

Maternal MHC Regulates Generation of Pathogenic Antibodies and Fetal MHC-Encoded Genes Determine Susceptibility in Congenital Heart Block

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Congenital heart block develops in fetuses of anti-Ro52 Ab-positive women. A recurrence rate of 20%, despite the persistence of maternal autoantibodies, indicates that there are additional, yet unidentified, factors critical for development of congenital heart block. In this study, we demonstrate that besides the maternal MHC controlling Ab specificity, fetal MHC-encoded genes influence fetal susceptibility to congenital heart block. 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, but not in LEW rats (RT1^b). Different anti-Ro52 Ab fine specificities were generated in RT1^{av1} versus RT1^b animals. Maternal and fetal influence was determined in an F₂ cross between LEW.AV1 and LEW strains, which revealed higher susceptibility in RT1^b than RT1^{av1} pups once pathogenic Ro52 Abs were present. This was further confirmed in that RT1^b pups more frequently developed heart block than RT1^{av1} pups after passive transfer of RT1^{av1} anti-Ro52 sera. Our findings show that generation of pathogenic Ro52 Abs is restricted by maternal MHC, whereas the fetal MHC locus regulates susceptibility and determines the fetal disease outcome in anti-Ro52-positive pregnancies. *The Journal of Immunology*, 2010, 185: 3574–3582.

Pregnancy in autoimmune conditions is often marred by complications, and the fetus may be directly affected by maternal autoantibodies to develop neonatal lupus, myasthenia, hypothyroidism, or learning disabilities (1–4). Congenital heart block is a potentially lethal manifestation of the neonatal lupus syndrome, which develops in fetuses of rheumatic women with Ro52 autoantibodies (5). In the rheumatic mothers, production of Ro52 Abs has been repeatedly linked to MHC class II DRB1*03 alleles (6, 7). During pregnancy, the maternal autoantibodies cross

the placenta and bind cardiomyocytes in the fetal heart, in which the atrioventricular conduction system is disrupted by inflammation with subsequent fibrosis and calcification leading to a complete atrioventricular block (AVB) (8, 9). Immunization of mice or rats with Ro52 or Ro52-derived peptides leads to AVB in the offspring, and these animal models have been used to define the nature and quality of the pathogenic autoantibodies that mediate heart block (10, 11).

The vast majority of mothers of infants with congenital heart block carry Ro52 Abs, but the risk for heart block in a single Ro-positive pregnancy is only 1–2% (12, 13). In mothers who already have a child with congenital heart block, the risk for an affected child is increased to ~20% in subsequent pregnancies (14); thus, heart block does not always develop despite persisting maternal anti-Ro52 autoantibodies (15). 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 (16–18).

To address the hypothesis that susceptibility may relate to the genetic makeup of the fetus itself, we used a 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 Abs with pathogenic specificity is restricted by maternal MHC, whereas a different set of MHC-encoded genes in the fetus confers increased susceptibility to congenital heart block and determines the disease outcome in Ro52-positive pregnancies.

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Abbreviations used in this paper: AVB, atrioventricular block; ECG, electrocardiogram; MaBP, maltose-binding protein.

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Materials and Methods

Experimental animals

DA (RT1av1), LEW.AV1 (RT1av1), and LEW (RT1l) rats were originally obtained from the Zentralinstitut für Versuchstierzucht (Prof. H. 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 (Blackthorn, U. K.). 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, Beverly, MA) as a fusion to maltose-binding protein (MaBP) or from the p6xHis vector and purified, as described (19, 20). Wild-type MaBP protein was expressed from the pMAL vector and purified, as described (19). Ro52 peptides p200, pZIP, and pOUT were purchased from Thermo BioSciences (Ulm, Germany).

Immunizations, serum transfer, and electrocardiogram recordings

Six-week-old rats were immunized with 100 μ g Ro52 protein in CFA and boosted three times with 50 μ g Ro52 protein in IFA. Rats were mated 2–4 wk 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 (21). ECGs were sampled for 5 s four times per minute with a sampling rate of 1000 Hz. The ECG was digitalized and analyzed with Pharnlab (AstraZeneca, Wilmington, DE). QRS complexes were averaged and used to calculate the PR interval. AVB I was defined as the average of PR intervals in control animals + 2 SD. PR values for each rat strain were normalized against the average of the control litters of that strain.

For serum transfer experiments, antisera were generated by the same protocol. Control sera were from nonimmunized 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 Abs

ELISA was performed, as described (15, 19), 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 μ g Ro52 protein in CFA at the base of the tail, and draining lymph nodes were removed on day 14 postimmunization. Single-cell suspensions were generated and cells were cultured with restimulation using p200 peptide (10 μ g/ml) and syngenic APCs in three rounds to generate clonal T cells, as previously described (22).

T cell proliferation and ELISPOT assays

T cell lines were plated with syngenic APCs in round-bottomed 96-well plates in culture medium with either Con A (5 μ g/ml), p200 peptide (10 μ g/ml), or p200 peptide with Ox-6 and/or Ox-17 (23) Abs (10 μ g/ml). After 48 h, either cells were pulsed for 16 h with 1 μ Ci [3 H]thymidine/well and proliferation measured as cpm using a microplate liquid scintillation counter (Wallac MicroBeta TriLux; PerkinElmer, Wellesley, MA), or IFN- γ production was analyzed in ELISPOT, as previously described (24). Cells were incubated in the anti-rat IFN- γ -precoated nitrocellulose plates for 20 h, and spots were analyzed using an ELISPOT plate reader (AID ELISPOT Reader System).

Molecular modeling of the peptide-binding clefts of RT1.B AV1 and RT1.B L

Sequence alignment was performed using the program BioEdit (www.mbio.ncsu.edu/BioEdit/). Molecular models of the peptide-binding clefts of RT1.B-AV1 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 (25). The crystal structures of HLA-DR4 (Protein Data Bank code 1D5Z), I-A^b (1MUJ), and I-A^u (2PXY) were used as templates for the modeling. 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, Valencia, CA). Reverse transcription was performed with random hexamer primers and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR was performed, as previously described (26), with 23 published individual TCRBV primers specific for TCRBV1–20 and a common 6-FAM-labeled CB primer (26). The product was separated in capillary electrophoresis and analyzed by Genescan software v3.7 (Applied Biosystems, Foster City, CA).

Polymorphic markers and genotyping of animals in the F₂ cross

Genomic DNA was prepared from tail tips (27). 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 single nucleotide polymorphism 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 (Dusseldorf, Germany). PCR amplification was performed, as previously described (29), with [γ -³³P]ATP end labeling 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 30 s, 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.

Statistical analysis

Statistical analysis was performed using Statistica 7.0 (Statsoft, Tulsa, OK). Nonparametric analysis with Mann-Whitney *U* test and ANOVA was used. The level of significance was set at $p < 0.05$.

Results

Maternal MHC regulates generation of pathogenic Ab specificities inducing heart block

Genetic influence has been implied in the development of congenital heart block (30), 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 was 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 experimental autoimmune encephalomyelitis (31) and collagen-induced arthritis (32, 33). Congenital heart block developed 3-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.AV1 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 (Fig. 1A), first-degree AVB 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 AVB (Fig. 1B). Rats from each strain immunized with control protein (MaBP) showed no heart block development (Fig. 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 ($p < 0.001$), whereas there was no significant difference in PR intervals between the three RT1^{av1}-carrying strains (Fig. 1C, 1D).

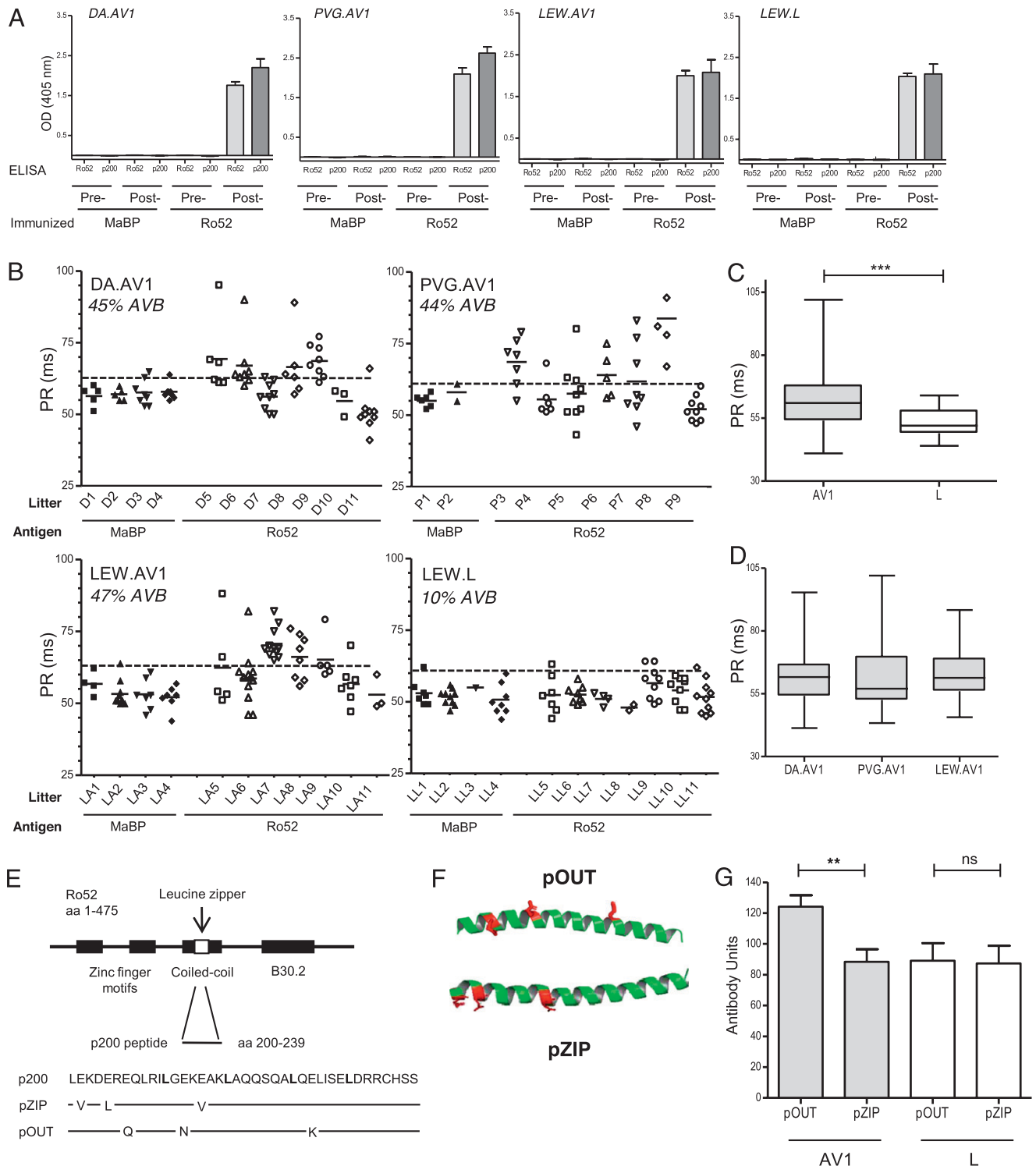


FIGURE 1. A Ro52 immunization rat model demonstrates association of RT1^{av1} with development of AVB. **A**, Ro52 and p200 Ab levels in DA (RT1^{av1}), PVG.AV1, LEW.AV1, and LEW (RT1^l) rats pre- and postimmunization with Ro52-MaBP or control MaBP protein. **B**, PR values for DA, PVG.AV1, LEW.AV1, and LEW rats by litter of rats immunized with Ro52-MaBP or control protein MaBP. Forty-five percent DA, 44% PVG.AV1, 47% LEW.AV1, and 10% LEW rat pups developed AVB. Hatched lines indicate +2 SD from the mean of each control group (PR mean \pm 2 SD: MaBP-DA, 57.3 \pm 3.2; MaBP-PVG.AV1, 55.6 \pm 2.7; MaBP-LEW.AV1, 53.4 \pm 4.6; MaBP-LEW, 52.0 \pm 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, $p < 0.001$. **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**, pOUT and pZIP peptide binding by RT1^{av1} and RT1^l anti-Ro52 rat sera. pZIP and pOUT binding is expressed normalized to p200 Ab levels for each serum. ** $p < 0.01$; *** $p < 0.001$.

Induction of congenital heart block depends on the presence of specific pathogenic autoantibodies binding to an epitope within the stretch of aa residues 200–239 (p200) of Ro52, which contains a leucine zipper motif important in dimer formation and protein-protein interaction (11, 34, 35). To analyze whether the difference in development of congenital heart block between RT1^{av1} and RT1^l strains related to the level or the specificity in the generated Ro52 Abs, sera from the pups and mothers were analyzed by ELISA using Ro52-p200 peptide and a set of mutated p200 peptides. The mutated peptides (pZIP and pOUT) were generated to distinguish binding specificity of Abs to different epitopes within the 40-aa p200 peptide (36). The peptide pZIP was designed to create an optimal leucine zipper motif with high dimer stability, thus inhibiting binding to the peptide dimer interface, and in pOUT negatively charged amino acids on the outer surface of the predicted zipper were substituted for positively or uncharged residues to alter the antigenicity while maintaining an intact structure. Mutated amino acids are indicated in Fig. 1E and 1F. Titers of Abs binding the p200 peptide of Ro52 did not differ significantly between RT1^{av1} and RT1^l strains or animals affected or not by heart block (Fig. 1A and data not shown). Notably, however, the specificity of Ro52-p200 Abs induced by immunization differed between rats with RT1^{av1} and RT1^l MHC alleles, as demonstrated by significantly differential binding to the mutated p200 peptides pZIP and pOUT (Fig. 1G). This indicates that although both RT1^{av1}- and RT1^l-bearing strains generate p200-binding Abs in response to Ro52 immunization, maternal MHC restricts the fine specificity of the generated Abs.

To investigate the mechanism relating to generation of different specificities in p200 immune responses, we first determined which of the AV1 MHC class II molecules was important for generating p200 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). To analyze the contribution of RT1.B and RT1.D molecules, we generated p200-specific T cell lines from LEW.AV1 rats and stimulated these with p200 peptide in the presence of Abs blocking RT1.B (Ox-6) or RT1.D (Ox-17). Using proliferation assays and IFN- γ production as readouts for T cell responses, we could clearly demonstrate a dominant role for RT1.B in activation of AV1-derived p200-specific clonal T cells, as blocking with Ox-6 significantly inhibited the T cell responses, whereas Ox-17 Abs had less effect on proliferation or IFN- γ production (Fig. 2A, 2B).

As RT1.B appeared crucial in generating pathogenic p200 immune responses, we focused further investigation on this MHC molecule. The RT1.B α - and β -chains for both AV1 and L have been sequenced (37, 38), and an alignment suggested several critical differences in both α - and β -chains (Fig. 2C). Molecular models of the peptide-binding clefts of RT1.B AV1 and L (Fig. 2D) were created based on their sequence homology to the MHC class II molecules I-A^u, I-A^b, and HLA-DR4 (39–41). A comparison of the molecular models of RT1.B AV1 and L revealed several important structural and electrostatic differences within the peptide-binding cleft, suggesting that the peptide repertoire bound and presented by RT1.B AV1 and RT1.B L will differ. The most important modifications are localized within and around the P1 pocket (Fig. 2D), used by most MHC class II molecules as a major anchoring site (42). The phenylalanine and the positively charged lysine residues at positions 28 and 35 of the α -chain of RT1.B AV1 are replaced by a histidine and a negatively charged glutamate, respectively, in the RT1.B L. Accordingly, the composition of the structural and electrostatic properties of the P1 pocket will differ between RT1.B AV1 and RT1.B L. Furthermore, the β -chain glycine residue at position 26 in RT1.B AV1 is modified to a larger

negatively charged aspartate that points toward 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 AV1 and L, affecting the pocket that interacts with the peptide residue p9. In this study, the negatively charged β -chain residue aspartate 57 in RT1.B AV1 is replaced by a serine in RT1.B L, whereas surrounding residues, such as α -chain residues T69 and L76 in RT1.B AV1, are substituted to two isoleucines in RT1.B L. Thus, 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 AV1 and L clearly suggest that the MHC-binding surface of the peptides bound to the two MHC class II molecules will be different, and that the conformation of most peptides bound in the two clefts also will 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 TCR usage by TCRBV spectratyping of the generated p200-specific T cell lines derived from LEW.AV1 and LEW rats ($n = 4$ and $n = 4$, respectively) including the TCR V β 1–20 genes. Our analysis showed that the TCRBV usage is different between p200-specific T cell lines derived from LEW.AV1 and LEW strains, respectively. The most striking difference was the dominant use of TCRBV1 in LEW.AV1-derived p200-specific T cell lines not observed in T cell lines from Lew.L animals, and a clear expansion of a single peak in TCRBV7 suggestive of a clonal expansion in Lew.AV1-derived lines that was not observed in the T cell lines from LEW animals (Fig. 2E, Supplemental Fig. 1). The TCRBV1 and seven spectratypes were normally distributed in naive T cells from both strains (Supplemental Fig. 1) (43). Other TCRBV genes investigated did not differ systematically between the strains (data not shown).

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 AVB in rat pups is dominantly inherited and associated with the MHC RT1^l haplotype

The specificities of generated anti-p200 Abs differed between RT1^{av1} and RT1^l strains, and the lower frequency of AVB in RT1^l pups could thus depend either on a lack of generation of pathogenic anti-Ro52 Ab specificities in RT1^l rat mothers, or fetal resistance in RT1^l pups to disease. 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 (Fig. 3A). F₀ LEW.AV1 and LEW rats were mated, and produced A1 (maternal AV1, paternal l) and La (maternal L, paternal av1) genotypes, which were mated in four different combinations (Fig. 3A). 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} F₂ 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} (Fig. 3A). ECG was performed at birth to detect heart block in the F₂ pups of Ro52-immunized mothers. Analysis of prolonged PR intervals with respect to the pup genotype interestingly revealed that a homozygous RT1^l or heterozygous RT1^{av1/l} genotype in the

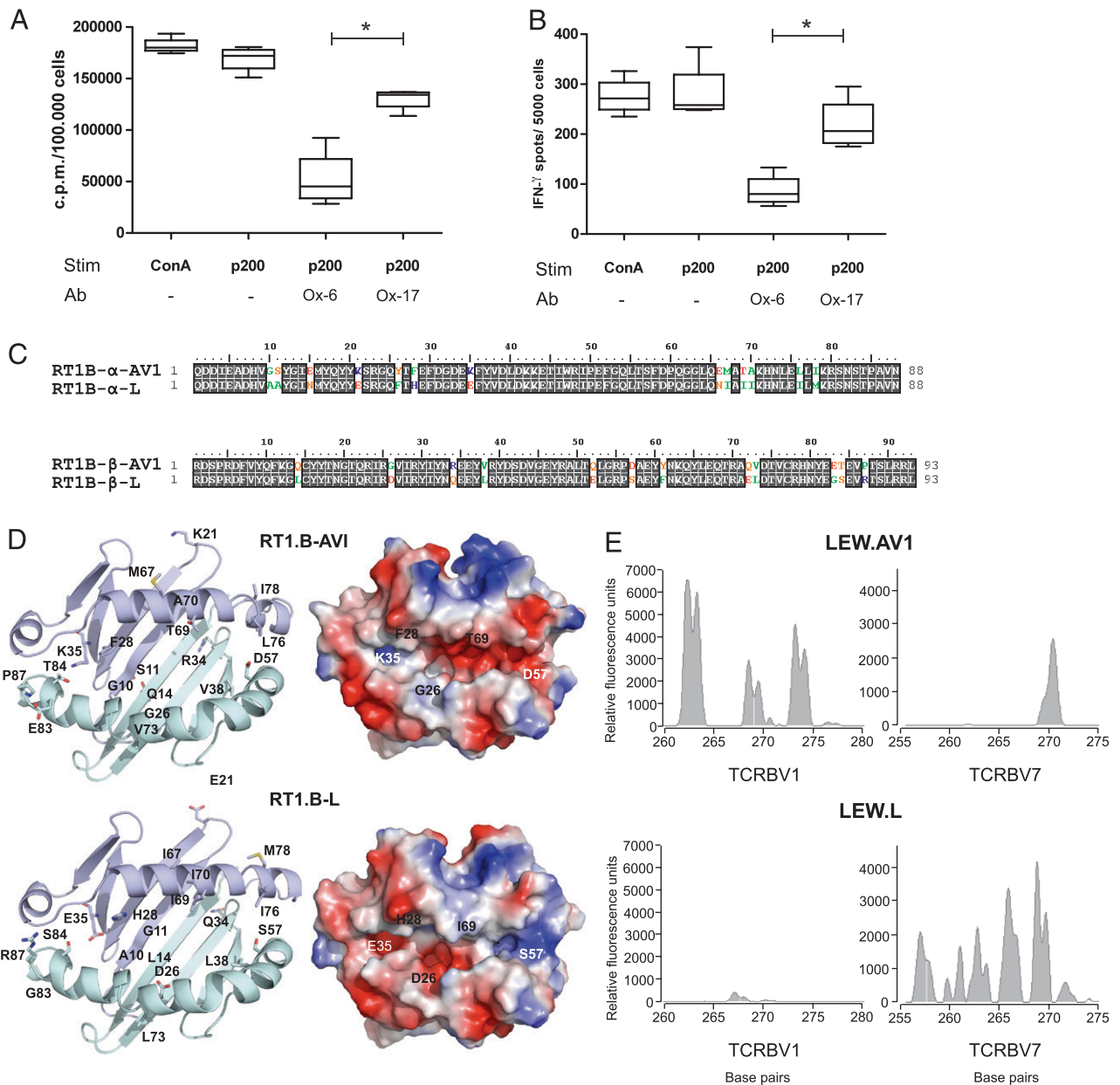


FIGURE 2. The RT1.B peptide-binding cleft and TCRBV usage in p200-specific clonal T cells differ between LEW RT1^{av1} and RT1^l rats. Proliferation (A) and IFN- γ spot formation (B) after p200 peptide (10 μ g/ml) stimulation of p200-specific T cell lines generated from LEW.AV1 rats. Addition of Abs Ox-6 (blocking RT1.B-mediated T cell activation) or Ox-17 (blocking RT1.D-mediated T cell activation) to stimulation cultures (10 μ g/ml) showed 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. C, Alignment of the amino acid sequences encoding the α - and β -chains of the MHC class II molecules RT1.B-AV1 and RT1.B-L. Conserved, identical amino acid blocks are shadowed gray, and amino acid positions that differ between the two strains are denoted in red (negatively charged), blue (positively charged), orange (polar), and green (hydrophobic). D, 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 suggests 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. E, TCRBV1 and seven spectratypes in p200-specific T cell lines of RT1^{av1} and RT1^l animals. * $p < 0.05$.

pup correlated with significantly higher PR intervals than in homozygous RT1^{av1} pups ($p < 0.05$, Fig. 3B, 3C). This indicates that the MHC haplotype RT1^l in the pups in fact confers a higher susceptibility to heart block induced by Ro52 Abs than an RT1^{av1} haplotype. 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 Ab specificity, and not on fetal resistance in LEW pups.

The p200 Ab-binding profile in the heterozygous RT1^{av1/l} Ro52-immunized rats in the F₁ generation (Fig. 3D) was similar to that of the RT1^{av1} rats on the three backgrounds, DA/PVG/LEW (Fig. 1G), demonstrating significantly differential binding to the mutated p200 peptides pZIP and pOUT. This indicates that generation of pathogenic heart block-inducing Abs is a dominant RT1^{av1}-linked trait. We also noted that F₂ pups descendant from F₁ parents with L/av1 \times L/av1 had more affected conduction than

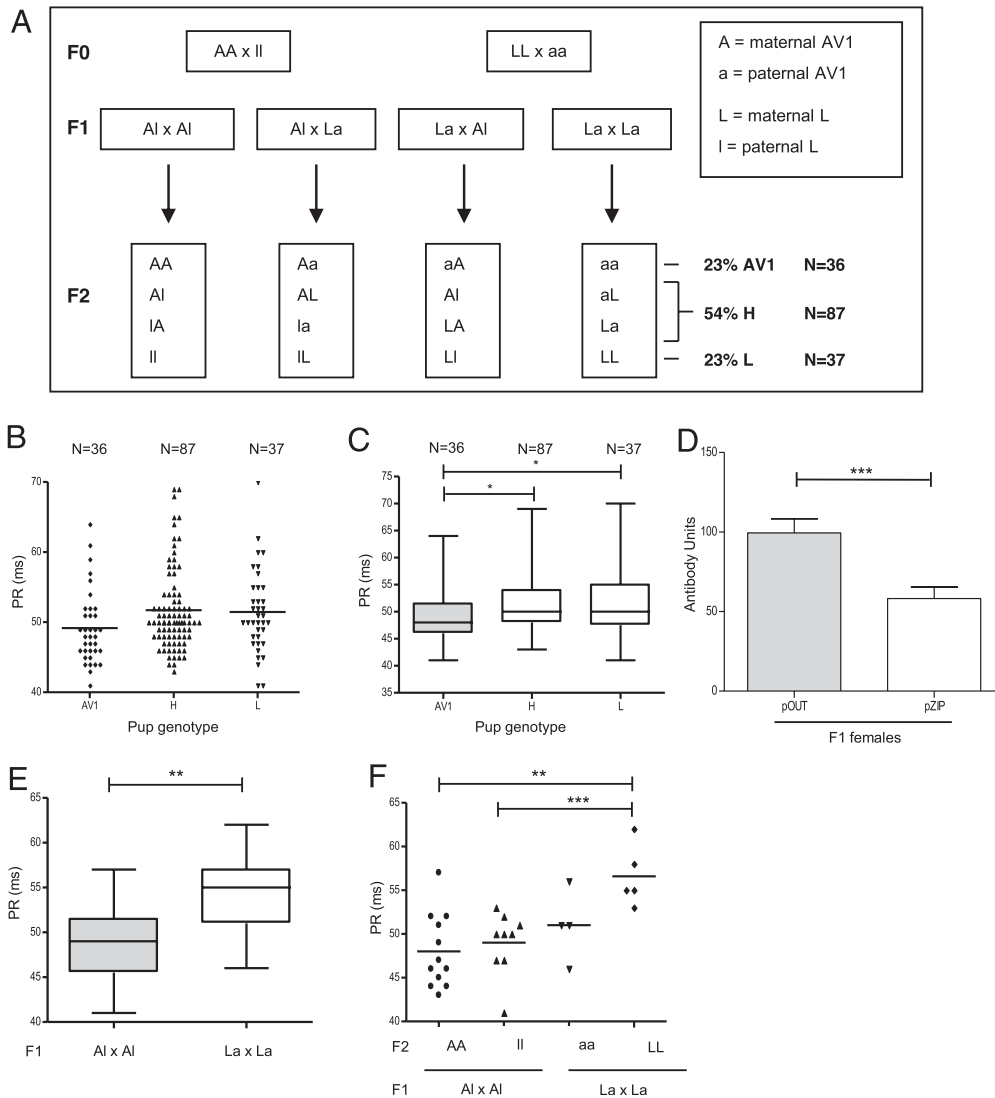


FIGURE 3. A reciprocal F₂ cross reveals MHC-encoded genes (RT1^I) confer fetal susceptibility to AVB. *A*, Schematic organization of the F₂ cross. Pups born were genotyped and found to be 23% RT1^{av1}, 23% RT1^I, and 54% heterozygous RT1^{av1/I}. *B*, Discrete PR values as measured by ECG at birth in pups with RT1^{av1}, heterozygous RT1^{av1/I}, or RT1^I genotype. *C*, Pups with an RT1^I 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 with 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 Ab-binding profile (pOUT > pZIP) as found in the RT1^{av1} rats from the immunization study ($p < 0.001$). *E*, Pups of Ro52-immunized L/AV1 × L/AV1 F₁ rats had significantly longer PR intervals compared with pups from Ro52-immunized AV1/L × AV1/L F₁ rats ($p < 0.01$). Only homozygous RT1^{av1} and RT1^I pups were included in the analysis. *F*, Significantly longer PR intervals were also observed if the RT1^I was inherited from the maternal founders (LL) than the paternal founders (II) ($p < 0.001$, 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. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

pups descendant from AV1/I × AV1/I F₁ parents (Fig. 3*E*, $p < 0.01$, RT1^{av1} and RT1^I homozygous pups only). When comparing the ECGs according to genotype of the F₂ pups, it was found that if the pups had inherited LL from the maternal founders (La × La F₁ mating) or LL from the paternal founders (Al × Al F₁ mating), there was a significant difference in PR intervals after autoantibody exposure in that pups that had inherited LL from the maternal founders (F₁: La × La; F₂: LL) had longer PR intervals (Fig. 3*A*, 3*F*, $p < 0.001$, Mann-Whitney *U* test), indicating that there may also be epigenetic effects on inheritance for susceptibility. In all, the data from the F₂ cross demonstrate that generation of maternal pathogenic heart block-inducing Ro52 Abs is a dominant trait linked to the RT1^{av1} MHC haplotype, and that pups with RT1^I alleles are more susceptible to the cardiopathogenic effects of these autoantibodies.

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

To directly investigate the increased susceptibility to develop heart block in pups with RT1^I alleles compared with 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 nonimmune serum from RT1^{av1} rats (DA) was used as a control in parallel transfer experiments. A total of 2 ml of serum was given i.p. to homozygous pregnant RT1^{av1} (DA) or RT1^I (LEW) rats on days 6 and 9 of pregnancy, and ECG was recorded from the pups at birth. Also in this experimental setting, using passive transfer of Ro52 Abs generated in RT1^{av1} rats, the RT1^I pups displayed higher susceptibility to pathogenic Ro52 Abs and had longer PR intervals than RT1^{av1} pups (Fig. 4*A*, 4*B*). Transfer of Ro52 Abs to

the two strains of pups was confirmed by ELISA using pup serum (Fig. 4C), also demonstrating Ab specificity for the p200 peptide of Ro52 (Fig. 4D). Transfer of increasing amounts of p200-specific Abs induced heart block also in AV1 pups (data not shown), but at the lower Ab levels used in our transfer experiments and compared with the immunization model (Fig. 4E, 4F), it was clear that pups with RT1^{av1} MHC are more susceptible to congenital heart block (Fig. 4A), and it should be noted that relatively low levels of Ro52 Abs were needed to induce PR prolongation in the more susceptible strain. In all, our results demonstrate that higher susceptibility to heart block development is linked to the RT1^{av1} MHC haplotype in the pups.

Discussion

Maternal autoantibodies are crucial in the development of congenital heart block, but the clinical observation of a recurrence rate

of only 20% in subsequent pregnancies suggests that there are additional factors critical for development of heart block. Hypothesizing that development of congenital heart block in the presence of maternal Ro52 autoantibodies may relate also to fetal susceptibility depending on genetic constitution, we used a 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 study confers strong evidence that allelic variants of genes within the MHC complex determine both the maternal ability to form pathogenic Abs as well as the fetal susceptibility to develop congenital heart block in response to these Abs. Our data further show that these traits are linked to different haplotypes in the mother and the child, respectively.

In our Ro52-immunization model, we demonstrated that the three rat strains sharing the RT1^{av1} haplotype (DA, PVG.AV1, LEW.AV1) had significantly longer PR intervals than did the

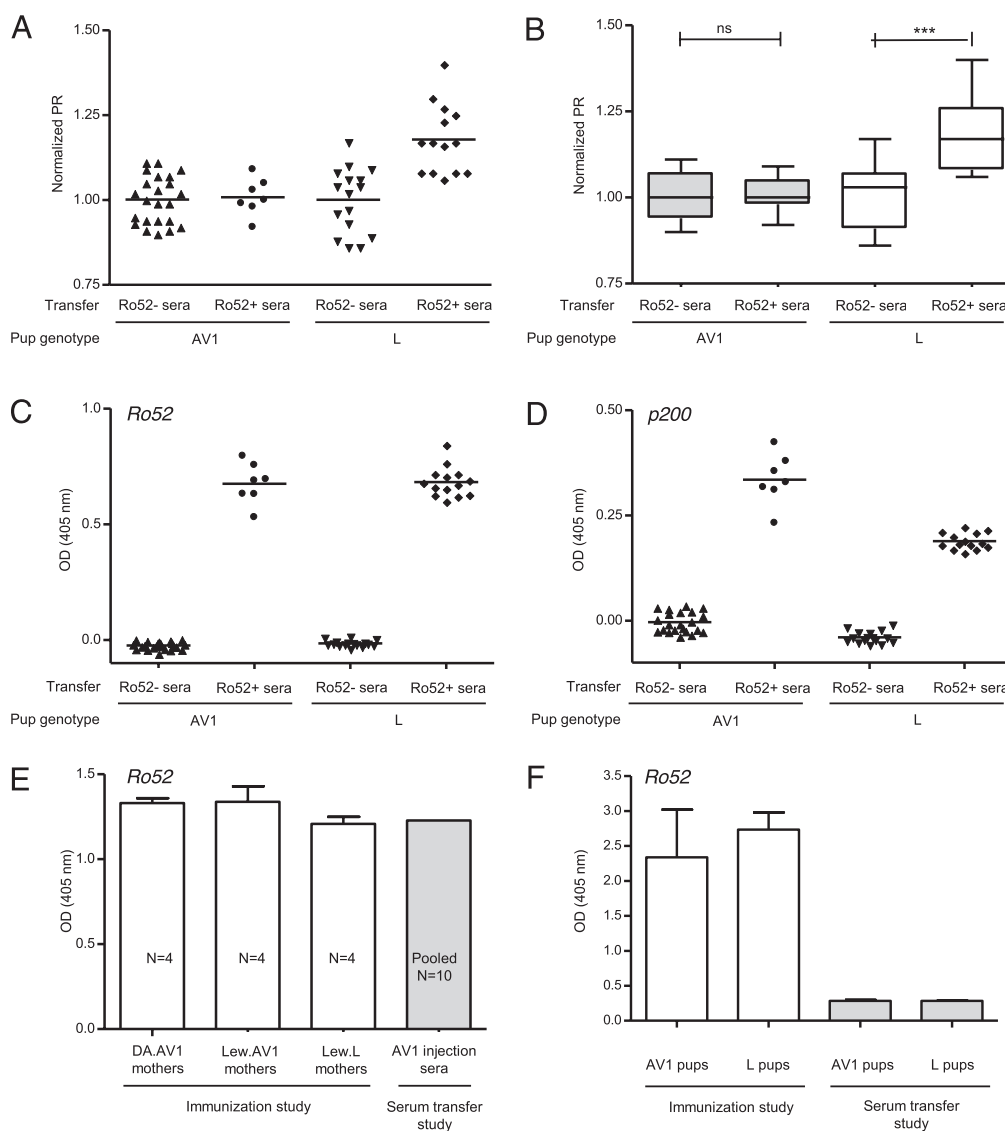


FIGURE 4. A Ro52⁺ serum transfer model confirms association of MHC-encoded genes with fetal susceptibility to AVB. Serum pooled from 10 Ro52-immunized RT1^{av1} rats (DA) and control-nonimmune RT1^{av1} (DA) rat serum were transferred i.p. to RT1^{av1} (DA) and RT1¹ (LEW) rats days 6 and 9 of pregnancy, and ECG was performed on pups at birth. **A**, Discrete values of normalized PR intervals for RT1^{av1} and RT1¹ rat pups, from control- and Ro52⁺ sera-injected mothers. **B**, RT1¹ pups had significantly longer PR intervals in pups born to rats injected with Ro52⁺ serum than those injected with control serum ($p < 0.001$). Ro52 (**C**) and p200 (**D**) Ab levels in RT1^{av1} and RT1¹ pups born to mothers injected with anti-Ro52–positive sera or control sera. **E**, Ro52 Ab levels in RT1^{av1} rat mothers from the immunization study and the pooled RT1^{av1} (DA) rat serum ($n = 10$) that was used in the transfer model. **F**, Levels of Ro52 Abs in representative RT1^{av1} (DA) ($n = 2$) and RT1¹ (LEW) pups ($n = 2$) from the immunization study and in pups born to mothers in the transfer study ($n = 6$ and $n = 8$, respectively). *** $p < 0.001$.

LEW (RT1^l) pups. This suggested either a maternal or a fetal MHC linkage to development of heart block. Maternal MHC linkage is essential in regulating the generation of pathogenic Abs through allelic differences in class II Ag-presenting properties, causing subtle differences in T cell specificities and fine specificity of Abs. Whereas congenital heart block occurs in 1–2% of human Ro-positive pregnancies, the presence of Abs specific for the p200 peptide of Ro52 in mothers is associated with an increased risk for the fetus to develop the heart block (44). In this study, we observed that Abs to Ro52-p200 induced by immunization in RT1^{av1} and RT1^l rats differed in their fine specificity, as shown by differential binding to mutated p200 peptides. Subsequently, they differed in their pathogenicity, as the incidence of AVB was higher in pups of RT1^{av1} mothers than in pups of RT1^l mothers.

The class II genes of the RT1^{av1} and RT1^l haplotypes display important structural and electrostatic differences within the peptide-binding cleft, suggesting that the peptide repertoire preferentially presented by RT1^{av1} and RT1^l MHC class II molecules will differ. The class II RT1-B molecule, which had a dominant role in the generation of Ro52-p200-specific responses in our study, has previously been described to show more allelic diversity than the RT1-D molecule, as characterized by different myelin basic protein peptide-binding profiles between the four different haplotypes, RT1^a, RT1^l, RT1ⁿ, and RT1^u (29), and to regulate the pathogenicity of the immune response following immunization with myelin Ags (45).

Whereas maternal MHC restricts the production of pathogenic Abs in our model of immunization-induced congenital heart block, we could also demonstrate in an F₂ cross between RT1^{av1} and RT1^l animals that the MHC haplotype in the pups was critical in regulating susceptibility to the development of heart block once pathogenic Abs were transferred. Inheriting one or both RT1^l alleles from the parents made the pups more susceptible to heart block than inheriting the RT1^{av1} haplotype. Furthermore, the origin of inheritance seemed to have an effect on the susceptibility to heart block in which maternal origin of LL alleles corresponded to significantly longer PR intervals than paternal inheritance of ll alleles, which suggests an additional layer of epigenetic regulation of congenital heart block, as previously indicated for other autoimmune conditions (46, 47). Development of the pathogenic Abs was dominantly linked as the heterozygotes (RT1^{av1/l}) in the F₂ cross displayed a similar p200 peptide-binding profile as the RT1^{av1} homozygotes in the immunization experiment.

Although the RT1^{av1} haplotype in the mother was important for production of pathogenic Abs, a separate MHC haplotype (RT1^l) seemed to influence susceptibility to congenital heart block. This was further demonstrated in a transfer experiment in which the pathogenic anti-Ro52 Abs from RT1^{av1} females were passively transferred to RT1^{av1} and RT1^l pups during gestation. Interestingly, we found that the RT1^l haplotype conferred an increased susceptibility to disease in the pups compared with the RT1^{av1} haplotype when the pathogenic Abs were transferred during gestation. This demonstrates that the RT1^{av1} haplotype is responsible for the generation of pathogenic Abs in the mothers, yet when preformed anti-Ro52 Abs are transferred to the RT1^l females during gestation, the pups are more susceptible to developing heart block than RT1^{av1} pups. This increased sensitivity of Lew.L pups compared with Lew.AV1 pups to develop AVB upon transfer of Ro52-specific Abs is also reflected by the fact that heart block developed in RT1^l pups at the lower levels of Abs after transfer compared with the levels obtained after immunization.

Our findings are reminiscent of the situation in human patients in which the production of anti-Ro52 Abs is strongly linked to the maternal MHC haplotype A1, B8, DR3B1*03 (6, 7, 48), whereas

this particular haplotype is rarely present in the children affected by the heart block (30, 49). Instead, fetal susceptibility has been suggested to relate to HLA Cw3 (30). At least 220 genes have been identified within the rat MHC (50), and the mechanism for the increased fetal susceptibility to develop AVB in response to maternally transferred autoantibodies may depend on either classical or nonclassical MHC molecules, or both. Additional studies will be needed to define the individual genes conferring the susceptibility and the mechanism by which they contribute to disease development, but it is tempting to speculate on a role for one of the many genes with immune-related functions in the MHC complex. In addition to the classical MHC genes, for which human fetal susceptibility has been suggested to relate to HLA Cw*03 (30), complement C4, complement factor B, and the proinflammatory cytokine TNF- α are encoded within the MHC complex. Complement deposition in autopsy specimens of deceased human fetuses affected by congenital heart block has been repeatedly described (51), and also the TNF- α -308 polymorphism has been implicated in the human disease (52), making these two genes attractive candidates for further investigation. Understanding the interaction between the passively obtained autoantibodies and the fetal MHC, and how this interaction leads to disease development may also increase our understanding of how the MHC locus regulates different traits in susceptibility in other autoimmune diseases, as it appears to contain several different susceptibility genes.

In summary, addressing the question of factors important for development of congenital heart block besides anti-Ro52 Abs per se, our findings demonstrate that generation of the pathogenic Ro52 Abs depends on and is restricted by maternal MHC, and that a different set of MHC-encoded genes in the fetus regulates susceptibility and determines the fetal outcome in Ro52-positive pregnancies.

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Disclosures

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