's adjuvant
CHB	Congenital heart block
CIA	collagen-induced arthritis
dsDNA	double-stranded deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
ECG	Electrocardiogram
ELISA	enzyme-linked immunosorbent assay
hYRNA	human y ribonucleic acid
HLA	human leukocyte antigen
Ig	Immunoglobulin
i.p.	intraperitoneal
MaBP	maltose binding protein
MHC	major histocompatibility complex
NLE	Neonatal lupus erythematosus
pSS	primary Sjögren's syndrome
RF	rheumatoid factor
RBCC	RING/B-box/coiled-coil
RNP	Ribonucleoprotein
s.c.	Subcutaneous
SLE	Systemic lupus erythematosus
SS	Sjögren's syndrome
ssDNA	single stranded deoxyribonucleic acid
Sm	Smith antigen
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TRIM	Tripartite motif
TCRBV	T cell receptor B variable
U_1nRNP	anti-nuclear ribonucleoprotein

1 INTRODUCTION

Our immune system has an ingenious ability to distinguish between what is body derived and what is harmful infectious agents like virus or bacteria. But, at certain occasions the immune system lose its tolerance to the body it is meant to protect and autoimmunity develops. In the worst case this may lead to the progress of an autoimmune disease. About 4-5% of the population is affected by an autoimmune disease (Naparstek 1993, Wandstrat 2001) and in general, the autoimmune disease incidence is higher in females compared to men (Lockshin 2006). The cause of this sex difference has been suggested to be due to sex hormones (Whitacre 2001), and the X chromosome has also been implicated (Invernizzi 2005, Selmi 2006) as well as that fetal-maternal (maternal-fetal) microchimerism may be of significance (Gleicher 2007).

Autoimmune diseases can be divided into cell-specific, organ-specific and systemic disease (Bach 1995). Rheumatic diseases is a large group of chronic inflammatory conditions with autoimmune features and a broad diversity of immunological manifestations. The following text will mainly focus on two rheumatic systemic conditions: Systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS).

1.1 SYSTEMIC LUPUS ERYTHEMATOSUS & SJÖGREN'S SYNDROME

Systemic lupus eryhematosus is a condition with many clinical and immunological manifestations, usually including inflammation and depositions of autoantibodies and immunocomplexes in multiple organs (Klippel 1997). The autoantibodies in SLE are directed towards ubiquitous autoantigens, and virtually any organ can be affected and many different symptoms and manifestations may occur in skin and mucous membranes, central nervous-, musculoskeletal-, cardiovascular-, respiratory-, abdominal- and renal systems and the eyes (Isenberg 2005). The cause of SLE development is thought to be due to a combination of susceptiblity genes and environmental factors. SLE mainly affects women - the female versus male ratio is 9:1. In addition, women are predominantly affected by SLE during their most fertile period, suggesting influence of disease development by hormonal factors (Petri 2002).

Patients may be diagnosed with either primary Sjögren's syndrome (pSS) and then occurs without any associated autoimmune disease or secondary Sjögren's syndrome (SS) when the syndrome coexist with another autoimmune disease. Characteristic signs of SS are destroyed and dysfunctional exocrine glands and deposits of lymphocytic infiltrates recognized as dryness in mouth and eyes, also described as sicca symptoms (Ramos-Casals 2007, Jonsson 2000). Typical symptoms for SS are fatigue, general malaise, low grade fever, myalgias and arthralgias. The prevalence of SS is between 0,1% and 3% depending on what classification criteria is used (Fox 1986, Jacobsson 1989, Bjerrum 1997, Dafni 1997, Gran 2002, Ramos-Casals 2007).

1.2 B CELLS, MHC CLASS II, AUTOANTIBODIES

There are two classes of MHC molecules: MHC class I molecules that collect peptides derived from proteins synthesized in the cytosol for cell surface presentation and MHC

class II molecules that bind and present peptides derived from proteins in intracellular vesicles such as macrophage vesicles or peptides internalized by phagozytic cells and B cells. The essential helper T cell recognizes the complex of bound antigenic peptide with the MHC class II molecule and activates the B cell, and the B cell can proliferate and differentiate into antibody producing plasma cells (Figure 1).

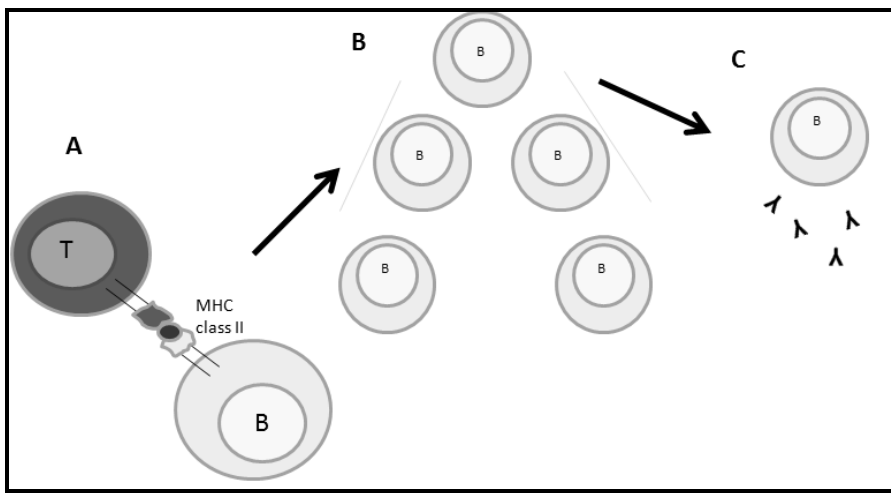


Figure 1. Antigen presentation by the MHC class II complex. The B cell is activated when T cell recognizes the antigen peptide MHC class II complex (A). The antigen recognition induces B cell proliferation (B) and differentiation to antibody-secreting plasma cells (C).

Unfortunately, sometimes the protective immune system lose its ability to just defend the body, but instead attack its protégé i.e. autoimmunity is developed. In autoimmune rheumatic diseases antibodies towards self antigens are developed – autoantibodies (Janeway 2001).
Important mediator molecules in the implication of immunology and autoimmunity are the so-called cytokines. The roles of cytokines are essential for immune cell development, immunoregulation and immune effector functions. Still there is much to understand in their role in autoimmunity. Increasing evidence support the fact that cytokines do not just function as either pro-inflammatory, inducing autoimmunity, or anti-inflammatory, suppressing autoimmunity. The cytokines seems to have a complicated role were combinations of cytokines exert variable effects during the development of an autoimmune disease. The cytokine interferon (IFN) is an example of giving such a pleiotropic effect on autoimmunity (O'Shea 2002).
An important cytokine in SS and SLE development and progression is IFNα (Bengtsson 2000, Båve 2005).

1.3 IMMUNOGLOBULIN ISOTYPES AND SUBCLASSES

The immunoglobulin (Ig) or antibodies are divided into five classes, IgA, IgD, IgE, IgG and IgM. IgA is secreted in saliva, breast milk and the intestine. The main role of IgD is

unknown besides that IgD and surface bound IgM represent the B cell receptor. IgG is the antibody isotype found in the circulation after antigen stimulation. During pregnancy IgG antibodies may transfer via active transport facilitated by F_c receptors into the fetal circulation. IgG antibodies are divided in four subclasses: IgG_1, IgG_2, IgG_3 and IgG_4.

1.4 AUTOANTIBODIES IN SLE AND SS

Different kinds of autoantibodies are associated with and may be found in the sera of both SLE and Sjögren's syndrome patients, but these autoantibodies may also be found in sera of patients with other diagnoses. Examples of autoantibodies found in SLE or SS patients are: rheumatoid factor (RF), which binds to the Fc region of IgG and anti-nuclear antibodies (ANA) with affinity to different intracellular antigens as anti-dsDNA, anti-ssDNA, anti-Sm (only SLE), anti-SSA/Ro (Ro52/Ro60), anti-SSB/La, and anti-U_1RNP (only SLE). ANA is an important diagnostic tool in SLE and SS (Griesmacher 2001).

1.5 RO52, RO60 AND LA

Ro/SSA and La/SSB proteins exist in all cells both in humans and mammals. These intracellular proteins bind/interact with hYRNA and together make a cytoplasmatic ribonucleoprotein (RNP) complex. It is not clarified what role this complex has or what function the complex associated proteins have.

1.5.1 La/SSB

There are studies suggesting that the La protein may have a function in transcriptation termination (Wolin 2002). In addition, La might be involved in virus replication, and play a key role in the induction of the immune system reaction against the RNP complex. This reaction is thought to be the source of a mechanism were the reaction against the La protein is followed by epitope spreading within the complex (Topfer 1995, Tseng 1997).

1.5.2 Ro/SSA

The Ro/SSA antigens consist of the non-homologous proteins Ro52 and Ro60. Ro60 functions in a discard pathway of defect 5SrRNA (O'Brian 1994, Labbe 1999). Animal studies on mice without Ro60 ($Ro60^{-/-}$) show symptoms similar to lupus together and develop autoantibodies (Xue 2003). Ro52 consists of 475 aa and belongs to the family RING/B-box/coiled-coil (RBCC) or tripartite motif proteins (TRIMS) (Reymond 2001) (Figure 2).

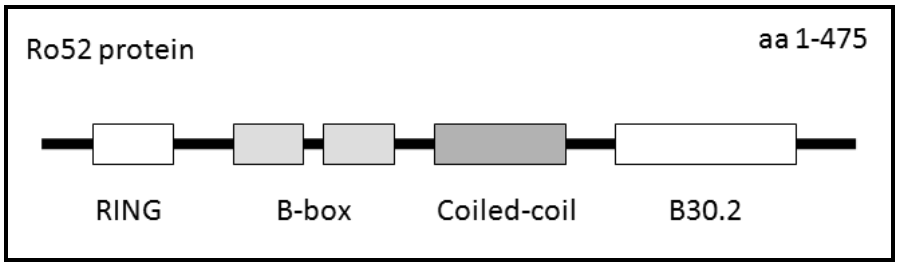


Figure 2. The common structure of the TRIM/RBCC proteins. The Ro52 protein consists of a RING finger, a B-box motif and a coiled-coil region.

The exact function/role for Ro52 is still not completely revealed, but a previous study suggests that Ro52 is an E3 ligase involved in the regulation of proliferation and cell death (Espinosa 2006). There are also results showing that the RING finger and B box domains are important in protein-protein interactions and it is common that ubiquitinating enzymes contain RING fingers (Joazeiro 2000). The coiled-coil region consists of a leucine zipper, a leucine rich alpha helix with a leucine positioned as every seventh amino acid in the polypeptide chain. This means that a leucine is pointing towards the same direction at each second turn in the helix, which is of significance due to its function in protein-protein interaction (Paris 2003). Ro52 is predominantly located in the cytoplasm, but may also be found in the nucleus, and has been suggested to shuttle between the two compartments (Simons 1994, Keech 1995, Pourmand 1998, Ohlsson 2002, Pourmand 1998). The Ro52 protein has also been associated to a regulating role in the transcription process (Wang 2001), but more recently was reported to downregulate activity of IRF transcription factors via ubiquitinations (Kong 2007, Higgs 2008, Espinosa 2009). Interestingly, Ro52 knockout mice develop tissue inflammation and systemic autoimmunity mediated by the IL-23/Th17 pathway (Espinosa 2009).

Since Ro52 is an intracellular protein it is a puzzle how it is initially exposed to the immune system with a following tolerance loss and auto-antibody development. Different proposals to the responsible mechanism have been suggested such as: cell stress via ultraviolet radiation (Zhang 2000, Saegusa 2002) or apoptosis followed by exposed intracellular proteins (Ohlsson 2002). There are also results showing that hormones like estradiol influence Ro52 expression (Wang 1996, Sakabe 1998, Zhang 2000), and that hormones together with ultraviolet radiation have a synergistic effect on Ro52 expression (Jones 1992). Furthermore, it has been shown that Ro52, besides association with the Ro/La RNP complex, binds to the heavy chain of immunoglobulin G (IgG) (Yang 1999, Rhodes 2002) and that calreticulin, a multifunctional intracellular protein, interacts with Ro52 (Cheng 1996).

In trials aimed to decide/define the autoantibody epitope on Ro52, it has been shown that the leucine zipper domain has a central role (Blange 1994, Buyon 1994, McCauliff 1994, Kato 1995, Dörner 1996) and results with synthetic peptides, overlapping the leucine zipper region, revealed that maternal Ro52 autoantibodies had a strong affinity to a peptide corresponding to aa 200-239, denoted p200; demonstrating a serologic marker for fetal risk of CHB (Salomonson 2002).

1.6 THE RO/SSA AND LA/SSB AUTOANTIGENS IN HEART BLOCK

Maternal Ro/SSA-La/SSB autoantibodies are supposed to bind a cross-reactive self-antigen on cardiomyocytes. Still no definite target for those antibodies has been identified, but different targets in cardiac tissue have been suggested such as α_{1C} and α_{1D} subunits of the L-type calcium channel (Boutjdir 2000, Qu 2005, Hu 2004), the T-type calcium channel (Hu 2004) and the atrial 5-HT_4 receptor (Eftekhari 2000, Eftekhari 2001, Kamel 2005).

1.7 SLE AND SS IMPLICATION DURING PREGNANCY

SLE and SS may be diagnosed at any age, but it is common among women that SLE has its onset during the child-bearing years (Petri 2002) and SS mainly develop at 30-50 years of age (Jonsson 2000), which indicates a close hormonal association. Pregnancy among the anti-SSA/Ro-SSB/La positive women is commonly associated with different kinds of complications such as renal involvement, proteinuria and preterm delivery (Alshohaib 2009, Wagner 2009). Studies indicate that regardless of symptomatic condition, lupus increases the fetal, neonatal and maternal risk during pregnancy. Even in inactive SLE, there is still an increased risk of complications during pregnancy (Alshohaib 2009, Wagner 2009). Other studies however do not show further complications besides CHB development of anti-SSA/Ro antibodies affecting the pregnancy outcomes (Brucato 2002).

1.8 NEONATAL LUPUS ERYTHEMATOSUS (NLE)

Neonatal lupus erythematosus is a passively transferred condition that influences the fetus. NLE includes numerous kinds of manifestations but the most common of NLE manifestations are skin rash and CHB. Other symptoms are cytopenias and liver involvment (Buyon 1998). The incidence of NLE is about 1-2% of children to mothers with SLE, and 15-20% of children to mothers diagnosed with SLE and Ro/SSA antibodies but also occurrs in the children of asymptomatic women (Spence 2006, Jaeggi 2005). Maternal Ro/La autoantibodies are strongly associated to the development of congenital heart block (CHB), which is the most serious condition of neonatal lupus erythematosus (McCauliffe 1995, Dörner 2000). About 50% of the NLE infants develop CHB (Lee 1990) and the condition is irreversible and fatal in its worst state. Apart from CHB, NLE symptoms are transient and the symptoms decline in parallel with the maternal antibody levels in the neonate circulation, and are dissolved approximately 6-8 months after delivery (Lee 1994).

There are studies suggesting different factors such as human leukocyte antigen (HLA) haplotypes, autoantibodies and isotype specificity as determining NLE symptom development in the infant (Kim 2001). It has also been suggested that CHB is predominantely correlated to Ro52 antibodies and that the transient conditions of NLE correlates with Ro60 antibodies (Lee 1994).

1.9 ELECTROCARDIOGRAM (ECG) INTERPRETATION

An electrocardiogram (ECG) consists of a P wave, a PR interval, a QRS wave, ST segment, T wave, QT interval, and possibly a U wave. The P wave represents activation of the atrium. The QRS complex signifies the ventricular depolarization and

the T wave (as well as the U wave) corresponds to the ventricular repolarization. The PR interval is measured from the P wave onset to the beginning of the QRS complex (Figure 3).

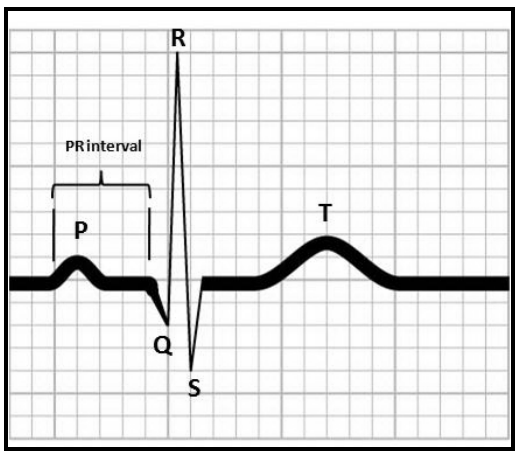


Figure 3. ECG showing the P, Q, R, S and T waves. PR prolongation and observation of missing QRS complexes in the ECG indicate AV block.

1.10 CONGENITAL HEART BLOCK (CHB)

Congenital heart block is a lethal condition with conduction abnormalities in a structurally normal heart (Buyon 2003), which develops in fetuses of Ro/SSA and La/SSB positive women. The mothers can be diagnosed with rheumatic diseases such as SLE and SS but may even be asymptomatic. Ro/SSA women have a 2% risk getting a child with CHB and the risk is about 20% among women who previously have had a child with CHB (Michaelson 1972, Julkunen 2001, Brucato 2001, Buyon 2009).

1.10.1 The three types of AV block

Congenital heart block is a condition with a disorder of the conduction system with an affected atrioventricular node in the fetal heart (John 2004). The congenital heart block which is hypothesized to develop via three stages, from a reversible first state with conduction prolongations to a third irreversible state with no conduction between the atria and the ventricles (Sonesson 2004). CHB is divided into AVB type I, AVB type II and AVB type III according to the severity grade. The three severity conditions can be distinguished as: AV block type I, which is the reversible state seen as a prolonged PR interval, AV block type II which is characteristic on the electrocardiogram (ECG) expressing absence of some conducted impulses through the AV node, and thus disappearance of some QRS complexes. AV block type II is also subdivided into three variations: 1) Type I (Wenckeback) second-degree AV block expressing a lengthening of the AV conduction before it is blocked, 2) Type II (Mobitz II) second-degree AV block is included by an abrupt or sporadic block without a preceding lengthening of the AV conduction time, 3) 2:1 AV block with a conduction of every second impulse through the AV node. In AV block type III there is no conducted impulses through the AV node at all, and this lethal condition demands a life-long pacemaker dependence (John 2004, Jaeggi 2005, Dubin 2003).

1.10.2 Incidence

The frequency of CHB in the population is 1/15 000 to 1/20 000 live births (Michaelsson 1972). CHB is most commonly detected between 18-24 weeks of gestation (Brucato 2001). Women positive to anti-Ro/SSA antibodies may therefore be examined by Doppler echocardiography every 2 weeks with start from the 16th week of gestational age. In that way there is an increased chance to detect early changes to the cardiac conduction that may develop into a complete atrioventricular block (AVB). There is today no available prophylactic therapy, but early detected conduction abnormalities might be treated with fluorinated steroids (Brucato 2008). There are different techniques available to survey and evaluate changes in fetal cardiac time intervals.

1.10.3 Cardiac pathology of congenital heart block

Histological investigations of the conduction system in fetal hearts have shown inflammation with deposits of antibodies, complement components and lymphocytic infiltrates with a developed calcification and fibrosis leading to imperfect signal conduction (Litsey 1985, Lee 1987, Clancy 2004). It has been suggested that macrophages have a major role in the sustained inflammatory reaction and fibrotic development because of a shift to a state of hypersecretion of proinflammatory cytokines such as TNFα (Miranda-Carus 2000). The fibrosis development observed in histologic samples is also believed to be dependent on TGFβ – a pro-fibrotic cytokine – secreted by macrophages (Clancy 2003). In addition, it is also suggested that macrophages increase the calcification in tissue by secreting alkaline phosphatase (Tintut 2002). Maternal myocardial cells have been found in fetal hearts of patients with NLS-CHB, and these findings are suggested to be an additional factor involved in the pathogenesis or progression of CHB (Stevens 2005).

1.11 BREAST-FEEDING AND MATERNAL AUTOANTIBODIES

The host defence is not fully developed in the new-born child, and transferred maternal antibodies are important in the fetal humoral defence. During the time in utero maternal IgG antibodies are transferred into the fetal circulation via placenta, and after birth the fetus is supplemented by secretory IgA in breast-milk (Hanson LÅ 2007). Also IgM is trasfered via the breast milk.

1.12 GENES INFLUENCE IN AUTOIMMUNITY

The autoimmune disease are chronic conditions associated with a reactive humoral and/or cellular immune system due to initial loss of tolerance to self antigens. In addition, besides the immunological reaction, it has been shown that the genetic background is of considerable importance due to disease susceptibility and there are associations with human leukocyte antigen (HLA)/major histocompatibility complex (MHC) and with non-HLA/non-MHC genes. The MHC gene region has a dominating role influencing the development of autoimmune diseases, but there is also a strong relation between chromosomal non-MHC regions and autoimmune disease. The region is divided into MHC class I, class II and class III (Ghodke 2005, Serrano 2006). The MHC complex is a gene dense region, located on human chromosome 6p12.31,

covering more than 120 expressed genes (1999). The corresponding MHC region in rats is called RT1 and is located on chromosome 20 (RN020). Besides the strong association of MHC-genes and autoimmunity, there are also non-MHC genes associated to autoimmune diseases which include *Cytotoxic T lymphocyte associated antigen 4* (CTLA4), *Protein tyrosine phosphatase* (PTPN22) and *Tumor necrosis factor-α* (TNFα).

Despite the strong association between maternal anti-Ro/SSA-La/SSB autoantibodies and CHB development, the risk is still relative low with its 2 %, which today makes it almost impossible to make a prospective prediction of the pregnancy outcome. Even women with a second pregnancy and a previous child with CHB are difficult to predict because 80% will deliver a baby without CHB. Today there are no suitable screening markers to apply in purpose preventing development of CHB. Due to the relative low incidence of CHB it is assumed that besides maternal autoantibodies also the fetal genetic background determines the susceptibility of AV-block development.

2 AIMS OF THE THESIS

Women positive for anti-Ro/SSA and La/SSB autoantibodies may risk getting a child with NLE, which in the worst case develops into an irreversible heart failure – congenital heart block (CHB). The heart block inducing autoantibodies are transferred to the fetus during pregnancy and affect the developing heart. A subsequent fetus may however not be affected, despite the presence of maternal autoantibodies. This indicates that there are additional, yet unidentified, factors critical for development of congenital heart block.

Based on these observations, the aim of my thesis project was

- To investigate Ro/SSA and La/SSB autoantibodies in congenital heart block.
- To identify additional factors contributing to development of congenital heart block.

3 RESULTS AND DISCUSSION

The strong association between anti-Ro/SSA-La/SSB antibodies and CHB is well known, but it is still not fully clarified what underlying pathogenic mechanism cause CHB development. It is well confirmed that maternal IgG autoantibodies against Ro/SSA-La/SSB antigen are transferred from the mother to the child via the placenta during pregnancy and that women with anti-Ro/SSA-La/SSB-antibodies have a risk of getting a child with NLE (Michaelsson 1972, Scott 1983, Ramsey-Goldman 1986, Lee 1990, Manthorpe 1992, Waltuck 1994, Brucato 2001). Because the incidence of CHB among children to Ro/SSA and La/SSB positive mothers is comparatively low and that surveillance of Ro/SSA La/SSB positive pregnancies for potential development of complete CHB in the fetus are expensive there is a need of a marker to distinguish mothers with increased risk getting a child with CHB. It has been shown that mothers to children with AV block have an autoantibody profile associated with a high level of antibodies directed against a definite peptide stretch, corresponding to aa 200-239, in the Ro52 protein, which is also called the leucine zipper region. This peptide region has been synthesized into a peptide denoted p200, which has been demonstrated as a serologic marker for fetal risk of CHB (Salomonson 2002).

3.1 PAPER I

This study investigated the context of transferred maternal Ro52-p200 autoantibodies during pregnancy and postnatally, and what implication breast-feeding have on NLE manifestations such as skin involvement or postnatal progression of AV-nodal disease, during the infants first year in life. Breast milk contains protecting antibodies but is also a possible source of Ro52 IgG, IgA and IgM autoantibodies. This study prospectively followed a cohort of 30 children with or without CHB, and 21 of these children were breast-fed.

3.1.1 Maternal – fetal autoantibody transfer

All the women in the study were Ro52/SSA positive and as in previous studies (Salomonsson 2002, Strandberg 2008) Ro52-p200 levels were statistically significantly higher in pregnancies with a development of AVB II and III than in pregnancies resulting in a normal AV conduction. Concerning Ro60 and La antibody levels no significant differences were detected. These results also correlate to a previous Finnish, Swedish and American cohort study (Strandberg 2008). Clancy and colleagues have claimed that p200 antibodies do not show an increased level or a specificity correlated to children with CHB. But on the other hand their assay was performed in a different way (Clancy 2005), which is of great importance because p200 loses its antigenecity if the fold and structure are disrupted (Ottoson 2005). On the other hand no significant differences in Ro52-p200 antibody levels were measured between mothers to children with AV block I or AV block II/III, which might be an indication of a similarity between these two groups concerning the initiation of AV block. However, the difference may be due to certain factors involved in the susceptibility of the immunological reaction taking place in the heart conduction system.

3.1.2 Maternal IgG autoantibodies

The majority of the maternal sera contained IgG antibodies recognizing Ro52, Ro60 and La and these antibodies were also in the fetal circulation at birth, confirming maternal IgG transfer to the fetus. Though considering the differences when comparing the ELISA results of maternal autoantibody Ro52 and p200 IgG levels, it might be due to the different assay protocols or a difficulty in calibrating the corresponding amount/concentration between full length Ro52 and the p200 peptide. In addition, the affinity may differ between the Ro52 protein and the biotin labeled peptide. The result is however consistent between the two antigens. The Ro60 and La autoantibody levels were not significantly different between mothers with AVB children and healthy children, which is in agreement with previous studies (Salomonsson 2002, Julkunen 2004, Fritsch 2005).

3.1.3 Fetal clearance of maternal autoantibodies

The fetal antibody titer was followed from birth and one year ahead. The test results show an apparent and relative fast clearance of IgG autoantibodies already at 4-5 weeks of age, which corresponds approximately to a normal maternal antibody half-life. This relative short time of possible detection of autoantibodies verifies that the major source of maternal autoantibodies is disrupted after placental detachment despite that more than 70% of the infants were breast-fed.

3.1.4 The implication of breast-feeding in NLE symptom

Askanase and colleagues have shown IgA and IgG autoantibodies specific to Ro52, Ro60 and La in breast milk (Askanase 2002). It is a disadvantage that our study has no data concerning immunoglobulin content from the breast milk of the participating mothers. On the other hand the maternal antibody levels in infant sera were of significantly lower levels indicating that autoantibodies in breast milk do not contribute substantially to titers established in utero. So, the symptoms associated to NLE are rather related to the placental IgG transfer and probably during a time in the development when the fetus is at its most vulnerable state.

3.1.5 Immunoglobulin clearance and reversal of AV block

NLE skin manifestations are associated with anti-La-antibodies and the mothers of children with NLE had significantly higher La IgG levels. We could see a normalization of the AV conduction time in three neonates with AV block type I, which was in parallel with the decrease of the maternal antibody levels in the infant sera. This additionally strengthens the meaning of placental transfer of maternal autoantibodies. The decrease of IgG titer may have nothing to do with the normalization of the AV conduction time among AV block I children - it is possible that the normalization already started in utero in the sense that these children did not express the specific susceptibility that is needed in the development leading to AV block III. So, perhaps the parallel occurrence of immunoglobulin clearance and the reverse of AV block I is due to confounding and should not at all be interpreted with the reverse of AV block I.

The placental transfer of maternal autoantibodies is the primary influencing factor in the development of CHB or other NLE manifestations, which is also established due to

the decrease of maternal autoantibody levels in sera belonging to breast-feed infants. Simultaneously, breast-feeding does not delay a conversion from AV block I to normal conduction. In other words, there are no reasons not to advise Ro/SSA-, La/SSB positive women to breast-feed their children.

We could not notice any significant difference in duration of breast-feeding regardless it was AV block children or healthy children. Although the number of participants in the cohort is too small to make any far reaching conclusion, the duration of breast-feeding indicates an interesting trend concerning that > 70% of the AV block II/III children were breast-feed in ≤ 2 months while > 70% of the healthy children were breast-feed in ≥ 2 months, which makes it tempting to speculate that the duration of breast-feeding correlates to the children's condition i.e. a healthy child will be breast-feed a longer time. The children with AV block I follow the same pattern as the healthy children.

3.2 PAPER II

Despite a strong association between anti-Ro/SSA-La/SSB antibodies and CHB, > 85 % of mothers to a child with AV-block are autoantibody positive, there is still a relative low risk (2 %) for these women to get child with CHB (Buyon 2004). It has been claimed there is a 33% incidence of AV block type I in utero, but due to the spontaneous reversal it is suggested that the clinical meaning is of less importance (Sonesson 2004). However, these results have been questioned by others (Buyon 2005, Rein 2005). Because some 20% of following pregnancies of Ro/SSA La/SSB positive mothers with a previous AV block child develops into a further CHB, there are strong indications that additional factors are involved in the development of CHB. The genetic importance in the development of CHB has been discussed (Siren 1999). This study point out how allelic variations of genes in the MHC complex determine both the maternal ability to form pathogenic antibodies as well as the fetal susceptibility to maternal autoantibodies in the context of CHB development.

3.2.1 Experimental animal model

To study the implication of different MHC and non-MHC genes in the development of CHB we used an established animal model with immunization of Ro52 protein in rats to induce CHB in the pups. The included rat strains have shown susceptibility for induction of other autoimmune diseases like experimental autoimmune encephalomyelitis (EAE) (Weissert 1998), and collagen-induced arthritis (CIA) (Lorentzen 1996, Griffiths 1993). This study was designed in purpose to investigate what influence MHC (RT1) and non-MHC genes have on different inbreed strains and MHC congenic rats. Three strains shared the same MHC haplotype $RT1^{avl}$ (DA, PVG.AV1 and LEW.AVl), and one strain (LEW) shared the background genes with the congenic LEW.AV1 strain, but distinguished in the MHC genes, because the native RT1 locus of the LEW strain is L ($RT1^{l}$). The immunizations of rats were according to an established model (Figure 4).

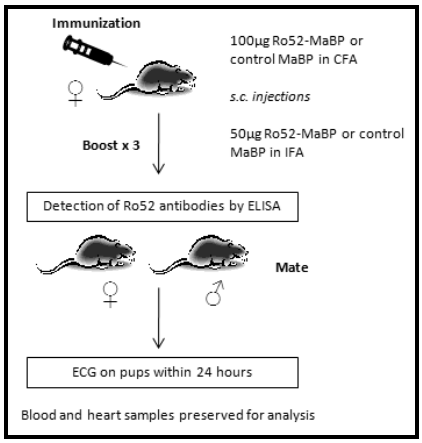


Figure 4. Ro52 immunization model. Rats were immunized and then mated 2-4 weeks after last boost. ECG was performed on pups within their first 24 hours.

After immunization of all rat strains (DA.AV1, PVG.AV1, LEWAV1 and LEW.L) Ro52-p200 antibodies were detected by ELISA, but with differing antigenic fine specificity. It has been shown that Ro52-p200 antibodies from mother of children with CHB have a different binding prophile to mutated p200 peptides than do Ro52-p200 antibodies from non-CHB mothers (Ottosson 2005). Similar results were shown in this study – $RT1^{av1}$ rats generated Ro52 antibodies with higher specificity to the same mutated p200 peptide pOUT, corresponding to the leucine zipper region, compared to rats of $RT1^{l}$ haplotype. That imply that Ro52 immunization generate antibodies towards Ro-52 and p200 irrespective of haplotype, but a decisive difference between $RT1^{avl}$ and $RT1^{l}$ haplotypes seems to be associated with the antibody fine specificity in AV block induced by Ro52 immunization. This was shown both due to the ability of antibody binding to mutated p200 peptides as well as their diverged pathogenic implication in the development of AV block - mothers of $RT1^{avl}$ got pups with developed PR prolongation, which did not happen to pups of $RT1^{l}$ mothers. This result confirmed previous studies regarding the ability of Ro52 autoantibody epitope binding specificity (Ottosson 2005), and a clear indication of the implication of maternal MHC. Because all rats with the same MHC haplotype $RT1^{av1}$, regardless of strain or different non-MHC genes (DA, PVG.AV1 och LEW.AV1), got pups with PR prolongation, and the frequency of PR prolongation between the strains is fairly equal showing a non-significant difference. This indicated that MHC haplotype AV1 determined the resulting significant PR prolongation compared to the MHC haplotype L.

3.2.2 Different binding properties between AV1 and L haplotypes

Rats express two different MHC class II molecules, RT1.B (orthologous to human HLA-DQ) and RT1.D (orthologous to human HLA-DR), and as we set out to decide each contribution of RT1.B and RT1.D molecules in the context of generating p200 T cell responses. In addition, the class II molecule RT1-B has a dominating role in generation of specific Ro52-p200 responses. It has also been shown that RT1-B express a larger allelic diversity compared to RT1-D (Saar 2008) and a greater ability to regulate the pathogenic development followed by an immunization (Strandberg 2008b). To decide what MHC molecule was dominating we used antibodies blocking either RT1.B (Ox-6) or RT1.D (Ox-17) in T cell lines from LEW.AV1 stimulated with p200

peptides. The proliferation- and IFNγ production assays demonstrated a dominant role for RT1.B. Since AV1 and L strains generated Ro52-p200 antibodies with diverging fine specificity the question was whether AV1 or L haplotype strains also express RT1.B MHC class II molecules with differing peptide binding ability. And the results showed a considerable difference in the peptide binding cleft, electrostatic as well as conformational, resulting in different peptide presentation. This could be one of the important factors that differ among Ro52 positive women – they all have Ro52 antibodies, but with different fine specificity i.e. heart block inducing properties.
When analyzing p200 specific T cells we confirmed the usage of different TCRBV among $RT1^{avl}$ and $RT1^{l}$ respectively. We could conclude that T cell lines of $RT1^{avl}$ and $RT1^{l}$ rats expressed different TCRBV; a dominance for TCRBV1 in AV1 and a dominance for TCRBV7 in L haplotype. This also indicates a diverging fine specificity since both B and T cell responses in Ro52 immunized LEW.AV1 and LEW.L rats differ, and the different TCRBV in each strain relates to different peptide presentation by the MHC class II. The difference in maternal MHC haplotype is of crucial significance in regulation of pathogenic antibody specificity, and these heart block inducing antibodies are linked to a dominant $RT1^{av1}$ trait.

3.2.3 An F2 cross reveals that the $RT1^{l}$ MHC haplotype increase the susceptibility to AV block in rat pups

Previous studies have shown that autoantibodies specific for p200 peptides in Ro52 are associated to an increased risk of fetal CHB (Strandberg 2008, Salomonsson 2002). But still, the risk is relative low and the associations to additional involved factors in CHB development strong, which makes it difficult to use the maternal p200 antibody profile as a prognosis tool. CHB development is associated to different haplotypes between the mother and the child. The allelic differences coding for class II antigen presenting properties, make important divergences in T cell specificity and specific antibody binding properties. The difference in p200 peptide specificity between $RT1^{av1}$ and $RT1^{l}$ strains and the lower frequency of AV block in $RT1^{l}$ pups could be due to insufficient pathogenic maternal $RT1^{l}$ Ro52 antibodies or a fetal resistance among $RT1^{l}$ pups. Due to evaluating whether heart block mainly is dependent on maternal or fetal factors we performed an F2 cross between AV block susceptible LEW.AV1 rats and heart block resistant LEW.L rats. Prolonged PR intervals were detected in F1 heterozygous $RT1^{av1/l}$ and monozygous $RT1^{l}$ pups, and the significant results show that the fetal haplotype is of significant importance due to susceptibility of maternal Ro52 antibodies. In addition, the p200 antibody profile of binding pOUT and pZIP was the same in immunized F1 heterozygous females as it was in the previous immunization study involving $RT1^{av1}$ rats, which confirmed consistency. This F2 cross study consisting of $RT1^{avl}$ and $RT1^{l}$ rats did show that the MHC haplotype in pups was of decisive importance in the implication of the regulating susceptibility of heart block development. Moreover, it appears to be of significance if the $RT1^{l}$ MHC is maternally rather than paternally inherited to the off-spring – pups of Ro52 immunized heterozygous F1 rats had significantly longer PR intervals if those rats consisted of maternal $RT1^{l}$ and paternal $RT1^{avl}$ compared to pups from Ro52 immunized rats

consisted of maternal $RT1^{avl}$ and paternal $RT1^{l}$. However, only monozygotic $RT1^{avl}$ and $RT1^{l}$ pups were analyzed.

3.2.4 Ro52 serum transfer confirms the association of MHC with fetal susceptibility to AV block

In order to confirm the association of MHC encoded genes with fetal susceptibility to AV block, $RT1^{av1}$ DA rat serum were transferred i.p. to $RT1^{av1}$ (DA) and $RT1^{l}$ (LEW) rats. The serum was pooled from Ro52 immunized $RT1^{av1}$ rats (DA) (n=10). The assay showed that maternal MHC restricts the production of specific pathogenic antibodies, but the fetal haplotype decides the AV block development once maternal antibodies are transferred into the fetal circulation.

Pups of $RT1^{av1}$ haplotype had the same heart rate as controls. Notably, maybe the assay should have had a control with no transferred serum at all just to have a complete normal baseline to compare with. According to the results of anti-Ro52- and p200 antibody levels, pups of both $RT1^{av1}$ and $RT1^{l}$ haplotypes showed high levels. The anti-Ro52-antibody levels were equal between $RT1^{av1}$ and $RT1^{l}$ while the anti-p200-antibody levels were slightly lower in pups with $RT1^{l}$ haplotype. These results may verify the suggestion - if the fetal genetic condition exists to develop AV block, the susceptibility is enough regardless of the Ro52 antibody level. When we compared Ro52 antibody levels in pups from the immunization study with Ro52 antibody levels in pups from the transfer study, much higher levels were measured in pup serum from the immunization study, which seems reasonable because immunization will influence the whole immunologic system in the rat. In addition, it also shows that the antibody level is not of that importance per se inducing AV block. The AV block development is rather dependent of a synergy between maternal anti-Ro52-antibody specificity and the fetal vulnerability as a consequence of the genetic susceptibility.

These results also support the theory of AV development described by Wahren-Herlenius and Sonesson (Wahren-Herlenius 2006) (Figure 5).

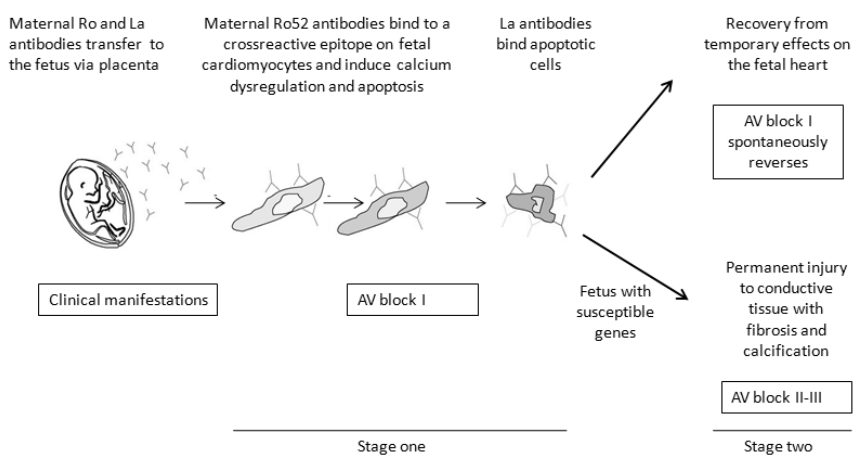


Figure 5. The two stage mechanism underlying the development of CHB (Adapted from Wahren-Herlenius 2006). In a first step, transplacentally transferred maternal Ro and La autoantibodies bind to cardiomyocytes and dysregulate calcium homeostasis leading to calcium overload and a following apoptosis. The apoptotic cells expose intracellular antigens to La autoantibodies. In a second step, genetically susceptible fetuses will develop a spread inflammatory reaction due to the antibody depositions and a further developed fibrosis and calcification into a resulting third-degree AV block (AV block III). Fetuses with no susceptible genes will not get any conduction damage and the clinical manifestations reverse spontaneously.

3.2.5 Upregulated genes in congenital heart block

As the genetic influence is assumed to be strongly associated to influencing the heart block development, AV block affected pup hearts were analyzed aiming to identify specific genes in the $RT1^{l}$ 20p12 MHC locus. There was a difference in 36/86 SNPs between AV1 and L haplotypes in the MHC locus.

Besides HLA-DMB, which is seen as a candidate gene in the MHC locus implicating a role in the regulation of the differential susceptibility(Litsey 1985, Clancy 2004), there are at least four more genes in the MHC locus: TRIM26, ATP6G2, Bat5 and G7c (Table 1).

Gene	Aliases	Function
HLA-DMB		A possible key component involved in the antigen presentation (Jensen 1999).
TRIM26	ZNF173, RNF95, AFP	Function in immune regulation and as a viral restriction factor (Nisole 2005).
ATP6G2		Transmembrane protein, subunit of the vacuolar ATPase H^{+} pump, regulate the pH of the cell (Nishi 2002).
Bat5	HLA-B, associated transcript 5	Transmembrane protein, α/β hydrolase, biological role unknown (Lehner 2004).
G7c	C6, f27	Unknown function, suggested role in extracellular interactions (Kummanovic 2001).

Table 1. Upregulated genes in the $RT1^{l}$ 20p12 MHC locus in congenital heart block. Identified upregulated genes in pup hearts after Ro52-positive transfer and suggested functions.

TRIM26 is a homologue of Ro52 (TRIM21), and the proteins share the tripartite motif with a ring domain, a B-box and a coiled-coil motif as well as a B30.2 domain (Chu

1995, Reymond 2001, Espinosa 2006, Ottosson 2006, Hennig 2005). TRIM26 is like Ro52 predicted as an E3 ligase, but the target for TRIM26-mediated ubquitination is still not identified.

The five above mentioned genes may be candidate genes involved in the pathogenesis of CHB either as targets for pathogenic heart block inducing autoantibodies or in the regulation of cellular mechanisms involved in the progress of heart block development. Exploring and identification of specific genes linked to experimental animal disease models increase the possibilities to find candidate genes in human patients. Since the techniques in exploring and interpret the function and the implication of genes and proteins are in progress, the methods to analyze are becoming faster, simpler and relative cheap to use, the easier it will be to identify new candidate genes. This is of importance in rare diseases because the populations to investigate are too small to give reliable information. Maybe these results could be part of the explanation why only a few neonates of Ro52 positive women get AV block – the origin of the MHC is one of the factors involved in the fetal susceptibility to develop AV block.

4 CONCLUSIONS

In the study comprising paper I, "Serologic follow-up of children born to mothers with Ro/SSA autoantibodies", we aimed to investigate the implication of maternal anti-Ro52-p200 antibodies and their association to NLE. Also, we studied if maternal Ro52 IgA and IgM autoantibodies in breast-milk influence the progress of NLE manifestations in the skin or the progress of AV-nodal disease.

We found that maternal autoantibodies decrease rapidly in the infant circulation during the first few weeks of life and that non-detectable levels were reached at one year of age. The autoantibody levels were rapidly decreased among breast-fed infants as well. This indicates that breast-feeding does not delay the reversal of AV block I to normal conduction.

We further found that refraining from breastfeeding does not prevent NLE skin lesions. Hence, we found no reason not to recommend breastfeeding to Ro/La autoantibody positive mothers.

Further, demonstrated in paper II, "Maternal MHC regulates generation of pathogenic antibodies and fetal MHC-encoded genes determine susceptibility in congenital heart block" our aims were to find additional risk factors contributing to CHB development in animals. We hypothesized that the susceptibility to CHB was related to the fetal genetic background.

We found that maternal MHC restricts the pathogenic specificity of anti-Ro52 antibodies, while the fetal susceptibility to develop CHB is determined by a different set of MHC-encoded genes. This indicates that the fetal genetic background is the main factor that determines the disease outcome in Ro52-positive pregnancies and explains why the majority of recurrent pregnancies do not lead to CHB affected children.

5 ACKNOWLEDGEMENTS

During my time as a PhD student at Karolinska Institutet I have had the joy to work with and meet excellent skillful and, of course, nice people. I would especially like to thank and show my gratitude to:

My supervisor: Professor *Marie Wahren-Herlenius,* for your great knowledge, enthusiasm, and your eagerness to convince me to really make this done.

My co-supervisor: Professor *Sven-Erik Sonesson,* for your humble attitude, your great competence, and your will to give me your time.

The head of the Rheumatology Unit: Professor *Lars Klareskog,* thank you for making the lab an excellent place for research.

Present and former members of the MWH group: *Alex Espinosa,* thank you for being a nice and skillful lab mate with a nice attitude. *Stina Salomonsson,* you have always been interested and supportive. *Lars Ottosson,* what to say? Always helpful with everything (not just the computers)… and to find things… *Aurélie,* you were a new member when I left, but you just give me positive vibes *Susanna Brauner,* your kindness and strong personality really corresponds to you. *Gabriella Dombos,* your soft and humble personality together with your will to help is in my memory. *Åse Elfving,* thank you for your warmness, care and all experimental help. Previous members which contribute to this great lab atmosphere include: *Karin Popovich, Amanda Skog, Monica Ek, Linn Strandberg, Wei Zhou and Vijole Dzikaite*.

All the co-authors!

All present and former members of the Rheumatology and Neuroimmunology group: Thank you for nice interactions, scientific input and friendship!

Administration: *Gunnel Bemerfeldt* and *Susanne Karlfeldt* for making problems no problems.

6 REFERENCES

Alshohaib S. Outcome of pregnancy in patients with inactive systemic lupus erythematosus and minimal proteinuria. Saudi J Kidney Dis Transpl. 2009;20(5):802-5.

Askanase AD, Miranda-Carus ME, Tang X, Katholi M, Buyon JP. The presence of IgG antibodies reactive with components of the SSA/Ro- SSB/La complex in human breast milk: implications in neonatal lupus. Arthritis Rheum. 2002;46:269-71.

Bach JF. Organ-specific autoimmunity. Immunol Today. 1995;16(7):353-5.

Bengtsson AA, Sturfelt G, Truedsson L, Blomberg J, Alm G, Vallin H, Rönnblom L. Activation of type I interferon system in systemic lupus erythematosus correlates with disease activity but not with antiretroviral antibodies. Lupus. 2000;9:664-71.

Bjerrum KB. Keratoconjunctivitis sicca and primary Sjögren's Syndrome in a Danish population aged 30-60 years. Acta Ophthalmol Scand. 1997;75(3):281-6.

Blange I, Ringertz NR, Pettersson I. Identification of antigenic regions of the human 52 kD Ro/SS-A protein recognised by patient sera. J Autoimmun. 1994;7:263-74.

Boutjdir M. Molecular and ionic basis of congenital complete heart block. Trends Cardiovasc Med. 2000;10:114-22.

Brucato A, Frassi M, Franceschini F, Cimaz R, Faden D, Pisoni MP, Muscara M, Vignati G, Stramba-Badiale M, Catelli L, Lojacono A, Cavazzana I, Ghirardello A, Vescovi F, Gambari PF, Doria A, Meroni PL, Tincani A. Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. Arthritis Rhem. 2001;44:1832-5.

Brucato A, Doria A, Frassi G, Castellino F, Franceschini D, Faden D, Pia Pisone M, Solerte L, Muscarà M, Lojacono A, Motta M, Cavazzana I, Ghirardello A, Vescove F, Tombini V, Cimaz R, Gambari PF, Meroni PL, Canesi B, Tincani A. Pregnancy outcome in 100 women with autoimmune diseases and anti-Ro/SSA antibodies: a prospective controlled study. Lupus. 2002;11:716-21.

Brucato A. Prevention of congenital heart block in children of SSA-positive mothers. 2008;47:35-37.

Buyon JP, Slade SG, Reveille JD, Hamel JC, Chan EK. Autoantibody responses to the "native" 52-kDa SS-A/Ro protein in neonatal lupus syndromes, systemic lupus erythematosus, and Sjögren's syndrome. J Immunol. 1994;152:3675-84.

Buyon JP, Hiebert R, Copel J, Craft J, Friedman D, Katholi M, Lee L, Provost TT, Reichlin M, Rider L, Rupel A, SaleebS, Weston WL, Skovron ML. Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence

rates obtained from a national neonatal lupus registry. J Am Coll Cardiol. 1998;31:1658-66.

Buyon JP, Clancy RM. Neonatal lupus syndromes. Curr Opin Rheumatol. 2003;15:535-41.

Buyon JP, Rupel A, Clancy RM. Neonatal syndromes. Lupus. 2004;13:705-12.
Buyon JP, Askanase AD, Kim MY, Copel JA, Friedman DM. Identifying an early marker for congenital heart block: when is a long PR interval too long? Comment on the article by Sonesson et al. Arthritis Rheum. 2005;52:1341-2.

Buyon JP, Clancy RM, Fridman DM. Autoimmune associated congenital heart block: integration of clinical and research clues in the management of the maternal/foetal dyad at risk. J Intern Med. 2009;265:653-62.

Båve U, Nordmark G, Lövgren T, Rönnelid J Cajander S, Eloranta ML, Alm GV, Rönnblom L. Activation of the type I interferon system in primary Sjogren's syndrome: a possible etiopathogenic mechanism. Arthritis Rheum. 2005;52:1185-95.

Cheng ST, Nguyen TQ, Yang YS, Capra JD, Sontheimer RD. Calreticulin binds hYRNA and the 52-kDa polypeptide component of the Ro/SS-A ribonucleoprotein autoantigen. J Immunol. 1996;156:4484-91.

Chu TW, Capossela A, Coleman R, Goci VL, Nallur G, Gruen JR. Cloning of a new "finger" protein gene (ZNF173) within the class I region of the human MHC. Genomics. 1995;29:229-39.

Clancy RM, Buyon JP. Clearance of apoptotic cells: TGF-β in the balance between inflammation and fibrosis. J Leukoc Biol. 2003;74:959-60.

Clancy RM, Kapur RP, Molad Y, Askanase AD, Buyon JP. Immunohistologic evidence supports apoptosis, IgG deposition, and novel macrophage/fibroblast crosstalk in the pathologic cascade leading to congenital heart block. Arthritis Rheum. 2004;50:173-182.

Clancy RM, Buyon JP, Ikeda K Nozawa K, Argyle DA, Friedman DM, Chan EK. Maternal antibody responses to the 52-kd SSA/Ro p200 peptide and the development of fetal conduction defects. Arthritis Rheum. 2005;52:3079-86.

Dafni UG, Tzioufas AG, Staikos P, Skopouli FN, Moutsopoulos HM. Prevalence of Sjögren's Syndrome in a closed rural community. Ann Rheum Dis. 1997;56(9):521-5.

Dörner T, Feist E, Wagenmann A, Kato T, Yamamoto K, Nishioka K, Burmester GR, et al. Anti-52 kDa Ro(SSA) autoantibodies in different autoimmune diseases preferentially recognize epitopes on the central region of the antigen. J Rheumatol. 1996;23:462-8.

Dörner T, Feist E, Pruss A Chaoui R, Goldner B Hiepe F. Significance of autoantibodies in neonatal lupus erythematosus. Int Arch Allergy Immunol. 2000;123:58-66.

Dubin D. Ion adventure in the heartland: exploring the heart's ionic-molecular microcosm. Vol.1.2003, Tampa, Florida: Cover Publishing Co. 26-54.

Eftekhari P, Salle L, Lezoualc'h F, Mialet J, Gastineau M, Briand JP, Isenberg DA, Fournie GJ, Argibay J, Fischmeister R et al. Anti-SSA/Ro52 autoantibodies blocking the cardiac 5-HT4 serotoninergic receptor could explain neonatal lupus congenital heart block. Eur J Immunol. 2000;30:2782-90.

Eftekhari P, Roegel JC, Lezoualc'h F, Fischmeister R, Imbs JL, Hoebeke J. Induction of neonatal lupus in pups of mice immunized with synthetic peptides derived from amino acid sequences of the serotoninergic 5-HT4 receptor.
Eur J Immunol. 2001;31:573-9.

Espinosa A, Zhou W, Ek M, Hedlund M, Brauner S, Popovic K, Horvath L, Wallerskog T, Oukka M, Nyberg F, Kuchroo VK, Wahren-Herlenius M. The Sjogren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death. J Immunol. 2006;176:6277-85.

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

Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV. Sjögren's Syndrome. Proposed criteria for classification. Arthritis Rheum. 1986;29(5):577-85.

Fritsch C, Hoebeke J, Dali H, Ricchiuti V, Isenberg DA, Meyer O, Muller S. 52-kDa Ro/SSA epitopes preferentially recognized by antibodies from mothers of children with neonatal lupus and congenital heart block. Arthritis Res Ther. 2005;8:R4.

Gleicher N, Barad DH. Gender as risk factor for autoimmune diseases. J Autoimmun. 2007;28:1-6.

Gordon P, Khamashta MA, Rosenthal E, Simpson JM, Sharland G, Brucato A, Franceschini F, De Bosschere K, Meheus L, Meroni PL, Hughes GR, Buyon JP. Anti-52 kDa Ro, anti-60 kDa Ro, and anti-La antibody profiles in neonatal lupus. J Rheumatol. 2004;31:2480-87.

Gran JT. Diagnosis and definition of primary Sjögren's Syndrome. Scand J Rheumatol. 2002;31(2):57-9.

Griesmacher A, Peichl P. Autoantibodies associated with rheumatic diseases. Clin Chem Lab Med. 2001;39:189-208.

Griffiths MM, Cannon GW, Leonard PA, Reese VR. Induction of autoimmune arthritis in rats by immunization with homologous rat type II collagen is restricted to the RT1avl haplotype. Arthritis Rheum. 1993;36:254-58.

Isenberg DA, Rahman A, Allen E, Farewell V, Akil M, Bruce IN, D'Cruz D, Griffiths B, Khamashta M, Maddison P, McHugh N, Snaith M, The LS, Yee CS, Zoma A, Gordon C. BILAG 2004. Development and initial validation of an updated version of the British Isles Lupus Assessment Group's disease activity index for patients with systemic lupus erythematosus. Rheumatology 2005;44:902–906.

Hanson LÅ. Session 1: Feeding and infant development Breast-feeding and immune function. Proc Nutr Soc. 2007;66:384-96.

Hennig J, . Structural organization and Zn2+-dependent subdomain interactions involving autoantigenic epitopes in the Ring-B-box-coiled-coil (RBCC) region of Ro52. J Biol Chem. 2005;280:33250-261.

Higgs R, Jefferies CA. Targeting IRFs by ubiquitination: regulating antiviral responses. Biochem Soc Trans. 2008;36:453-8.

Hu K, Qu Y, Yue Y, Boutjdir M. Functional basis of sinus bradycardia in congenital heart block. Circ Res. 2004;94:32-8.

Invernizzi P, Miozzo M, Selmi C, Persani L, Bettezzati PM, Zuin M, et al. Chromosome monosomy: a common mechanism for autoimmune diseases. J Immunol. 2005;175:575-8.

Jacobsson LT, Axell TE, Hansen BU, Henricsson VJ, Larsson A, Lieberkind K, Lilja B, Manthorpe R. Dry eyes or mouth - an epidemiological study in Swedish adults, with special reference to primary Sjogren's syndrome. J Autoimmun. 1989;2:521-7.

Jaeggi ET, Nii M. Fetal brady- and tachyarrhythmias: new and accepted diagnostic and treatment methods. Semin Fetal Neonatal Med. 2005;10:504-14.

Janeway CA, Travers P, Walport M, Shlomchik MJ. Immuno biology 5th edition, 2001.

Jensen PE, Weber DA, Thayer WP, Chen X, Dao CT. HLA-DM and the MHC class II antigen presentation pathway. Immunol Res. 1999;20:195-205.

Joazeiro CA, Weissman AM. RING finger proteins: mediators of ubiquitin ligase activity. Cell. 2000;102:549-52.

John AD, Fleicher L. Electocardiography. Int Anesthesiol Clin. 2004;42:1-12.

Jones SK. Ultraviolet radiation (UVR) induces cell-surface Ro/SSA antigen expression by human keratinocytes in vitro: a possible mechanism for the UVR induction of cutaneous lupus lesions. Br J Dermatol. 1992;126:546-53.

Jonsson R, Haga HJ, Gordon TP. Current concepts on diagnosis, autoantibodies and therapy in Sjogren's syndrome. Scand J Rheumatology. 2000;29:341-8.

Julkunen H, Eronen M. The rate of recurrence of isolated congenital heart block: a population-based study. J Rheumatol. 2004a;31:183-189.

Julkunen H, Miettinen A, Walle TK, Chan Ek, Eronen M. Autoimmune responses in mothers of children with congenital and postnatally diagnosed isolated heart block: a population based study. J Rheumatol. 2004b;31:183-9.

Kamel R, Eftekhari P, Clancy R, Buyon JP, Hoebeke J. Autoantibodies against the serotoninergic 5-HT4 receptor and congenital heart block: a reassessment. J Autoimmun. 2005, 25:72-6.

Kato T, Sasakawa H, Suzuki S, Shirako M, Tashiro F, Nishioka K, Yamamoto K. Autoepitopes of the 52-Kd SS-A/Ro molecule. Arthritis Rheum. 1995;38:990-8.

Keech CL,Gordon TP, McCluskey J. Cytoplasmic accumulation of the 52 kDa Ro/SS-A nuclear antigen in transfected cell lines. J Autoimmun. 1995;8:699-712.

Kim J, Smith KJ, Skelton H. Neonatal lupus erythematosus: factors which may lead to clinical disease in the foetus even in the absence of disease in the mother. Clin Exp Dermatol. 2001;26:423-6.

Kleinman D, Katz VL, Kuller JA. Perinatal outcomes in women with systemic lupus erythematosus. J Perinatol. 1998;18(3):178-82.

Kong HJ, Anderson DE, Lee CH, Jang MK, Tamura T, Tailor P, Cho HK, Cheong J, Xiong H, Morse HC 3rd, Ozato K. Cutting edge: autoantigen Ro52 is an interferon inducible E3 ligase that ubiquitinates IRF-8 and enhances cytokine expression in macrophages. J Immunol. 2007;179:26-30.

Kummanovic A, Lindahl KF. G7c in the lung tumor susceptibility (Lts) region of the Mhc class III region encodes a von Willebrand factor type A domain protein. Immunogenetics. 2001;53:64-8.

Labbe JC, Hekimi S, Rokeach LA. The levels of the RoRNP-associated Y RNA are dependent upon the presence of ROP-1, the Caenorhabitis elegans Ro60 protein. Genetics. 1999;151:143-50.

Lee LA, Coulter S, Erner S, Chu H. Cardiac immunoglobulin deposition in congenital heart block associated with maternal anti-Ro autoantibodies. Am J Med. 1987;83:793-6

Lee LA. Maternal autoantibodies and pregnancy--II: The neonatal lupus syndrome. Baillieres Clin Rheumatol. 1990;4:69-84.

Lee LA, Frank MB, McCubbin V, Reichlin M. Autoantibodies of neonatal lupus erythematosus. J Invest Dermatol. 1994;102:963-66.

Lehner B, Semple JI, Brown SE, Counsell D, Campbell RD, Sanderson CM. Analysis of a high-throughput yeast two-hybrid system and its use to predict the function of intracellular proteins encoded within the human MHC class III region. Genomics. 2004;83:153-67.

Litsey SE, Noonan JA, O'Connor WN, Cottrill CM, Mitchell B. Maternal connective tissue disease and congenital heart block. Demonstration of immunoglobulin in cardiac tissue. N Engl J Med. 1985;312:98-100.

Lorentzen JC, Klareskog L. Susceptibility of DA rats to arthritis induced with adjuvant oil or rat collagen is determined by genes both within and outside the major histocompatibility complex. Scand J Immunol. 1996;44:592-8.

Manthorpe T, Manthorpe R. Congenital complete heart block in children of mothers with primary Sjogren's syndrome. Lancet. 1992;340:1359-60.

McCauliffe DP, Yin H, Wang LX, Lucas L. Autoimmune sera react with multipl epitopes on recombinant 52 and 60 kDa Ro(SSA) proteins. J Rheumatol. 1994;21:1073-80.

McCauliffe DP. Neonatal lupus erythematosus: a transplacentally acquired autoimmune disorder. Semin Dermatol. 1995;14:47-53.

Michaelsson M, Engle MA. Congenital complete heart block: an international study of the natural history. Cardiovasc Clin. 1972;4:85-101.

Miranda-Carus ME, Askanase AD, Clancy RM, Di Donatu F, Chou TM, Libera MR, Chan EK, Buyon JP. Anti- SSA/Ro and anti-SSB/La autoantibodies bind the surface of apoptotic fetal cardiocytes and promote secretion of TNF-α by macrophages. J Immunol. 2000;165:5345-51.

Naparstek Y, Plotz PH. The role of autoantibodies in autoimmune disease. Annu Rev Immunol. 1993;11:79-104.

Nishi T, Forgac M. The vacuolar (H^+)-ATPases – nature's most versatile proton pumps. Nat Rev Mol Cell Biol. 2002;3:94-103.

Nisole S, Stoye JP, Saib A. TRIM family proteins: retroviral restriction and antiviral defence. Nat Rev Microbiol. 2005;3:799-808.

O'Brian CA, Wolin SL. A possible role for the 60-kD Ro autoantigen in a discard pathway for defective 5SrRNA. Genes Dev. 1994;8:2891-903.

Ohlsson M, Jonsson R, Brokstad KA. Subcellular redistribution and surface exposure of the Ro52, Ro60 and La48 autoantigens during apoptosis in human ductal

epithelialcells: a possible mechanism in the pathogenesis of Sjögren's syndrome. Scand J Immunol. 2002;56:456-69.

O'Shea JJ, Ma A,Lipsky P. Cytokines and autoimmunity. Nat Rev Immunol. 2002;2:37-45.

Ottosson L, Salomonsson S, Hennig J, Sonesson SE, Dörner T, Raats J, Kuchroo VK, Sunnerhagen M, Wahren-Herlenius M. Structurally derived mutations define congenital heart block-related epitopes within the 200-239 amino acid stretch of the Ro52 protein. Scand J Immunol. 2005;61:109-18.

Ottosson L, Hennig J, Espinosa A, Brauner S, Wahren-Herlenius M, Sunnerhagen M. Structural, functional and immunologic characterization of folded subdomains in the Ro52 protein targeted in Sjogren's syndrome. Mol Immunol. 2006;43:588-98.

Paris S, Longhi R, Santambrogio P, de Curtis I. Leucine-zipper-mediated homo- and hetero- dimerization of GIT family p95-ARF GTPase-activating protein, PIX, paxillin-interacting proteins 1 and 2. Biochem J. 2003;372:391-8.

Petri M. Epidemiology of systemic lupus erythematosus. Best Pract Res Clin Rheumatol. 2002;16:847-8.

Pourmand N, Blange I, Ringertz N, Pettersson I. Intracellular localization of the Ro 52 kD auto-antigen in HeLa cells visualized with green fluorescent protein chimeras. Autoimmunity. 1998;28:225-33.

Ramos-Casals M, Brito-Zerón P. Emerging biological therapies in primary Sjögren's Syndrome. Rheumatology (Oxford). 2007;46(9):1389-96.

Ramsey-Goldman R, Hom D, Deng JS, Ziegler GC, Kahl LE, Steen VD, LaPorte RE, Medsger TA, jr. Anti-SS-A antibodies and fetal outcome in maternal systemic lupus erythematosus. Arthritis Rheum. 1986;29:1269-73.

Rein AJ, Mevorach D, Perles Z, Shovali A, Elchalal U. Fetal first-degree heart block, or where to set the confidence limit: comment on the article by Sonesson et al. Arthritis Rheum. 2005;52:366; author reply 366-8.

Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, et al. The tripartite motif family identifies cell compartments. Embo J. 2001;20:2140-51.

Saegusa J, Kawano S, Koshiba M, Hayashi N, Kosaka H, Funasaka Y, Kumagai S. Oxidatative stress mediates cell surface expression of SS-A/Ro antigen on keratinocytes. Free Radic Biol Med. 2002;32:1006-16.

Sakabe K, Yoshida T, Furuya H, Kayama F, Chan EK. Estrogenic xenobiotics increase expression of SS-A/Ro autoantigens in cultured human epidermal cells. Acta Derm Venereol. 1998;78:420-3.

Salomonsson S, Dörner T, Theander E, Bremme K, Larsson P, Wahren-Herlenius M. A serologic marker for fetal risk of congenital heart block. Arthritis Rheum. 2002;46:1233-41.

Saar K et al. SNP and haplotype mapping for genetic analysis in the rat. Nat Genet. 2008;40:560-6.

Scott JS, Maddison PJ, Taylor PV, Esscher E, Scott O, Skinner RP. Connective-tissue disease antibodies to ribonucleoprotein, and congenital heart block. N Engl J Med. 1983;309:209-12.

Selmi C, Invernizzi P, Gershwin ME. The X chromosome and systemic sclerosis. Curr Opin Rheumatol 2006;18:601-5.

Simons FH, Pruijn GJ, van Venrooij WJ. Analysis of the intracellular localization and assembly of Ro ribonucleoprotein particles by microinjection into Xenopus laevis oocytes. J Cell Biol. 1994;125:981-8.

Siren MK, Julkunen H, Kaaja R, Ekblad H, Koskimies S. Role of HLA in congenital heart block: susceptibility alleles in children. Lupus. 1999;8:60-7.

Sonesson SE, Salomonsson S, Jacobsson LA, Bremme K, Wahren-Herlenius M. Signs of first degree heart block occur in one-third of fetuses of pregnant women with anti-SSA/Ro 52-kd antibodies. Arthritis Rheum. 2004;50:1253-61.

Spence D, Hornberger L, Hamilton R, Silverman ED. Increased risk of complete congenital heart block in infants born to women with hyperthyroidism and anti-Ro and/or anti-La antibodies. J Rheumatol. 2006;33:167-70.

Stevens AM, Hermes HM, Lambert NC, Nelson JL, Meroni PL, Cimaz R. Maternal and sibling microchimerism in twins and triplets discordant for neonatal lupus syndrome-congenital heart block. Rheumatology. 2005;44:187-91.

Strandberg L, Salomonsson S, Bremme K, Sonesson SE, Wahren-Herlenius M. Ro52, Ro60 and La autoantibody levels and Ro52 IgG subclass profiles longitudinally throughout pregnancy in congenital heart block pregnancies. Lupus. 2006;15:346-53.

Strandberg L, Winqvist O, Sonesson SE, Mohseni S, Salomonsson S, Bremme K, Buyon JP. Julkunen H, Wahren-Herlenius M. Antibodies to amino acid 200-239 (p200) of Ro52 as serologic markers for the risk of developing congenital heart block. Clin Exp Immunol. 2008;154:30-37.

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

Tintut Y, Patel J, Territo M, Saini T, Parhami F, Demer LL. Monocyte/macrophage regulation of vascular calcification in vitro. Circulation. 2002;105:650-5.

Topfer F, Gordon T, McCluskey J. Intra- and intermolecular spreading of autoimmunity involving the nuclear self-antigens La (SS-B) and Ro (SS-A). Proc Natl Acad Sci USA. 1995;92:875-9.

Tseng CE, Chan EK, Miranda E, Gross M, Di Donato F, Buyon JP. The 52.kd protein as a target of intermolecular spreading of the immune response to components of the SS-A/Ro-SS-B/La complex. Arthritis Rheum. 1997;40:936-44.

Wagner SJ, Craici I, Reed D, Norby S, Bailey K, Wiste HJ, Wood CM, Moder KG, Liang KP, Liang KV, Rose C, Rozkos T, Sitina M, Grande JP, Garovic VD. Maternal and foetal outcomes in pregnant patients with active lupus nephritis. Lupus. 2009;18(4):342-7.

Wahren-Herlenius M, Sonesson SE. Specificity and effector mechanisms of autoantibodies in congenital heart block. Curr Opin Immunol. 2006;18:690-6.

Waltuck J, Buyon JP. Autoantibody-associated congenital heart block: outcome in mothers and children. Ann Intern Med. 1994;120:544-51.

Wandstrat A, Wakeland E. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. Nat Immunol. 2001;2:802-9.

Wang D, Chan EK. 17-β-estradiol increases expression of 52-kDa and 60-kDa SS-A/Ro autoantigens in human keratinocytes and breast cancer cell line MCF-7. J Invest Dermatol. 1996;107:610-4.

Wang D, Buyon JP, Yang Z, Di Donato F, Miranda-Carus ME, Chan EK. Leucine zipper domain of 52 kDa SS-A/Ro promotes protein dimer formation and inhibits in vitro transcription activity. Biochim Biophys Acta. 2001;1568:155-61.

Weissert R, Wallström E, Storch MK, Stefferl A, Lorentzen J, Lassmann H, Linington C, Olsson T. MHC haplotype-dependent regulation of MOG-induced EAE in rats. J Clin Invest. 1998;102:1265-73.

Wolin SL, Cedervall T. The La protein. Annu Rev Biochem. 2002;71:375-403.
Xue D, Shi H, Smith JD, Chen X, Noe DA, Cedervall T, Yang DD, et al. A lupus-like syndrome develops in mice lacking the Ro 60-kDa protein, a major lupus antigen. Proc Natl Acad Sci USA. 2003;100:7503-8.

Zhang J, Xu Z, Jin J, Zhu T, Ma S. Induction of Ro/SSA antigen expression on keratinocyte cell membrane by heat shock and phorbol 12-myristate 13-acetate as well as estradiol and ultraviolet B. J Dermatol Sci. 2000;24:92-8.

Qu Y, Baroudi G, Yue Y, Boutjdir M. Novel molecular mechanism involving alpha1D (Cav1.3) L-type calcium channel in autoimmune-associated sinus bradycardia. Circulation. 2005;111:3034-41.

I

Lupus (2009) 18, 792–798
http://lup.sagepub.com

PAPER

Serologic follow-up of children born to mothers with Ro/SSA autoantibodies

R Klauninger[1], A Skog[1], L Horvath[1], O Winqvist[2], A Edner[3], K Bremme[3], SE Sonesson[3] and M Wahren-Herlenius[1]
[1]Rheumatology Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden; [2]Clinical Immunology Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden; and [3]Department of Women and Child Health, Karolinska Institutet, Stockholm, Sweden

Neonatal lupus erythematosus (NLE) develops in foetuses of mothers with Ro/SSA and La/SSB antibodies and may include foetal atrioventricular block and dermatologic manifestations. In this study, we investigated postnatal Ro and La IgG, IgA and IgM antibody levels up to 1 year of age in 32 children born to Ro/SSA positive mothers. Antibody levels were correlated with NLE manifestations, and the role of breast feeding in transfer of autoantibodies from mother to child was evaluated. Ro52, Ro60 and La IgG antibodies all transferred from the mothers to their foetus *in utero* and were present in the infant at birth as detected by enzyme-linked immunosorbent assay using recombinant antigens and a synthetic peptide. A significant decrease in Ro52, Ro60 and La IgG autoantibody levels of the infants was observed from birth to 4–5 weeks of age ($P < 0.05$, $P < 0.05$ and $P < 0.01$). Ro- and La-specific IgA and IgM antibodies were detected in the serum from a subset of mothers. However, Ro- and La-specific IgA and IgM antibody levels were low or non-detectable in children raised both with and without breastfeeding. Furthermore, NLE skin lesions developed independently of breastfeeding. Our findings support a role for placental materno-foetal transfer of IgG autoantibodies in the pathogenesis of NLE and indicate that refraining from breastfeeding does not protect from NLE skin involvement. *Lupus* (2009) **18,** 792–798.

Key words: congenital heart block; neonatal lupus; Ro52; SSA

Introduction

Pregnant women with Ro/SSA and La/SSB autoantibodies are at risk of having children with neonatal lupus erythematosus (NLE).[1] The syndrome may present with skin rash, liver involvement and cytopenias, as well as isolated congenital heart block.[2,3] The mothers are often diagnosed with systemic lupus erythematosus (SLE) or Sjögren's syndrome but may also be asymptomatic. The foetal atrioventricular (AV) block that develops between 18th and 24th week of gestation after placental transfer of Ro/SSA autoantibodies from the mother to the foetus[4] may be initiated as a first degree block.[5] Immunoglobulin and complement deposits are found in the foetal heart, and after mononuclear cell infiltration, fibrosis and calcification of the cardiac tissue, the block progresses to a complete third degree AV block (AVB).[6,7]

Both Ro60 and La antibodies have been implicated in the pathogenesis of congenital heart block, but there is accumulating evidence that the Ro52 antibodies are the initiating autoantibodies mediating the heart block.[8–12] More specifically, antibodies recognising amino acid (aa) 200–239 (p200) of Ro52 were shown to bind the cell surface of cardiomyocytes and induce dysregulation of intracellular calcium and eventually induce cell death.[13] Further, prolongation of the foetal AV-time relates to the level of maternal antibodies specific for the 200–239 aa stretch of Ro52.[13]

The complete congenital heart block develops in 2% of foetuses of Ro/SSA positive mothers[14] and is associated with a substantial mortality of 20–30%.[4,15,16] The less severe first degree AVB is detected in up to one-third of foetuses of Ro52-positive pregnancies and may revert spontaneously during the third trimester or the first few weeks after birth.[5] The levels of Ro52-p200 antibodies in the child postnatally have not been investigated. Further, the contribution of breastfeeding to Ro52 IgA and IgM autoantibody levels postnatally in the child has not been analysed, or whether breastfeeding puts the

Correspondence to: Marie Wahren-Herlenius, Rheumatology Unit, Department of Medicine, CMM L8:04, Karolinska Institutet, SE-171 76 Stockholm, Sweden. Email: Marie.Wahren@ki.se

Robert Klauninger and Amanda Skog authors contributed equally.

Received 6 October 2008; accepted 20 January 2009

10.1177/0961203309103188

child at risk for Ro/SSA- and La/SSB-related manifestations such as skin involvement or postnatal progression of AV-nodal disease. To evaluate neonatal lupus symptoms and autoantibody levels postnatally in infants born to mothers with Ro/SSA antibodies, we prospectively followed a cohort of 32 children with or without congenital heart block with frequent serum sampling and clinical follow-up during their first year of life.

Patients and methods

Patients

A total of 30 mothers and their 32 children were included in the study. Two mothers had two consecutive pregnancies with resulting children included. In all, 10 mothers were diagnosed with Sjögren's syndrome,[17] 18 mothers with SLE,[18] 1 with rheumatoid arthritis[19] and 3 did not fulfil criteria for rheumatic disease. The pregnancies were followed with Doppler echocardiography at the Pediatric Cardiology Unit at Astrid Lindgren Children's Hospital as previously described.[5,20] In all, 27 pregnancies were recruited for foetal surveillance based on the presence of maternal Ro/SSA antibodies and risk of congenital heart block, whereas 5 pregnancies were referred after signs of complete AVB had already been observed.[21] All newborn infants were followed up with at least a clinical cardiac examination and an electrocardiogram (ECG) after birth. Healthcare records were available from 30 infants and used to collect data on skin involvement and breastfeeding. A child was considered as breastfed when this was the sole intake of nutrition.

Serum was sampled from the mothers during late pregnancy ($n = 30$) or at the time of labour and from the infants/children at birth from the cord ($n = 16$), at 3 days of age ($n = 16$), at 4–5 weeks ($n = 22$), at 6 months ($n = 3$) and at 1 year ($n = 14$). Sera were stored in aliquots at −70 °C until use. The human ethics committee at the Karolinska University Hospital approved the study, and informed consent was obtained from the parents.

Recombinant proteins and synthetic peptide

Recombinant Ro52, Ro60 and La proteins were expressed in *Escherichia coli* as previously described using the pMAL vector (New England Biolabs, Beverly, Massachusetts, USA) with maltose-binding protein (MaBP) as fusion partner.[22] Wild type MaBP was used as control. Full-length proteins and MaBP were purified on amylose columns according to the manufacturer's instructions (New England Biolabs). Protein concentrations were determined with Bradford protein assay (Bio-Rad, Richmond, Virginia, USA). Synthetic p200 peptide including aa 200–239 of Ro52 was synthesised by Thermo Biosciences (Ulm, Germany) with biotin conjugated at the N-terminal end. Peptide purity was confirmed by high performance liquid chromatography and mass spectrometry.

Enzyme-linked immunosorbent assay for detection of maternal and infant antibodies to recombinant Ro52, Ro60 and La proteins

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described[23] with minor modifications. In short, medium-binding 96-well plates (Nunc, Odense, Denmark) were coated overnight (1 μg/well) with recombinant full-length Ro 52, Ro 60, La or wild-type vector MaBP protein diluted in carbonate buffer (pH 9.6). Plates were blocked with phosphate buffered saline (PBS)-0.05% Tween (TPBS)/5% milk powder, and sera were tested at 1:1000 dilutions in TPBS/1% milk powder. Bound antibodies were detected by affinity-purified alkaline phosphatase (AP)-conjugated anti-human IgG antibodies (Dakopatts, Glostrup, Denmark). Phosphatase substrate tablets (Sigma, St. Louis, Missouri, USA) dissolved in diethanolamine buffer (pH 9.6) were used as substrate. The absorbance was measured at 405 nm. All steps were performed at room temperature except coating, which was performed at 4 °C. An index was calculated based on a ratio with the serum from one high titre patient selected as positive control and with serum from one selected negative control; recombinant protein-index = [(OD sample − OD negative control)/(OD positive control − OD negative control)] × 100.

ELISA for detection of maternal and infant antibodies to peptide p200

ELISA for detection of p200 binding antibodies was performed as previously described.[24] In brief, high-binding 96-well plates (Nunc) were coated with 100 μL of 3 μg/mL streptavidin diluted in water. Plates were incubated at +4 °C for 2 days and then dried at 37 °C and stored at +4 °C until use. Plates were washed four times with wash buffer (0.15 M NaCl, ×0.006 M $NaH_2PO_4.H_20$, 20% NaN_3/0.05% Tween-20/2% BSA) and unspecific binding blocked with 200 μL 4% BSA in PBS. Plates were washed once with PBS and coated at least 6 h at room temperature with 100 μL of 3 μg/mL biotin-p200 peptide

in coating buffer (0.03 M Na_2CO_3, 0.07 M $NaHCO_3$, 0.1% NaN_3). Plates were washed four times with wash buffer. A total of 100 μL serum was added per well at a dilution of 1:300, and plates were incubated shaking at room temperature for 2 h. Plates were washed four times and affinity-purified AP-conjugated, rabbit anti-human IgG antibodies (Dakopatts) were added at a dilution of 1:1000. Plates were washed four times with wash buffer. Phosphatase substrate tablets (Sigma) were dissolved in diethanolamine pH 9.8, and 100 μL/well was used for detection of IgG. The absorbance was measured at 405 nm using a Sunrise absorbance reader (Tecan, Männedorf, Switzerland) and the Magellan V 3.11 software (Tecan, Männedorf, Switzerland). A p200 index was calculated based on a ratio with one high-titre patient selected as standard where the p200 index = [(OD sample − OD negative control)/(OD positive control − OD negative control)] × 100. All p200 assays in this study were run in the same laboratory, Department of Clinical Immunology, Karolinska University Hospital.

Statistical analysis

Statistical analysis was performed using Statistica 7.0 (Statsoft, Tulsa, Oklahoma, USA). The Mann–Whitney *U*-test was used to compare different groups, and the Wilcoxon test was used to analyse longitudinal data within individuals. The level of significance was set at $P < 0.05$.

Results

Pregnancy and transfer of maternal Ro52, Ro60 and La autoantibodies to the foetus

During the 32 included pregnancies, second or third degree heart block developed in 6 children, first degree heart block was detected in 7 children and in 19 children no cardiac involvement was registered. The majority of these cases have been clinically described before.[21] Of the six foetuses with AVB II/III, two responded to steroid treatment and were born with a first degree AVB. Of these, one had a normal ECG at 1 week of age, whereas the other still had an AVB I at 8 months. Of the seven foetuses with signs of AVB I *in utero*, four had a normal ECG at birth and three had an AVB I. The three neonates born with AVB I had reverted to normal conduction within 4 weeks of age.

Maternal autoantibodies relate to the development of congenital heart block, and to examine the correlation between maternal and foetal anti-p200, anti-Ro52, anti-Ro60 and anti-La IgG antibody levels, sera were collected from mothers and children at birth. As previously noted, p200 antibody levels were higher in sera from mothers of children affected by heart block than in sera from mothers of children without cardiac involvement (Figure 1A and B). Ro60 and La antibodies were also detected in most, but not all, mothers of children with first or second/third degree congenital heart block (Figure 1C and D). Investigating sera from the neonates by antigen-specific ELISAs showed that p200, Ro52, Ro60 and

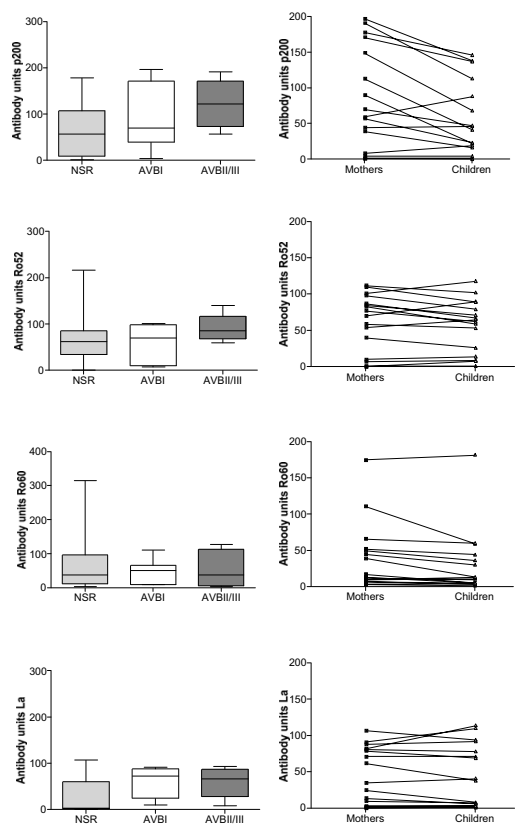


Figure 1 Autoantibodies to Ro52, Ro60 and La antigens in mothers and their children. Antibody levels to p200 (A), Ro52 (B), Ro60 (C) and La (D) in the mothers were determined using ELISA with recombinant antigen and synthetic peptides. Levels of p200-Ro52 antibodies were higher in mothers of children with AVB II/III than in mothers of children with normal sinus rhythm ($P < 0.05$), whereas Ro60 and La antibody levels were not significantly different. All autoantibody specificities were transferred from the mother to the foetus (E) p200, (F) Ro52, (G) Ro60 and (H) La as determined by ELISA with recombinant antigen and synthetic peptides.

La IgG antibodies were all transferred to the children (Figure 1 E–H).

Clearing of maternal IgG Ro52, Ro60 and La antibodies from the circulation of the infant

The infants were followed clinically and serologically during their first year of life. Serum was sampled at birth, at 3–4 days of age, at 4–5 weeks, at 6 months and at 1 year of age. Transferred maternal p200, Ro52, Ro60 and La IgG antibodies were continuously depleted from the circulation of the infant (Figure 2). At 4–5 weeks of age, p200 antibody levels were significantly lower than at birth ($P < 0.001$). Also Ro52, Ro60 and La antibody levels had decreased significantly at 4–5 weeks of age compared with levels at birth ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively). In the infants with first degree heart block at birth, this decrease in autoantibody levels was parallel with normalisation of the AV interval. Antibody levels continued to decrease, and at 1 year of age, no child had detectable maternal autoantibodies (Figure 2).

Ro52, Ro60 and La IgA and IgM antibodies in mothers and children

IgG is the dominating immunoglobulin isotype of Ro52, Ro60 and La antibodies, but IgA and IgM Ro/La antibodies have also been shown in patients.[25,26] Although IgG is actively transported across the placenta and reaches the foetus *in utero*, IgA and IgM isotypes do not cross the placenta. However, immunoglobulin transfer from the mother to the infant via breast milk during lactation is a further potential source for autoantibodies in the child after birth, including autoantigen-specific IgA and IgM. In Sweden, the tradition for breastfeeding is strong and the majority (21/30) of mothers in our study fed their babies by breast milk alone for the first 8 weeks, and most of them longer (Figure 3). We observed that there was no significant difference in the duration of breastfeeding in relation either to foetal diagnosis ($P > 0.05$) or to maternal diagnosis ($P > 0.05$).

Serum samples from the mothers and their children at 4–5 weeks of age were analysed by ELISA for IgA- and IgM-specific antibodies recognising Ro52, Ro60 and La antigens to investigate the degree of foetal uptake via breast milk and whether breastfeeding increased the risk for postnatal neonatal lupus symptoms. Several mothers had IgA or IgM antibodies recognising the Ro52, Ro60 or La autoantigens, but all infants had low or non-detectable levels of the corresponding IgA and IgM autoantibodies at 4–5 weeks of age (Figure 4). Serum levels were equally low in breastfed and non-breastfed children. However, IgA or IgM Ro52, IgA Ro60 and IgA and IgM La antibody levels were significantly higher in mothers of foetuses with AVB I-III compared with mothers of foetuses with normal conduction ($P < 0.03$, in all cases).

NLE skin lesions developed in five infants (16%). Maternal IgA and IgM Ro52, Ro60 or La antibody levels were not significantly associated with development of skin lesions in the neonate. Interestingly, however, anti-La IgG antibody levels were significantly

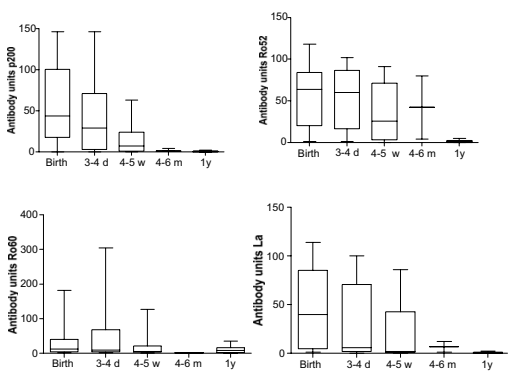


Figure 2 Clearing of Ro and La autoantibodies from the circulation of the infant. Levels of p200, Ro52, Ro60 and La antibodies were investigated in samples taken at birth (cord blood), at 3–4 days, 4–5 weeks, 4–6 months and 1 year using ELISA with recombinant antigens and synthetic p200 peptide.

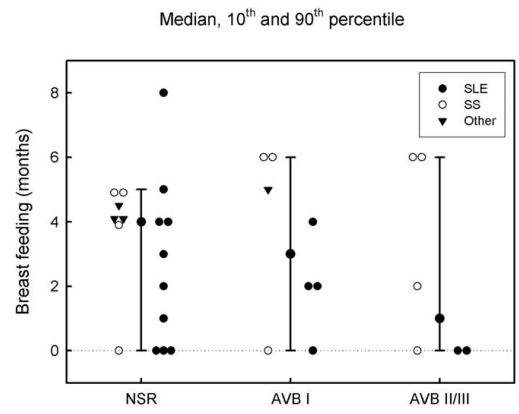


Figure 3 Breastfeeding period of infants born to Ro52-antibody positive mothers. Period (months) of breastfeeding postnatally of children included in the study. Foetal cardiac diagnosis is indicated as atrioventricular block (AVB) I and AVB II/III. Maternal rheumatic diagnosis is indicated as SLE, Sjögren's syndrome (SS) or other. NSR: normal sinus rhythm.

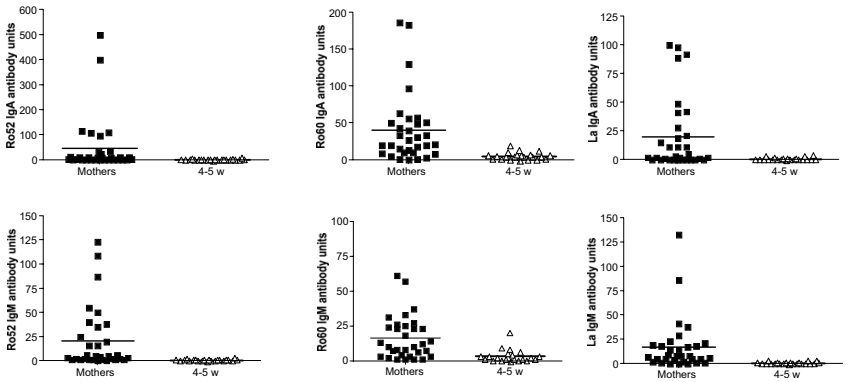


Figure 4 Ro52, Ro60 and La IgM and IgA autoantibodies in mothers and their infants. Levels of Ro52, Ro60 and La IgA and IgM autoantibodies were determined by ELISA in maternal samples and samples from infants at 4–5 weeks of age.

higher in mothers of children who developed skin lesions ($P < 0.02$). Of the infants who developed NLE skin lesions, one child was breastfed for 4 months, two were breastfed for 2 months and two were not breastfed. Thus, skin lesions appear not to depend on the baby being breastfed.

Discussion

Foetal exposure to Ro/SSA and La/SSB autoantibodies may lead to development of NLE. During pregnancy, maternal IgG autoantibodies are transported to the foetus via placenta, whereas breast milk is a potential source of autoantibodies for the infant after birth. To investigate levels of postnatal autoantibodies in infants born to Ro/SSA positive mothers and their correlation to NLE manifestations, we followed a cohort of 32 children from birth to 1 year of age with frequent serum sampling and clinical follow-up.

All included mothers were Ro/SSA positive, and as has been previously shown,[11,24] Ro52-p200 levels were higher in pregnancies where AVB II or III developed than in pregnancies where the foetus had normal AV conduction. Sera from the majority of the mothers contained IgG autoantibodies recognising all three heart block-associated autoantigens Ro52, Ro60 and La. All three autoantibody specificities were transferred to the neonate, and they were present in the circulation of the infants at birth. Transfer of Ro52 autoantibodies of IgG subclasses has previously been described, and a predominance of IgG1 was shown in the foetus.[27] After birth, the foetal levels of Ro and La IgG autoantibodies, including p200 antibody levels, rapidly decreased and were significantly lower at 4–5 weeks of age. The infantile decrease of IgG autoantibodies is in accordance with the half-life of circulating immunoglobulins, suggesting that after placental detachment, the source of immunoglobulins is markedly decreased or ended. The decrease in autoantibody levels occurred despite that breast milk was the only ingest for at least 1 month in more than 70% of the infants.

Breast milk may also contain IgA and IgM antibodies and has been shown to contain IgA and IgG autoantibodies specific for Ro52, Ro60 and La.[28] In our study, a subset of mothers had IgA or IgM antibodies binding Ro52, Ro60 and La antigens. Although breast milk from Ro/La antibody positive mothers has been described to contain IgA and IgG specific for Ro52, Ro60 and La,[28] the levels of Ro52-, Ro60- and La-specific IgA and IgM were low or not detectable in infants of our study at 4–5 weeks of age. At the later time points of infant serum sampling in our study, all specific IgA and IgM antibody levels were non-detectable (data not shown). Thus, although the maternal milk may contain autoantigen-specific IgA and IgM antibodies, it appears that the dose or foetal uptake is not enough to make the infant seropositive. Further, also IgG antibodies specific for Ro and La antibodies decreased rapidly in all infants although the majority of the children were breastfed, emphasising the predominant role of placental materno-foetal transfer of antibodies in supplying the foetus with specific immunoglobulins. However, a significant correlation of IgA or IgM Ro52, IgA Ro60 and IgA and IgM La antibody levels was noted in mothers of foetuses with AVB I-III compared with mothers of foetuses with normal conduction. As IgA and IgM antibodies do not cross the placenta and are not

present in the neonate at birth, the correlation of autoantigen-specific maternal IgA and IgM antibodies with heart block in the foetus is probably rather a reflection of the maternal autoreactive immune system per se than directly causative of congenital heart block.

Because NLE skin lesions occurred in five of the children, we correlated the skin manifestations with breastfeeding. We found that NLE skin lesions developed both in breastfed and in non-breastfed infants, as was observed also by Askanase *et al*.[28] Even if the numbers are limited, these observations suggest that breast feeding is not involved in the development of skin manifestations. These findings are in line with our findings that breast milk is not a predominant source of transfer of heart block-associated autoantibodies. Therefore, the pathogenesis of NLE skin lesions is likely to be coupled to the placental transfer of autoreactive antibodies recognising skin-specific epitopes, and our data indicate that La antibodies may be involved in inducing NLE skin lesions as mothers of infants with NLE skin lesions had significantly higher La IgG levels.

The decrease in autoantibody levels in three neonates affected by first degree AVB at birth without a history of higher degree of block was paralleled by normalisation of AV conduction time. This is in accordance with the findings of Cimaz *et al*.,[29] who described concomitant disappearance of ECG abnormalities and Ro52 and Ro60 autoantibodies in infants without congenital heart block (CHB) born to Ro/SSA positive mothers. These findings further underscore a role of materno-foetal transfer of autoantibodies in affecting the foetal cardiac conduction system because disappearance of the maternal autoantibodies from the foetal circulation led to normalised foetal conduction in cases were a permanent damage had not developed.

Our results show that maternal autoantibodies in the infant decrease rapidly during the first few weeks of life and have reached non-detectable levels at 1 year of age. The antibody levels decrease also in breastfed infants, and breast feeding does not appear to delay conversion from AVB I to normal conduction. Further, refraining from breastfeeding did not prevent NLE skin lesions, and our interpretation is that there is no reason not to recommend breastfeeding to mothers with Ro/La autoantibodies.

Acknowledgements

This study was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, Karolinska Institutet, King Gustaf V:s 80-year Foundation, the Heart-Lung Foundation, and the Swedish Rheumatism Association.

References

1 Scott JS, Maddison PJ, Taylor PV, Esscher E, Scott O, Skinner RP. Connective-tissue disease, antibodies to ribonucleoprotein, and congenital heart block. *N Engl J Med* 1983; **309**: 209–212.
2 Buyon JP, Clancy RM. Neonatal lupus: basic research and clinical perspectives. *Rheum Dis Clin North Am* 2005; **31**: 299–313.
3 Wahren-Herlenius M, Sonesson SE. Specificity and effector mechanisms of autoantibodies in congenital heart block. *Curr Opin Immunol* 2006; **18**: 690–696.
4 Buyon JP, Hiebert R, Copel J, *et al*. Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. *J Am Coll Cardiol* 1998; **31**: 1658–1666.
5 Sonesson SE, Salomonsson S, Jacobson L-A, Bremme K, Wahren-Herlenius M. Signs of first-degree heart block occur in one-third of fetuses of pregnant women with anti-SSA/Ro52-kd antibodies. *Arthritis Rheum* 2004; **50**: 1253–1261.
6 Litsey SE, Noonan JA, OConnor WN, Cottrill CM, Mitchell B. Maternal connective tissue disease and congenital heart block. Demonstration of immunoglobulin in cardiac tissue. *N Engl J Med* 1985; **312**: 98–100.
7 Clancy RM, Kapur RP, Molad Y, Askanase AD, Buyon JP. Immunohistologic evidence supports apoptosis, IgG deposition, and novel macrophage/fibroblast crosstalk in the pathologic cascade leading to congenital heart block. *Arthritis Rheum* 2004; **50**: 173–182.
8 Buyon JP, Winchester RJ, Slade SG, *et al*. Identification of mothers at risk for congenital heart block and other neonatal lupus syndromes in their children. Comparison of enzyme-linked immunosorbent assay and immunoblot for measurement of anti-SS-A/Ro and anti-SS-B/La antibodies. *Arthritis Rheum* 1993; **36**: 1263–1273.
9 Julkunen H, Kurki P, Kaaja R, *et al*. Isolated congenital heart block. Long-term outcome of mothers and characterization of the immune response to SS-A/Ro and to SS-B/La. *Arthritis Rheum* 1993; **36**: 1588–1598.
10 Dörner T, Chaoui R, Feist E, Goldner B, Yamamoto K, Hiepe F. Significantly increased maternal and fetal IgG autoantibody levels to 52 kD Ro (SS-A) and La(SS-B) in complete congenital heart block. *J Autoimmun* 1995; **8**: 675–684.
11 Salomonsson S, Dorner T, Theander E, Bremme K, Larsson P, Wahren-Herlenius M. A serologic marker for fetal risk of congenital heart block. *Arthritis Rheum* 2002; **46**: 1233–1241.
12 Fritsch C, Hoebeke J, Dali H, *et al*. 52-kDa Ro/SSA epitopes preferentially recognized by antibodies from mothers of children with neonatal lupus and congenital heart block. *Arthritis Res Ther* 2006; **8**: R4.
13 Salomonsson S, Sonesson SE, Ottosson L, *et al*. Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block. *J Exp Med* 2005; **201**: 11–17.
14 Brucato A, Frassi M, Franceschini F, *et al*. Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. *Arthritis Rheum* 2001; **44**: 1832–1835.
15 Schmidt KG, Ulmer HE, Silverman NH, Kleinman CS, Copel JA. Perinatal outcome of fetal complete atrioventricular block: a multicenter experience. *J Am Coll Cardiol* 1991; **17**: 1360–1366.
16 Waltuck J, Buyon JP. Autoantibody-associated congenital heart block: outcome in mothers and children. *Ann Intern Med* 1994; **120**: 544–551.
17 Vitali C, Bombardieri S, Jonsson R, *et al*. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002; **61**: 554–558.
18 Tan EM, Cohen AS, Fries JF, *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271–1277.

19 Arnett FC, Edworthy SM, Bloch DA, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; **31**: 315–324.
20 Andelfinger G, Fouron JC, Sonesson SE, Proulx F. Reference values for time intervals between atrial and ventricular contractions of the fetal heart measured by two Doppler techniques. *Am J Cardiol* 2001; **88**: 1433–1436.
21 Skog A, Wahren-Herlenius M, Bremme K, Sonesson S-E. Outcome and growth of infants fetally exposed to heart block-associated maternal anti-Ro52/SSA autoantibodies. *Pediatrics* 2008; **121**: e803–e809.
22 Elagib KEE, Tengnér P, Levi M, *et al*. Immunoglobulin variable genes and epitope recognition of human monoclonal anti-Ro 52-kd in primary Sjögren's syndrome. *Arthritis Rheum* 1999; **42**: 2471–2478.
23 Ottosson L, Salomonsson S, Hennig J, *et al*. Structurally derived mutations define congenital heart block related epitopes within the 200-239 amino acid stretch of the Ro52 protein. *Scand J Immunol* 2005; **61**: 109–118.
24 Strandberg L, Winqvist O, Sonesson S-E, *et al*. Antibodies to amino acid 200-239 (p200) of Ro52 as serologic markers for the risk of developing congenital heart block. *Clin Exp Immunol* 2008; **154**: 30–37.
25 Wahren M, Ringertz NR, Pettersson I. IgM and IgG subclass distribution of human anti-Ro/SSA 60 kDa autoantibodies. *Scand J Immunol* 1994; **39**: 179–183.
26 Tengnér P, Halse A-K, Haga H-J, Jonsson R, Wahren-Herlenius M. Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjögren's syndrome. *Arthritis Rheum* 1998; **41**: 2238–2248.
27 Strandberg L, Salomonsson S, Bremme K, Sonesson SE, Wahren-Herlenius M. Ro52, Ro60 and La autoantibody levels and Ro52 IgG subclass profiles longitudinally throughout pregnancy in congenital heart block pregnancies. *Lupus* 2006; **15**: 346–353.
28 Askanase AD, Miranda-Carus ME, Tang X, Katholi M, Buyon JP. The presence of IgG antibodies reactive with components of the SSA/Ro- SSB/La complex in human breast milk: implications in neonatal lupus. *Arthritis Rheum* 2002; **46**: 269–271.
29 Cimaz R, Meroni PL, Brucato A, *et al*. Concomitant disappearance of electrocardiographic abnormalities and of acquired maternal autoantibodies during the first year of life in infants who had QT interval prolongation and anti-SSA/Ro positivity without congenital heart block at birth. *Arthritis Rheum.* 2003; **48**: 266–268.

II

Maternal MHC regulates generation of pathogenic antibodies and fetal MHC-encoded genes determine susceptibility in congenital heart block

Linn Strandberg[1], Aurelie Ambrosi[1], Maja Jagodic[2], Vijole Dzikaite[1], Peter Janson[3], Mohsen Khademi[2], Stina Salomonsson[1], Lars Ottosson[1], Robert Klauninger[1], Ulrika Ådén[4], Sven-Erik Sonesson[4], Maria Sunnerhagen[5], Katrien L. de Graaf[6], Adnane Achour[7], Ola Winqvist[3], Tomas Olsson[2], Marie Wahren-Herlenius[1]

[1] Rheumatology Unit, Department of Medicine, [2] Neuroimmunology Unit, Department of Clinical Neuroscience, [3] Clinical Allergy Research Unit, Department of Medicine, [4] Department of Woman and Child Health, Karolinska Institutet, [5] Molecular Biotechnology, Linköping University, [6] Hertie Institute for clinical Brain Research, University of Tübingen, Germany, [7] Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Stockholm, Sweden.

Total character count: 18,651 characters
Key words: autoimmunity, congenital heart block, Ro52, MHC
Abbreviations: congenital heart block (CHB), atrioventricular block (AVB)
Short title: Fetal susceptibility in congenital heart block

Corresponding author and reprint requests:
Marie Wahren-Herlenius
Rheumatology Unit, Department of Medicine
CMM L8:04, Karolinska Institutet
SE-171 76 Stockholm, SWEDEN
Telephone: +46-8-51773431, Fax: +46-8-51775562
E-mail: Marie.Wahren@ki.se

ABSTRACT

Congenital heart block develops in fetuses of Ro52-antibody positive women. The heart block inducing autoantibodies are transferred to the fetus during pregnancy and affect the developing heart. A subsequent fetus may however not be affected, despite the presence of maternal autoantibodies. This indicates that there are additional, yet unidentified, factors critical for development of congenital heart block. Here we demonstrate that besides the maternal MHC controlling antibody specificity, congenital heart block depends on fetal susceptibility, which is determined by fetal MHC-encoded genes. Using MHC congenic rat strains we show that heart block develops in rat pups of three strains carrying MHC haplotype $RT1^{av1}$ (DA, PVG.AV1 and LEW.AV1) after maternal Ro52 immunization, while pups from LEW rats ($RT1^{l}$) rarely developed block. Different anti-Ro52 antibody fine-specificities were generated in $RT1^{av1}$ versus $RT1^{l}$ animals. Maternal and fetal influence was determined in an F2 cross between LEW.AV1 and LEW strains, which revealed higher susceptibility in $RT1^{l}$ than $RT1^{av1}$ pups once pathogenic Ro52-antibodies were present. This was further confirmed in that $RT1^{l}$ pups more frequently developed heart block than $RT1^{av1}$ pups after passive transfer of $RT1^{av1}$ anti-Ro52 sera, and by microarray analysis of fetal hearts that revealed increased expression of distinct MHC-encoded genes in $RT1^{l}$ compared to $RT1^{av1}$ pups. Our findings suggest that generation of the pathogenic Ro52 antibodies is restricted by maternal MHC, and that a different set of genes encoded by the fetal MHC locus regulate susceptibility and determine the fetal disease outcome in anti-Ro52 positive pregnancies.

INTRODUCTION

Pregnancy in autoimmune conditions is often marred by complications, and the fetus may be directly affected by maternal autoantibodies to develop neonatal lupus, myastenia or hypothyroidism. Congenital heart block is a potentially lethal manifestation of the neonatal lupus syndrome, which develops in fetuses of rheumatic women with Ro52 autoantibodies (1). In the rheumatic mothers, production of Ro52-antibodies has been repeatedly linked to MHC class II DRB1*03 alleles (2, 3). During pregnancy, the maternal autoantibodies cross the placenta and bind cardiomyocytes in the heart, in which the atrioventricular (AV) conduction system is disrupted by inflammation with subsequent fibrosis and calcification leading to a complete AV block (4, 5). Immunization of mice or rats with Ro52 or Ro52-derived peptides leads to AV block in the offspring, and these animal models have been used to define the nature and quality of the pathogenic autoantibodies that mediate heart block (6, 7).

The vast majority of mothers of infants with congenital heart block carry Ro52 antibodies, but the risk for heart block in a single Ro-positive pregnancy is only 1-2% (8, 9). In mothers who have children with congenital heart block, the risk for an affected child is increased to 20% in subsequent pregnancies (10), but heart block does not always develop despite persisting maternal anti-Ro52 autoantibodies (11). Although maternal autoantibodies are crucial in the development of fetal heart block, the clinically observed recurrence rate of 20% suggests that additional factors are critical for establishing the heart block. Maternal disease severity and infections during pregnancy have been investigated as potential risk factors, but were not found to contribute to the congenital heart block (12-14).

To address the hypothesis that susceptibility may relate to the genetic make-up of the fetus itself, we used the Ro52 immunization-induced animal model of congenital heart block to study the

role of different MHC (RT1) and non-MHC genes in susceptibility to the heart block in various rat strains. Our data demonstrate that production of Ro52-antibodies with pathogenic specificity was restricted by maternal MHC, while a different set of MHC-encoded genes in the fetus conferred increased susceptibility to congenital heart block and determined the disease outcome in Ro52-positive pregnancies.

RESULTS AND DISCUSSION

Maternal MHC regulates generation of pathogenic antibody-specificities inducing heart block

Genetic influence has been implied in the development of congenital heart block (15), but has not been explored experimentally. To define the contribution of MHC and non-MHC genes, various rat strains that differed in MHC and background genes were immunized with Ro52 and fetal heart block assessed in the newborn pups by ECG. In a pilot study, two strains, DA ($RT1^{av1}$) and LEW ($RT1^{l}$), differing in both MHC and non-MHC genes were investigated. The strains have previously been shown to differ in their susceptibility to other induced autoimmune diseases such as EAE (16) and collagen-induced arthritis (17, 18). Congenital heart block developed three-fold more often in pups of the DA strain than in LEW pups (data not shown), and a study was therefore designed to dissect the influence of MHC and non-MHC genes by use of several inbred strains and MHC congenic rats (Supplemental Table 1).

Rats of four different strains were included. Three shared the same MHC haplotype $RT1^{av1}$ (DA, PVG.AV1 and LEW.AV1), and the fourth strain (LEW) shared the background genes with the congenic LEW.AVI strain, but differed in the MHC genes, as the native RT1 locus of the LEW strain is L ($RT1^{l}$). After Ro52-immunization of rat females and mating (Figure 1A), first-degree AV block developed in 45% of the DA ($RT1^{av1}$) pups, 44% of the PVG.AV1 and 47% of the LEW.AV1 pups. However, only 10% of pups in the LEW ($RT1^{l}$) group developed AV block (Figure 1B). Rats from each strain immunized with control protein (maltose-binding protein, MaBP) showed no heart block development (Figure 1B). The pups of Ro52 immunized animals in the three rat strains sharing the $RT1^{av1}$ haplotype (DA, PVG.AV1, LEW.AV1) had significantly longer PR intervals than did the

LEW ($RT1^{l}$) pups ($p<0.001$), while there was no significant difference in PR-intervals between the three $RT1^{av1}$ carrying strains (Figure 1C and D).

Induction of congenital heart block depends on the presence of specific pathogenic autoantibodies binding to an epitope within the stretch of amino acid (aa) residues 200-239 (p200) of Ro52 (7). To analyze whether the difference in development of congenital heart block between $RT1^{av1}$ and $RT1^{l}$ haplotype carrying strains related to the level or specificity in produced Ro52-binding antibodies, sera from the pups and mothers were analyzed using ELISA with p200 peptide and mutated p200 peptides (Figure 1E and F). The mutated peptides denoted pZIP and pOUT were generated to distinguish binding specificty of antibodies to different epitopes within the 40 aa p200 peptide (7), and mutated amino acids are indicated in Figure 1E and F. Titers of antibodies binding the p200 peptide of Ro52 did not differ between strains or animals affected or not by heart block (Figure 1G, H), but notably the binding specificity of Ro52-p200 immunization-induced antibodies differed between rats with $RT1^{av1}$ and $RT1^{l}$ MHC alleles as demonstrated by differential affinity to the mutated p200 peptides pZIP and pOUT (Figure 1I). This indicates that although both $RT1^{av1}$ and $RT1^{l}$ bearing strains generate p200 binding antibodies in response to Ro52 immunization, maternal MHC restricts the fine specificity of the generated antibodies.

To investigate the mechanism relating to generation of different p200 immune responses in the LEW $RT1^{av1}$ and $RT1^{l}$ strains, we first determined the relative role of MHC class II molecules in AV1 carrying strains in generating T cell responses. The rat has two major MHC class II molecules; RT1.B (orthologous to human HLA-DQ) and RT1.D (orthologous to human HLA-DR). p200-specific T cell lines from LEW.AV1 rats were stimulated by p200 in the presence of antibodies blocking RT1.B (Ox-6) or RT1.D (Ox-17). Using proliferation assays and IFN-γ formation in ELISPOT, we could clearly demonstrate a dominant role for RT1.B in activation of

AV1-derived p200-specific clonal T cells, since blocking with Ox-6 significantly inhibited the T cell responses and Ox-17 antibodies had little effect on proliferation or IFN-γ production (Figure 2A and B).

The RT1.B α and β chains for AVI and L have been sequenced (19, 20), and an alignment suggested several critical differences in both α and β chains (Figure 2C). Molecular models of the peptide binding clefts of RT1.B AV1 and L (Figure 2D) were created based on their sequence homology to the MHC class II molecules I-A^u , I-A^b and HLA-DR4 (21-23). A comparison of the molecular models of RT1.B AVI and L reveal several important structural and electrostatic differences within the peptide-binding cleft, suggesting that the peptide repertoire bound and presented by RT1.B AVI will differ from RT1.B L. The most important modifications are localized within and around the P1 pocket (Figure 2D), used by most MHC class II molecules as a major anchoring site (24). The phenylalanine and the positively charged lysine residues at position 28 and 35 of the α-chain of RT1.B AVI are replaced by a histidine and a negatively charged glutamate, respectively, in the RT1.B L. Thus, the composition of the structural and electrostatic properties of the P1-pocket will differ between RT1.B AVI and RT1.B L. Furthermore, the β-chain glycine residue at position 26 in RT1.B AVI is modified to a larger negatively charged aspartate that points towards the middle section of the peptide binding cleft, most probably interacting with peptide residue p4 in the antigenic peptide. Finally, important structural and electrostatic modifications are present in the C-terminal part of the peptide binding clefts of RT1.B AVI and L, affecting the pocket that interacts with the peptide residue p9. Here, the negatively charged β-chain residue aspartate 57 in RT1.B AVI is replaced by a serine in RT1.B L, while surrounding residues such as α-chain residues T69 and L76 in RT1.B AVI are substituted to two isoleucines in RT1.B L. In conclusion, the structural and electrostatic differences observed in N- and C-termini as well as within the middle section of the

peptide binding clefts of RT1.B AVI and L suggest that the peptide-binding motifs of the epitopes bound to the two MHC class II molecules will be different, and that the conformation of most peptides bound in the two clefts will also differ. Ultimately the sequential and structural comparison suggests that different peptides will be preferentially presented by the AV1 and L RT1.B MHC class II molecules.

To analyze the functional impact of these potentially important differences in peptide presentation we performed an analysis of the T cell receptor usage by TCRBV spectratyping of p200 specific T cell lines from LEW.AV1 and LEW rats. Our analysis showed that the TCRBV usage is different between LEW.AV1 and LEW strains in p200 specific T cell lines. The most striking difference was the dominant use of TCRBV1 in LEW.AV1-derived p200 specific T cell lines not observed in T cells lines from Lew.L animals, and a clear expansion of a single peak in TCRBV7 suggestive of a clonal expansion in Lew.AV1 which was not observed in the T cell lines from LEW animals (Figure 2E and Supplemental Figure 1). The TCRBV1 and 7 spectratypes were normally distributed in naïve T cells from both strains (Supplementary Figure 1), and constitute less than 1% and 2% respectively of the total TCRBV repertoire in naïve Lewis rats (25).

From these experiments we conclude that the fine specificities of both B and T cell responses in Ro52-immunized LEW.AV1 and LEW rats differ, and that generation of T cells with different specificity and TCRBV usage most probably relates to differential peptide presentation by the MHC in LEW.AV1 and LEW strains.

Increased susceptibility to AV block in rat pups is dominantly inherited and associated with the MHC RT1^{l} allele

The specificities of generated anti-p200 antibodies differed between RT1^{av1} and RT1^{l} strains,

and the lower frequency of AV block in $RT1^{l}$ pups could thus depend either on a lack of generation of pathogenic anti-Ro52 antibody specificities in $RT1^{l}$ rat mothers, or fetal resistance in $RT1^{l}$ pups to disease. In order to differentiate whether the observed MHC-linked effects in the development of heart block were due to maternal or fetal factors, we performed an F2 cross between the susceptible LEW.AV1 rat strain and the resistant LEW rat strain (Figure 3A). LEW.AV1 and LEW rats were mated, and the F1 heterozygous females ($RT1^{av1/l}$) were immunized with Ro52. These F1 females were then mated with heterozygous F1 males ($RT1^{av1/l}$) to produce homozygous $RT1^{av1}$, $RT1^{l}$ or heterozygous $RT1^{av1/l}$ offspring. Genotyping of the F2 generation pups revealed that RT1 haplotype frequencies were 23% $RT1^{av1}$, 23% $RT1^{l}$, and 54% heterozygous $RT1^{av1/l}$. ECG was performed at birth to detect heart block in the pups of Ro52-immunized mothers. Analysis of prolonged PR intervals with respect to the pup genotype revealed that a homozygous $RT1^{l}$ or heterozygous $RT1^{av1/l}$ genotype in the pup correlated with significantly higher PR intervals than in homozygous $RT1^{av1}$ pups ($p<0.05$) (Figure 3B, C). This indicates that the MHC allele $RT1^{l}$ in the pups in fact confers a higher susceptibility to heart block induced by Ro52 antibodies than $RT1^{av1}$ alleles in the pup. From this follows that the findings of the immunization study of DA, PVG.AV1, LEW.AV1 and LEW rats did indeed depend on a difference in maternal MHC haplotype regulation of pathogenic antibody specificity, and not fetal resistance in LEW pups.

The p200 antibody binding profile in the heterozygous $RT1^{av1/l}$ Ro52-immunized rats in the F1 generation was similar to that of the $RT1^{av1}$ rats on the three backgrounds DA/PVG/LEW (Figure 3D), indicating that generation of pathogenic heart block inducing antibodies is a dominant $RT1^{av1}$-linked trait. We also noted that pups descendent from F1 parents with L/AV1 x L/AV1 had more affected conduction than pups descendent from AVI/L x AV1/L parents (Figure 3E, $p<0.01$, $RT1^{av1}$ and $RT1^{l}$ homozygous pups only), and that pups which inherit LL from the maternal founder rather

than ll from the paternal founder have longer PR intervals (Figure 3F, $p<0.001$ Mann-Whitney U-test). This suggests an additional layer of epigenetic regulation of congenital heart block, as previously indicated for other autoimmune conditions (26, 27).

In all, the data from the F2 cross demonstrate that generation of maternal pathogenic heart block-inducing Ro52 antibodies is a dominant trait linked to the $RT1^{av1}$ MHC haplotype, and that pups with $RT1^{l}$ alleles are more susceptible to the cardio-pathogenic effects of these autoantibodies.

Congenital heart block is induced in $RT1^{l}$ pups following transfer of $RT1^{av1}$ Ro52-immune serum to pregnant $RT1^{l}$ rats

To directly investigate the increased susceptibility to develop heart block in pups with $RT1^{l}$ alleles compared to pups with $RT1^{av1}$ alleles, we generated Ro52-immune serum in $RT1^{av1}$ rats (DA) for transfer experiments. Sera from 10 immunized animals were pooled for transfer, and non-immune serum from $RT1^{av1}$ rats (DA) was used as a control in parallel transfer experiments. Two ml of serum were given i.p. to homozygous pregnant $RT1^{av1}$ (DA) or $RT1^{l}$ (LEW) rats on day 6 and 9 of pregnancy, and ECG was recorded from the pups at birth. Also in this experimental setting, using passive transfer of Ro52 antibodies generated in $RT1^{av1}$ rats, the $RT1^{l}$ pups displayed higher susceptibility to pathogenic Ro52 antibodies and had longer PR intervals than $RT1^{av1}$ pups (Figure 4A). Transfer of Ro52 antibodies to the pups was confirmed by ELISA using pup serum (Figure 4C and D). Transfer of increasing amounts of p200 specific antibodies induced heart block also in AV1 pups (data not shown), but at the lower antibody levels used in our transfer experiments and compared to the immunization model (Figure 4E and F), it was clear that pups with $RT1^{l}$ MHC are more susceptible to congenital heart block (Figure 4A). In all, our results demonstrate that higher susceptibility to heart block development is linked to the $RT1^{l}$ MHC haplotype in the pups.

Specific genes linked to fetal susceptibility for congenital heart block

To identify specific genes linked to fetal susceptibility for congenital heart block, we first analyzed the genetic differences in the L and AV1 RT1 loci. Using microsatellites, the LEW.AV1 congenic rat was defined by a <7Mb region at the telomeric end of chromosome 20 originating from the AV1 haplotype (Figure 5A), and the L and AV1 loci differed in 36 of 86 investigated SNPs within this interval (28), Figure 5B. The identified polymorphisms are not randomly dispersed throughout the RT1 locus, but 3-4 clusters were observed, Figure 5B. This suggests that factors relating to the different susceptibility in the two strains may be encoded by genes located in, or not far from, the SNP clusters.

To identify specific MHC-encoded genes with altered expression linked to the difference in fetal susceptibility in AV1 and L animals, we performed microarray mRNA expression analyses of hearts from LEW and LEW.AV1 pups of mothers that had received normal non-immune or anti-Ro52 positive serum (AV1-derived) in passive transfer. A comparison of the expression of MHC-encoded genes in $RT1^{l}$ and $RT1^{av1}$ pups where the mother received control serum only, revealed that the non-classical HLA-DMb and RT1-T24-1 genes were upregulated in LEW pups compared to LEW.AV1 pups at $p<0.0005$ (data not shown). HLA-DMb was the most highly upregulated gene with a ratio of 4.1 LEW/LEW.AV1, emphasizing that even at the basal level there are important differences in the expression of these MHC-encoded genes between the two strains. The difference in expression of these two genes further increased in hearts from $RT1^{l}$ and $RT1^{av1}$ pups of mothers that had received anti-Ro52 positive immune serum, and HLA-DMb was five-fold higher expressed in LEW compared to Lew.AV1 ($p<0.0005$) in pups from mothers receiving anti-Ro52 positive serum (Figure 5C). We also identified four additional non-class I or II MHC-encoded genes (Bat5, TRIM26, G7c, ATP6v1G2) with an increased expression at $p<0.0005$ in LEW pups compared to LEW.AV1

pups, though the highest difference was still in the expression of HLA-DMb (Figure 5C). One gene, Clic2, was downregulated in LEW pups compared to LEW.AV1 pups after transfer of Ro52-positive serum. All genes with increased expression in $RT1^{l}$ pups after either normal serum or Ro52-positive serum treatment observed by microarray analysis were re-confirmed also by quantitative RT-PCR (Figure 5D and E). Down-regulation of Clic2 was not confirmed by the PCR analysis (Figure 5E).

In addition to MHC-encoded genes, a set of genes outside the 20p12 locus were also differentially expressed between the two strains. These were functionally annotated and predominantly belonged to the immune system, signaling and metabolic processes (Supplemental Figure 2), and may reflect secondary changes in expression patterns after disease onset. Notably, HLA-DMb however remained the most differentially expressed gene between the two strains at $p<0.0005$, even at the genome-wide analysis. These data functionally identify HLA-DMb as one of the possible candidate genes within the MHC locus that may regulate the differential susceptibility observed between the LEW and LEW.AV1 pups in mediating congenital heart block development. However, there are at least five additional genes within the MHC locus not belonging to classical MHC class I or II, that are differentially expressed in the two strains in response to the pathogenic anti-Ro52 antibodies. All of these genes are potential candidates that may be mediating differential susceptibility.

In conclusion, our study confers strong evidence that allelic variants of genes within the MHC complex both affect the maternal ability to form pathogenic antibodies, as well as the fetal susceptibility to develop congenital heart block in response to these antibodies. Our data further show that these traits are linked to different alleles in the mother and the child, respectively. We demonstrate that maternal MHC-linkage is essential in generation of pathogenic antibodies by allelic differences in class II antigen presenting properties, also causing subtle differences in T cell

specificities. The class II genes of the $RT1^{av1}$ and $RT1^{l}$ haplotypes have also previously been characterized with regard to their different peptide binding spectra of both the RT1.B and D molecules (29), and the pathogenicity of the immune response following immunization with myelin antigens (30). Fetal susceptibility to the passively transferred antibodies related to a different MHC haplotype, and interestingly also in human patients the production of Ro52-antibodies is strongly linked to specific maternal MHC - A1, B8, DR3B1*03 (2, 3, 31) - while these particular maternal alleles are rarely present in the affected children (15, 32). Rather, human fetal susceptibility has been suggested to relate to HLA-Cw*03 (15). In addition to MHC-mediated effects, our studies also indicate an epigenetic influence in fetal susceptibility to congenital heart block, as has been observed for other autoimmune conditions (26, 27).

The rat MHC-complex encodes at least 220 genes (33), and we identified a specific upregulation of HLA-DMb along with five more genes in the susceptible $RT1^{l}$ strain in heart block development. HLA-DMb facilitates removal of the CLIP peptide and stabilizes the peptide-empty MHC class II molecules while the repertoire of peptides is selected for binding, and may thus be of great importance in the developing fetal inflammation which disrupts the atrioventricular conduction system in the affected heart and leads to the complete AV block (4, 5).

In summary, our findings show that generation of the pathogenic Ro52 antibodies is restricted by maternal MHC, while a different set of MHC-encoded genes in the fetus regulate susceptibility and determine the fetal outcome in Ro52-positive pregnancies.

MATERIALS AND METHODS

Experimental animals

DA (RT1av1), LEW.AV1 (RT1av1) and LEW (RT1l) rats were originally obtained from the Zentralinstitut for Versuchstierzucht (Prof. Hans Hedrich, Hannover, Germany). After introgressing the DA RT1av1 into the LEW strain, animals were backcrossed for 16 generations. PVG.AV1 (RT1av1) rats were originally obtained from Harlan UK Limited (Blackthorn, UK). Animals were kept and bred in the animal facility at the Center for Molecular Medicine at the Karolinska Institutet. All experimental protocols were approved by the Stockholm North Ethics Committee.

Recombinant proteins and synthetic peptides

Recombinant Ro52 protein was expressed from the pMAL vector (New England Biolabs) as a fusion to maltose binding protein (MaBP) or from the p6xHis vector and purified as described (34, 35). Wild-type MaBP protein was expressed from the pMAL vector and purified as described (34). Ro52 peptides p200, pZIP and pOUT were purchased from Thermo BioSciences.

Immunizations, serum transfer and ECG recordings

Six-week-old rats were immunized with 100 μg Ro52 protein in complete Freund's adjuvant and boosted three times with 50 μg Ro52 protein in incomplete Freund's adjuvant. Rats were mated 2-4 weeks after last boost. On the day of delivery, three-lead electrocardiograms (ECGs) were recorded from conscious pups using four microelectrodes attached to a body clip (36). ECGs were sampled for 5 seconds four times per minute with a sampling rate of 1000 Hz. The ECG was digitalized and analyzed with Pharmlab (AstraZeneca). QRS-complexes were averaged and used to

calculate the PR-interval. AV block I was defined as the average of PR-intervals in control animals +2SD.

For serum transfer and microarray experiments, anti-sera were generated by the same protocol. Control sera were from non-immunized animals. Rats were injected i.p. with 2 ml serum on day 6 and 2 ml serum on day 9 of pregnancy.

ELISA for detection of Ro52 protein and peptide antibodies

ELISA was performed as described (7, 34) using p200 peptide and His-tagged Ro52. Rat sera were tested at a dilution of 1:500 (immunization studies) or 1:50 (serum transfer experiments), and all at 1:50 when comparing levels.

Generation of T cell lines

Female LEW.AV1 (n=4) or LEW rats (n=4) were immunized with 100 μg Ro52 protein in complete Freund's adjuvant at the base of the tail and draining lymph nodes removed on day 14 post immunization. Single cell suspensions were generated and cells cultured with restimulation using p200 peptide (10 μg/ml) and syngenic APCs in three rounds interspersed with IL-2 supplemented T cell medium to generate clonal T cells as previously described (37).

T cell proliferation and ELISPOT assays

T cell lines were plated with syngenic APCs in parallel sets of round-bottomed 96-well plates in culture medium with either concavalin A (5 μg/ml), p200 peptide (10 μg/ml) or p200 peptide with Ox-6 and/or Ox-17 (38) antibodies (10 μg/ml). After 48h cells were either pulsed for 16 h with 1 μCi [3H]thymidine per well and proliferation measured as counts per minute using a microplate liquid

scintillation counter (Wallac MicroBeta TriLux; PerkinElmer), or IFN-γ production analyzed in ELISPOT as previously described (39). Cells were incubated in the anti-rat IFN-γ pre-coated nitrocellulose plates for 20h, and spots were analyzed using an Elispot plate-reader (AID Elispot Reader System).

Molecular modelling of the peptide binding clefts of RT1.B AVI and RT1.B L

Sequence alignment was performed using the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/). Molecular models of the peptide binding clefts of RT1.B-AVI and RT1.B-L were created on the basis of their sequence homology to classical MHC class II molecules, using the SWISS-MODEL Protein Modeling Server (40). The crystal structures of HLA-DR4 (PDB code 1D5Z), I-A^b (1MUJ) and I-A^u (2PXY) were used as templates for the modelling. The coordinates of both models will be provided upon request. All figures were created using the program PYMOL (http://pymol.sourceforge.net/).

TCRBV spectratyping

Total RNA was extracted from T cell lines using an RNeasy mini kit (Qiagen). Reverse transcription was performed with random hexamer primers and Superscript reverse transcriptase (Invitrogen). The PCR was performed as previously (41)with 23 published individual TCRBV primers specific for TCRBV1-20 and a common 6-FAM labelled CB primer (41). The product was separated in capillary electrophoresis and analyzed by the Genescan software v3.7 (Applied Biosystems).

Polymorphic markers and genotyping of animals in the F2 cross

Genomic DNA was prepared from tail tips (42). D20Wox17, D20Wox18, D20Rat21, D20UW1, D20Rat41, D20Rat45, D20Mgh4, D20Rat50, D20Rat31, D20Rat33, D20Rat7 and D20Rat6 (http://rgd.mcw.edu/), covering 27.4 Mb of telomeric end of chromosome 20, were used to establish the borders of AV1 congenic interval and for subsequent typing of F2 rats. The SNP map of the AV1 and L RT1 loci was adapted from (28). Genotyping in the F2 cross was performed using three markers polymorphic for the MHC region between AV1 and L (D20Rat41, D20UW1 and D20Rat21). Primers were purchased from Proligo. PCR amplification was performed as previously described (43) with [γ-^{33}P]ATP end-labelling of the forward primer and using the following thermocycling protocol: initial denaturation at 94°C for 13 min, followed by 30 cycles of 94°C for 30s, 55°C for 1 min and 72°C for 1.5 min, followed by a final extension period at 72°C for 7 min. PCR products were size fractionated on 6% polyacrylamide gels and visualized by autoradiography. All genotypes were evaluated manually by two independent observers.

Microarray analyses

RNA from fetal hearts was purified using the TRIzol reagent (Invitrogen) followed by RNeasy Mini kits (Qiagen). The Agilent 2100 Bioanalyzer (Agilent Technologies) was used to confirm integrity of the RNA. RNA was prepared for microarray analysis using the whole transcript sense target labeling protocol (Affymetrix) and Rat Gene 1.0 ST arrays used to profile mRNA levels from individual subjects. The scanned output files were analyzed using the Affymetrix GeneChip Command Console. In the Expression consol (v1.1), background correction was performed by PM-GCBG, data normalized by Global Median and resulting data were summarized using the PLIER software. Gene expression was compared by student's unpaired t-test and the level of significance set to $p<0.0005$.

Real-time PCR

RNA from fetal hearts was purified using the TRIzol reagent (Invitrogen) followed by RNeasy Mini kits (Qiagen). The Agilent 2100 Bioanalyzer (Agilent Technologies) was used to confirm integrity of the RNA. Reverse transcription was performed with random hexamer primers and Superscript reverse transcriptase (Invitrogen). The primers used are defined in Supplemental Table 2. A SYBR Green–based protocol was applied (44), and PCR products were analyzed by melting curve analysis to confirm a single product. The mRNA levels were calculated using the standard curve method and normalized to HPRT mRNA.

Statistical analysis

Statistical analysis for experiments other than microarray analysis was performed using Statistica 7.0 (Statsoft, Tulsa, OK, USA). Non-parametric analysis with Mann-Whitney U-test and ANOVA was used. The level of significance was set at $p < 0.05$.

Online supplemental material

Table S1 details strains and animal number used in the study, Table S2 specifies non-classical MHC encoded genes identified in micoarray experiments and defines primers used in RT-PCR. Figure S1 illustrates TCRVB1 in naïve T cells and T cell lines from $RT1^{av1}$ and $RT1^{l}$ derived Ro52-p200 specific T cell lines. Figure S2 shows analysis of microarray data and functional annotation of genes.

ACKNOWLEDGMENTS

We sincerely thank Kristina Becanovic for assistance with immunizations, Marika Rönnholm and David Brodin for excellent technical assistance and computation at the Affymetrix core facility at Novum, BEA, Bioinformatics and Expression Analysis. The study was supported by the Swedish Research Council, the Swedish Foundation for Strategic Research, the Karolinska Institute, the Heart-Lung Foundation, the Swedish Rheumatism Association and EURATools (LSHG-CT-2005-019015). The authors have no conflicting financial interests.

REFERENCES

1. Wahren-Herlenius, M., and S.E. Sonesson. 2006. Specificity and effector mechanisms of autoantibodies in congenital heart block. *Curr Opin Immunol* 18:690-696.
2. Gottenberg, J.E., M. Busson, P. Loiseau, J. Cohen-Solal, V. Lepage, D. Charron, J. Sibilia, and X. Mariette. 2003. In primary Sjogren's syndrome, HLA class II is associated exclusively with autoantibody production and spreading of the autoimmune response. *Arthritis Rheum* 48:2240-2245.
3. Nakken, B., R. Jonsson, K.A. Brokstad, K. Omholt, A.H. Nerland, H.J. Haga, and A.K. Halse. 2001. Associations of MHC class II alleles in Norwegian primary Sjogren's syndrome patients: implications for development of autoantibodies to the Ro52 autoantigen. *Scand J Immunol* 54:428-433.
4. Litsey, S.E., J.A. Noonan, W.N. O'Connor, C.M. Cottrill, and B. Mitchell. 1985. Maternal connective tissue disease and congenital heart block. Demonstration of immunoglobulin in cardiac tissue. *N Engl J Med* 312:98-100.
5. Clancy, R.M., R.P. Kapur, Y. Molad, A.D. Askanase, and J.P. Buyon. 2004. Immunohistologic evidence supports apoptosis, IgG deposition, and novel macrophage/fibroblast crosstalk in the pathologic cascade leading to congenital heart block. *Arthritis Rheum* 50:173-182.
6. Miranda-Carus, M.E., M. Boutjdir, C.E. Tseng, F. DiDonato, E.K. Chan, and J.P. Buyon. 1998. Induction of antibodies reactive with SSA/Ro-SSB/La and development of congenital heart block in a murine model. *J Immunol* 161:5886-5892.

7. Salomonsson, S., S.E. Sonesson, L. Ottosson, S. Muhallab, T. Olsson, M. Sunnerhagen, V.K. Kuchroo, P. Thoren, E. Herlenius, and M. Wahren-Herlenius. 2005. Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeostasis, and mediate congenital heart block. *J Exp Med* 201:11-17.

8. Brucato, A., M. Frassi, F. Franceschini, R. Cimaz, D. Faden, M.P. Pisoni, M. Muscara, G. Vignati, M. Stramba-Badiale, L. Catelli, A. Lojacono, I. Cavazzana, A. Ghirardello, F. Vescovi, P.F. Gambari, A. Doria, P.L. Meroni, and A. Tincani. 2001. Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. *Arthritis Rheum* 44:1832-1835.

9. Cimaz, R., D.L. Spence, L. Hornberger, and E.D. Silverman. 2003. Incidence and spectrum of neonatal lupus erythematosus: a prospective study of infants born to mothers with anti-Ro autoantibodies. *J Pediatr* 142:678-683.

10. Solomon, D.G., A. Rupel, and J.P. Buyon. 2003. Birth order, gender and recurrence rate in autoantibody-associated congenital heart block: implications for pathogenesis and family counseling. *Lupus* 12:646-647.

11. Strandberg, L., S. Salomonsson, K. Bremme, S. Sonesson, and M. Wahren-Herlenius. 2006. Ro52, Ro60 and La IgG autoantibody levels and Ro52 IgG subclass profiles longitudinally throughout pregnancy in congenital heart block risk pregnancies. *Lupus* 15:346-353.

12. Lockshin, M.D., E. Bonfa, K. Elkon, and M.L. Druzin. 1988. Neonatal lupus risk to newborns of mothers with systemic lupus erythematosus. *Arthritis Rheum* 31:697-701.

13. Derksen, R.H., and J.F. Meilof. 1992. Anti-Ro/SS-A and anti-La/SS-B autoantibody levels in relation to systemic lupus erythematosus disease activity and congenital heart block. A

longitudinal study comprising two consecutive pregnancies in a patient with systemic lupus erythematosus. *Arthritis Rheum* 35:953-959.

14. Eronen, M., A. Miettinen, T.K. Walle, E.K. Chan, and H. Julkunen. 2004. Relationship of maternal autoimmune response to clinical manifestations in children with congenital complete heart block. *Acta Paediatr* 93:803-809.

15. Siren, M.K., H. Julkunen, R. Kaaja, H. Ekblad, and S. Koskimies. 1999. Role of HLA in congenital heart block: susceptibility alleles in children. *Lupus* 8:60-67.

16. Weissert, R., E. Wallstrom, M.K. Storch, A. Stefferl, J. Lorentzen, H. Lassmann, C. Linington, and T. Olsson. 1998. MHC haplotype-dependent regulation of MOG-induced EAE in rats. *J Clin Invest* 102:1265-1273.

17. Lorentzen, J.C., and L. Klareskog. 1996. Susceptibility of DA rats to arthritis induced with adjuvant oil or rat collagen is determined by genes both within and outside the major histocompatibility complex. *Scand J Immunol* 44:592-598.

18. Griffiths, M.M., G.W. Cannon, P.A. Leonard, and V.R. Reese. 1993. Induction of autoimmune arthritis in rats by immunization with homologous rat type II collagen is restricted to the RT1av1 haplotype. *Arthritis Rheum* 36:254-258.

19. Holmdahl, R., M. Karlsson, K. Gustafsson, and H. Hedrich. 1993. Structural polymorphism of six rat RT1Ba genes. *Immunogenetics* 38:381.

20. Ettinger, R.A., A.K. Moustakas, and S.D. Lobaton. 2004. Open reading frame sequencing and structure-based alignment of polypeptides encoded by RT1-Bb, RT1-Ba, RT1-Db, and RT1-Da alleles. *Immunogenetics* 56:585-596.

21. Zhu, Y., A.Y. Rudensky, A.L. Corper, L. Teyton, and I.A. Wilson. 2003. Crystal structure of MHC class II I-Ab in complex with a human CLIP peptide: prediction of an I-Ab peptide-binding motif. *J Mol Biol* 326:1157-1174.

22. Bolin, D.R., A.L. Swain, R. Sarabu, S.J. Berthel, P. Gillespie, N.J. Huby, R. Makofske, L. Orzechowski, A. Perrotta, K. Toth, J.P. Cooper, N. Jiang, F. Falcioni, R. Campbell, D. Cox, D. Gaizband, C.J. Belunis, D. Vidovic, K. Ito, R. Crowther, U. Kammlott, X. Zhang, R. Palermo, D. Weber, J. Guenot, Z. Nagy, and G.L. Olson. 2000. Peptide and peptide mimetic inhibitors of antigen presentation by HLA-DR class II MHC molecules. Design, structure-activity relationships, and X-ray crystal structures. *J Med Chem* 43:2135-2148.

23. Feng, D., C.J. Bond, L.K. Ely, J. Maynard, and K.C. Garcia. 2007. Structural evidence for a germline-encoded T cell receptor-major histocompatibility complex interaction 'codon'. *Nat Immunol* 8:975-983.

24. Jones, E.Y., L. Fugger, J.L. Strominger, and C. Siebold. 2006. MHC class II proteins and disease: a structural perspective. *Nat Rev Immunol* 6:271-282.

25. Wu, H., G.Y. Zhang, and J.F. Knight. 2001. T cell receptor BV gene usage in interstitial cellular infiltrates in active Heymann nephritis. *Nephrol Dial Transplant* 16:1374-1381.

26. Encinas, J.A., M.B. Lees, R.A. Sobel, C. Symonowicz, H.L. Weiner, C.E. Seidman, J.G. Seidman, and V.K. Kuchroo. 2001. Identification of genetic loci associated with paralysis, inflammation and weight loss in mouse experimental autoimmune encephalomyelitis. *Int Immunol* 13:257-264.

27. Teuscher, C., R. Noubade, K. Spach, B. McElvany, J.Y. Bunn, P.D. Fillmore, J.F. Zachary, and E.P. Blankenhorn. 2006. Evidence that the Y chromosome influences autoimmune disease in male and female mice. *Proc Natl Acad Sci U S A* 103:8024-8029.

28. The STAR consortium. 2008. SNP and haplotype mapping for genetic analysis in the rat. *Nat. Genet. 40, in press*.

29. de Graaf, K.L., R. Weissert, P. Kjellen, R. Holmdahl, and T. Olsson. 1999. Allelic variations in rat MHC class II binding of myelin basic protein peptides correlate with encephalitogenicity. *Int Immunol* 11:1981-1988.

30. Forsthuber, T.G., C.L. Shive, W. Wienhold, K. de Graaf, E.G. Spack, R. Sublett, A. Melms, J. Kort, M.K. Racke, and R. Weissert. 2001. T cell epitopes of human myelin oligodendrocyte glycoprotein identified in HLA-DR4 (DRB1*0401) transgenic mice are encephalitogenic and are presented by human B cells. *J Immunol* 167:7119-7125.

31. Siren, M.K., H. Julkunen, R. Kaaja, P. Kurki, and S. Koskimies. 1999. Role of HLA in congenital heart block: susceptibility alleles in mothers. *Lupus* 8:52-59.

32. Miyagawa, S., K. Shinohara, K. Kidoguchi, T. Fujita, T. Fukumoto, Y. Yamashina, K. Hashimoto, A. Yoshioka, S. Sakurai, O. Nishihara, and T. Shirai. 1997. Neonatal lupus erythematosus: HLA-DR and -DQ distributions are different among the groups of anti-Ro/SSA-positive mothers with different neonatal outcomes. *J Invest Dermatol* 108:881-885.

33. Hurt, P., L. Walter, R. Sudbrak, S. Klages, I. Muller, T. Shiina, H. Inoko, H. Lehrach, E. Gunther, R. Reinhardt, and H. Himmelbauer. 2004. The genomic sequence and comparative analysis of the rat major histocompatibility complex. *Genome Res* 14:631-639.

34. Salomonsson, S., T. Dorner, E. Theander, K. Bremme, P. Larsson, and M. Wahren-Herlenius. 2002. A serologic marker for fetal risk of congenital heart block. *Arthritis Rheum* 46:1233-1241.

35. Espinosa, A., W. Zhou, M. Ek, M. Hedlund, S. Brauner, K. Popovic, L. Horvath, T. Wallerskog, M. Oukka, F. Nyberg, V.K. Kuchroo, and M. Wahren-Herlenius. 2006. The

Sjogren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death. *J Immunol* 176:6277-6285.

36. Eftekhari, P., J.C. Roegel, F. Lezoualc'h, R. Fischmeister, J.L. Imbs, and J. Hoebeke. 2001. Induction of neonatal lupus in pups of mice immunized with synthetic peptides derived from amino acid sequences of the serotoninergic 5-HT4 receptor. *Eur J Immunol* 31:573-579.

37. Munder, M., E. Bettelli, L. Monney, J.M. Slavik, L.B. Nicholson, and V.K. Kuchroo. 2002. Reduced self-reactivity of an autoreactive T cell after activation with cross-reactive non-self-ligand. *J Exp Med* 196:1151-1162.

38. de Graaf, K.L., S. Barth, M.M. Herrmann, M.K. Storch, C. Otto, T. Olsson, A. Melms, G. Jung, K.H. Wiesmuller, and R. Weissert. 2004. MHC class II isotype- and allele-specific attenuation of experimental autoimmune encephalomyelitis. *J Immunol* 173:2792-2802.

39. Weissert, R., A. Lobell, K.L. de Graaf, S.Y. Eltayeb, R. Andersson, T. Olsson, and H. Wigzell. 2000. Protective DNA vaccination against organ-specific autoimmunity is highly specific and discriminates between single amino acid substitutions in the peptide autoantigen. *Proc Natl Acad Sci U S A* 97:1689-1694.

40. Kopp, J., and T. Schwede. 2006. The SWISS-MODEL Repository: new features and functionalities. *Nucleic Acids Res* 34:D315-318.

41. de Graaf, K.L., G.P. Berne, M.M. Herrmann, G.K. Hansson, T. Olsson, and R. Weissert. 2005. CDR3 sequence preference of TCRBV8S2+ T cells within the CNS does not reflect single amino acid dependent avidity expansion. *J Neuroimmunol* 166:47-54.

42. Laird, P.W., A. Zijderveld, K. Linders, M.A. Rudnicki, R. Jaenisch, and A. Berns. 1991. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res* 19:4293.

43. Jacob, H.J., D.M. Brown, R.K. Bunker, M.J. Daly, V.J. Dzau, A. Goodman, G. Koike, V. Kren, T. Kurtz, A. Lernmark, and et al. 1995. A genetic linkage map of the laboratory rat, Rattus norvegicus. *Nat Genet* 9:63-69.

44. Strandberg, L., A. Ambrosi, A. Espinosa, L. Ottosson, M.L. Eloranta, W. Zhou, A. Elfving, E. Greenfield, V.K. Kuchroo, and M. Wahren-Herlenius. 2007. Interferon-alpha Induces Up-regulation and Nuclear Translocation of the Ro52 Autoantigen as Detected by a Panel of Novel Ro52-specific Monoclonal Antibodies. *J Clin Immunol* Epub ahead of print Dec 11; 10.1007/s10875-007-9157-0

FIGURE LEGENDS

Figure 1. A Ro52 immunization rat model demonstrates association of RT1^{av1} with development of AV block. (A) Ro52 and p200 antibody levels in DA (RT1^{av1}), PVG.AV1, LEW.AV1 and LEW (RT1^{l}) rats pre- and post-immunization with Ro52-MaBP or control MaBP protein. (B) PR values for DA, PVG.AV1, LEW.AV1 and LEW rats by litter in rats immunized with Ro52-MaBP or control protein MaBP. 45% DA, 44% PVG.AV1, 47% LEW.AV1 and 10% LEW rat pups developed AVB I. Hatched lines indicate +2SD from the mean of each control group (PR mean±SD: MaBP-DA 57.3±3.2; MaBP-PVG.AV1 55.6±2.7; MaBP-LEW.AV1 53.4±4.6; MaBP-LEW 52.0±4.2). (C) Pups from Ro52-immunized rats with MHC haplotype AV1 had significantly longer PR intervals than pups from Ro52-immunized rats with MHC haplotype L, $p<0.0001$. (D) There was no significant difference in PR intervals of pups from Ro52-immunized mothers of the three strains with the same MHC haplotype (AV1) but different non-MHC genes. (E) Schematic representation of the Ro52 protein and p200 peptide, with the p200 derived pZIP and pOUT peptide amino acid replacements indicated. (F) Ribbon-structure representation of the predicted pZIP and pOUT secondary structure fold visualizing the position of mutated amino acids (represented in red). (G) p200 antibody levels in Ro52-immunized AV1 and L rats and (H) p200 antibody units in healthy or AVB I affected pups. (I) pOUT and pZIP peptide binding in RT1^{av1} and RT1^{l} rats. pZIP and pOUT binding is expressed normalized to p200 antibody levels for each serum. * $p<0.05$, ** $p<0.01$.

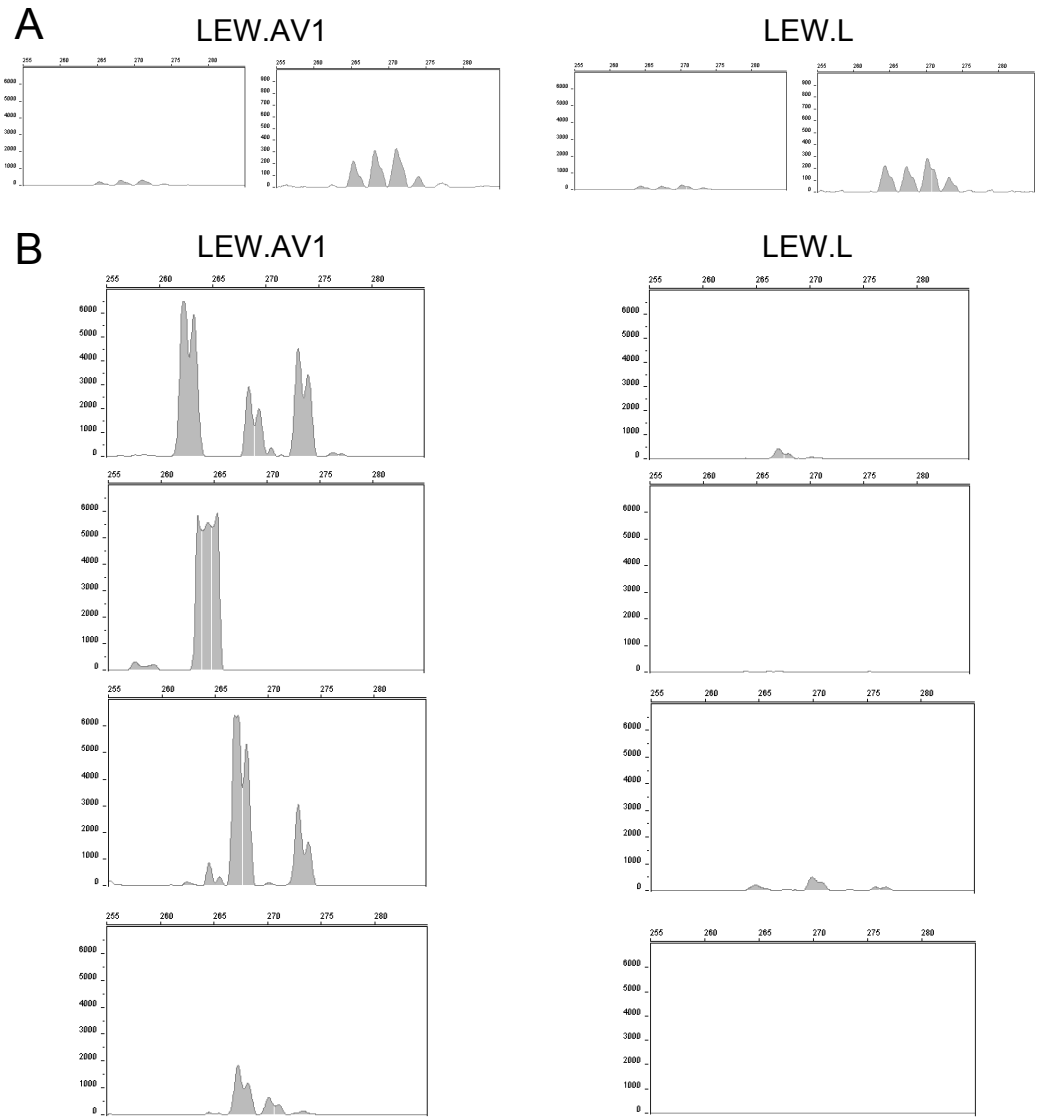
Figure 2. The RT1.B peptide binding cleft and TCRBV usage in p200 specific clonal T cells differ between LEW RT1^{av1} and RT1^{l} rats. (A) Proliferation and IFN-γ spot formation after p200 peptide (10 μg/μl) stimulation of p200-specific T cell lines generated from LEW.AV1 rats.

Antibodies Ox-6 (blocking RT1.B-mediated T cell activaton) or Ox-17 (blocking RT1.D-mediated T cell activation) were added to stimulation cultures (10 μg/μl), showing that the activation of p200-specific clonal T cells is predominantly dependent on presentation via RT1.B. Data from p200 specific T cell lines derived from four individual LEW.AV1 rats tested in triplicates are shown. (B) Alignment of the amino acid sequences encoding the α- and β-chains of the MHC class II molecules RT1.B-AVI and RT1.B-L. Conserved, identical amino acid blocks are shadowed grey, and amino acid positions that differ between the two strains are denoted in red (negatively charged), blue (positively charged), orange (polar) and green (hydrophobic). (C) Molecular models of the peptide-binding clefts of the MHC class II molecules RT1.B-AVI and RT1.B-L are presented in the upper and lower panels, respectively, using schematic (left) and surface electrostatic (right) representations. Negatively and positively charged regions of the surfaces are given in red and blue, respectively, and a comparison of the molecular models suggest that large structural and electrostatic differences are present within the respective peptide-binding clefts of the AV1 and L RT1.B molecules within the P1, P4 and P9 pockets. Residues that potentially contact bound peptides and that result in the most important structural and charge differences within the peptide binding cleft of RT1.B-AVI and RT1.B-L are indicated. (D) TCRBV1 and 7 spectratypes in p200-specific T cell lines of $RT1^{av1}$ and $RT1^{l}$ animals.

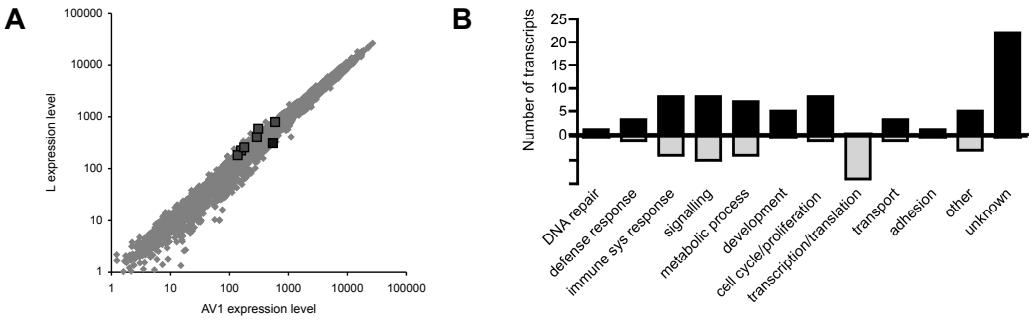
Figure 3. A reciprocal F2 cross reveals MHC encoded genes ($RT1^{l}$) confer fetal susceptibility to AV block. (A) Schematic organization of the F2 cross. Pups born were genotyped and found to be 23% $RT1^{av1}$, 23% $RT1^{l}$ and 54% heterozygous $RT1^{av1/l}$. (B) Discrete PR values as measured by ECG at birth in pups with $RT1^{av1}$, heterozygous $RT1^{av1/l}$ or $RT1^{l}$ genotype. (C) Pups with an $RT1^{l}$ haplotype have significantly longer PR intervals than pups with the $RT1^{av1}$ haplotype ($p<0.05$, Mann-

Whitney U-test) and pups with a heterozygous genotype also have significantly prolonged PR intervals compared to the $RT1^{av1}$ pups ($p<0.05$, ANOVA and Mann-Whitney U-test). (D) ELISA with p200, pZIP and pOUT peptides with sera from F1 immunized female rats shows that F1 heterozygous mothers have the same antibody binding profile (pOUT > pZIP) as found in the $RT1^{av1}$ rats from the immunization study. (E) Pups of Ro52 immunized L/AV1 x L/AV1 F1 rats had significantly longer PR intervals compared to pups from Ro52 immunized AV1/L x AV1/L F1 rats ($p<0.01$). Only homozygous $RT1^{av1}$ and $RT1^{l}$ pups were included in the analysis. (F) Significantly longer PR intervals were also observed if the $RT1^{l}$ was inherited from the maternal founders (LL) than the paternal founders (ll) ($p<0.001$, Mann-Whitney U-test). There is however no difference in PR intervals when the $RT1^{av1}$ haplotype is inherited from the maternal or paternal founder. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Figure 4. A $Ro52^{+}$ serum transfer model confirms association of MHC encoded genes with fetal susceptibility to AV block. Serum pooled from ten Ro52 immunized $RT1^{av1}$ rats (DA) and control non-immune $RT1^{av1}$ (DA) rat serum were transferred i.p. to $RT1^{av1}$ (DA) and $RT1^{l}$ (LEW) rats day 6 and 9 of pregnancy and ECG performed on pups at birth. (A) Discrete values of normalized PR intervals for $RT1^{av1}$ and $RT1^{l}$ rat pups, from control and $Ro52^{+}$ sera injected mothers. (B) $RT1^{l}$ pups had significantly longer PR intervals in pups born to rats injected with $Ro52^{+}$ serum than those injected with control serum ($p<0.001$). (C) Ro52 and (D) p200 antibody levels in $RT1^{av1}$ and $RT1^{l}$ pups born to mothers injected with anti-Ro52 positive sera or control sera. (E) Ro52 antibody levels in $RT1^{av1}$ rat mothers from the immunization study and the pooled $RT1^{av1}$ (DA) rat serum (n=10) which was used in the transfer model. (F) Levels of Ro52 antibodies in representative $RT1^{av1}$ (DA)



Supplemental Figure 1, Strandberg et al. TCRBV1 spectratype analyses in (A) naive LEW.AV1 and LEW.L T cells presented both in the same scale as in (B), and enlarged to visualize normal distribution. (B) TCRBV1 spectratype analyses in p200 specific T cell lines derived from LEW.AV1 and LEW.L animals (n=4 and n=4, respectively).



Supplemental Figure 2, *Strandberg et al.* (A) Scatterplot of average gene expression of all genes analyzed by the Rat Gene 1.0 ST array in LEW (n=4) by LEW.AV1 (n=6) pup hearts after transfer of Ro52-positive serum. The non-classical MHC-encoded genes with an increased (red) or decreased (blue) expression ($p<0.0005$) in $RT1^{l}$ compared to $RT1^{av1}$ are indicated by squares. (B) Functional annotation with grouping according to the Gene Ontology Annotation database (http://www.ebi.ac.uk/GOA/) of the genes from (A) with an increased or decreased expression in pup hearts at $p<0.0005$ in $RT1^{l}$ compared to $RT1^{av1}$ after Ro52-positive serum transfer.

Supplemental Table 1. Rat strains and MHC genotype in immunization, transfer and F2 cross studies. Both congenic and native RT1 loci are indicated.

Ro52 immunization study	**# pups / strain.RT1**				
Rats immunized with	**DA.AV1**	**PVG.AV1**	**LEW.AV1**	**LEW.L**	**Total**
Ro52	49	48	51	49	197
Control protein	26	8	28	26	88
Serum transfer					
Rats injected with					
AV1 Ro52-immune serum	7			18	25
AV1 non-immune serum	23			16	39

F2 cross LEW.AV1 x LEW.L	**# pups / F1 mating combination** RT1 genotype of pups (AV1, AVI/L, L)				
Rats immunized with	**AV1/L x AV1/L**	**L/AV1 x AV1/L**	**AV1/L x L/AV1**	**L/AV1 x L/AV1**	**Total**
Ro52	45 (13,24,8)	46 (12,25,9)	48 (7,26,15)	21 (4,12,5)	160 (36,87,37)

Supplemental Table 2.

MHC-encoded genes with increased or decreased expression ($p<0.0005$) in congenital heart block and primers used for quantitative real-time PCR.

Symbol	Name	Gene Id	Forward primer Reverse primer
Atp6v1g2	ATPase, H+ transporting, V1 subunit G isoform 2	368044	5'-CAAGCAGCAGGCGGCCATGG-3' 5'-GAAGCTGAGTCAGGACTCGC-3'
Bat5	HLA-B associated transcript 5	361796	5'-GAAGCTGGTGATCTGC-3' 5'-AATGGTACCCCCGTGC-3'
Clic2	chloride intracellular channel 2	294141	5'-CTCGCTCCTCCAAGGTATCC-3' 5'-CGTTTAAATTCTCTAAGCAGAG-3'
G7c	G7c protein	309611	5'-CTCCTTGCTGCCTCGCTACC-3' 5'-CACTGAGCTCCAGAAGCACG-3'
Hla-dmb	major histocompatibility complex, class II, DM beta	294273	5'-GACCCACAGAACCAGAACGC-3' 5'-CAAGCTGCCCGTTCTTCATCC-3'
RT1-T24-1	histocompatibility 2, T region locus 24	361787	5'-CTTTACCCGAACTGACCCTCC-3' 5'-GTCTCCACAAGCTCCATGTCC-3'
Trim26	tripartite motif protein 26	309586	5'-TGGTGGAGAAGGCTGC-3' 5'-TGCAGCTTCGTCAGTGC-3'