

Identification of Candidate Loci at 6p21 and 21q22 in a Genome-Wide Association Study of Cardiac Manifestations of Neonatal Lupus

Robert M. Clancy,¹ Miranda C. Marion,² Kenneth M. Kaufman,³ Paula S. Ramos,² Adam Adler,³ the International Consortium on Systemic Lupus Erythematosus Genetics, John B. Harley,⁴ Carl D. Langefeld,² and Jill P. Buyon¹

Objective. Cardiac manifestations of neonatal lupus, comprising atrioventricular conduction defects and cardiomyopathy, occur in fetuses exposed to anti-Ro/SSA antibodies, and carry substantial mortality. There is strong evidence of a genetic contribution to the risk. This study was undertaken to evaluate single-nucleotide polymorphisms (SNPs) for associations with cardiac neonatal lupus.

Methods. Children of European ancestry with cardiac neonatal lupus (n = 116) were genotyped using the Illumina 370K SNP platform and merged with 3,351

controls. Odds ratios (ORs) and 95% confidence intervals (95% CIs) for association with cardiac neonatal lupus were determined.

Results. The 17 most significant associations with cardiac neonatal lupus were found in the HLA region. The region near the MICB gene showed the strongest variant (rs3099844; $P_{\text{dom}} = 4.52 \times 10^{-10}$, OR 3.34 [95% CI 2.29–4.89]), followed by a missense variant within C6orf10 (rs7775397; $P_{\text{dom}} = 1.35 \times 10^{-9}$, OR 3.30), which lies between NOTCH4 and BTNL2, and several SNPs near the tumor necrosis factor α gene, including rs2857595 ($P_{\text{add}} = 1.96 \times 10^{-9}$, OR 2.37), rs2230365 ($P_{\text{add}} = 1.00 \times 10^{-3}$, OR 0.46), and rs3128982 ($P_{\text{add}} = 6.40 \times 10^{-6}$, OR 1.86). Outside the HLA region, an association was detected at 21q22, upstream of the transcription regulator ets-related isoform 1 (rs743446; $P = 5.45 \times 10^{-6}$, OR 2.40). HLA notwithstanding, no individual locus previously implicated in autoimmune diseases achieved genome-wide significance.

Conclusion. These results suggest that variation near genes related to inflammatory and apoptotic responses may promote cardiac injury initiated by passively acquired autoantibodies.

Since the first reports of congenital heart block as an autoimmune disease published >30 years ago (1–3), the consistency with which the presence of maternal antibodies directed against components of the Ro/SSA–La/SSB RNP complex has been demonstrated is remarkable. Injury to the fetal heart most often occurs during weeks 18–24 of gestation and is presumed to be dependent on IgG transport by neonatal Fc receptor of maternal IgG autoantibodies (4,5). Maternal health status accompanying the production of the putative autoantibodies is not a risk factor for fetal disease, since

Supported by NIH grants AR-42455 and N01-AR-42271 and by American Heart Association grant-in-aid 0655938T. Ms Marion and Drs. Ramos and Langefeld's work was supported by the Wake Forest University Health Sciences Center for Public Health Genomics. Drs. Kaufman and Harley are recipients of US Department of Veterans Affairs Merit Awards. Dr. Harley's work was also supported by NIH grants P01-AI-083194-01, P20-RR-020143-06, U19-AI-082714-01, P01-AR-049084-07, N01-AR-62277, R01-DE-018209-01A1, R37-AI-024717-21, and R01-AR-042460-15. The International Consortium on Systemic Lupus Erythematosus Genetics was funded by the Alliance for Lupus Research.

¹Robert M. Clancy, PhD, Jill P. Buyon, MD: New York University Langone School of Medicine, New York, New York; ²Miranda C. Marion, MA, Paula S. Ramos, PhD, Carl D. Langefeld, PhD: Wake Forest University, Winston Salem, North Carolina; ³Kenneth M. Kaufman, PhD, Adam Adler, BS: Oklahoma Medical Research Foundation, Oklahoma City; ⁴John B. Harley, MD: Oklahoma Medical Research Foundation, University of Oklahoma, and US Department of Veterans Affairs Medical Center, Oklahoma City.

The International Consortium on Systemic Lupus Erythematosus Genetics comprises lupus researchers who are dedicated to genetic discoveries in systemic lupus erythematosus. Its members include John B. Harley, MD, Marta E. Alarcon-Riquelme, MD, Lindsey A. Criswell, MD, MPH, Chaim O. Jacob, MD, PhD, Robert P. Kimberly, MD, Kathy L. Moser, PhD, Betty P. Tsao, PhD, Timothy J. Vyse, MA, MRCP, PhD, and Carl D. Langefeld, PhD.

Address correspondence and reprint requests to Robert M. Clancy, PhD, New York University Langone School of Medicine, 301 East 17th Street, New York, NY 10003. E-mail: bobdclancy@aol.com.

Submitted for publication February 18, 2010; accepted in revised form July 8, 2010.

many mothers are clinically asymptomatic at the time heart block is detected, and only then are antibodies sought and identified (6). Although advanced conduction abnormalities are the signature phenotype of cardiac neonatal lupus, the spectrum of injury can extend to the myocardium and endocardium (7,8). Third-degree block is not reversible and carries a significant risk of mortality (20–30%, primarily fetal and neonatal) and morbidity (with >60% of surviving children requiring permanent pacing before adulthood) (9).

Fetal exposure to maternal anti-Ro/SSA antibodies is a necessary but not sufficient condition for cardiac neonatal lupus. Three prospective studies of women with the candidate antibodies have estimated the risk of cardiac neonatal lupus to be 2% if the mother has had no previously affected pregnancies (10–12). Accordingly, other factors must be required to promote cardiac injury. Evidence of a fetal genetic factor in the development of cardiac disease is empirically supported by an estimated recurrence rate in subsequent pregnancies that is ~10-fold higher than the risk to the anti-Ro/SSA-positive mother with no affected children (13). The estimated sibling recurrence risk ratio, λ_s , is ~3,000; that is, siblings of affected infants have a 3,000 times higher risk than the population prevalence of cardiac neonatal lupus per se, which is ~1:15,000 live births (14–16). Since the maternal anti-Ro/SSA antibodies are required for disease expression, conditioning on maternal anti-Ro/SSA antibodies yields a λ_s of ~10. Although supportive, data on concordance in monozygotic twins are very limited. Of 6 twin sets in which maternal anti-Ro/SSA antibodies were reported to be present and monozygosity stated, 2 (33%) were concordant for cardiac neonatal lupus (9,17–20).

We posit that genetic polymorphisms influence the fetal response to maternal autoantibodies. Herein, we report the first genome-wide association study in children with cardiac neonatal lupus associated with maternal anti-Ro/SSA antibodies. The identification of fetal genes predisposing to cardiac scar might provide insight into the pathogenesis of the disease and delineate risk profiles.

PATIENTS AND METHODS

Patients. All cases included in this study ($n = 116$) were members of families enrolled in the US-based Research Registry for Neonatal Lupus (RRNL) (9). A fetus, neonate, or child was considered to have cardiac neonatal lupus if the following criteria were met: 1) the presence of a conduction defect (first-degree, second-degree, or third-degree heart block) documented by electrocardiogram (EKG) (in the case

of first-degree block, a prolonged PR interval detected solely by in utero echocardiogram was insufficient), echocardiogram, history of pacemaker, or statement in the medical record; and/or presence of cardiac injury which specifically included autopsy evidence of a mononuclear infiltrate in the endocardium, myocardium, and pericardium; and/or endocardial fibroelastosis on echocardiogram always associated with cardiac dysfunction and 2) presence of antibodies to the 52-kd Ro/SSA, 60-kd Ro/SSA, or 48-kd La/SSB RNPs in the maternal serum, as determined by enzyme-linked immunosorbent assay using recombinant proteins or by a commercial laboratory (90% of cases were tested in the research laboratory of JPB and RMC). The 116 cases with cardiac neonatal lupus were compared with 3,351 Caucasian controls genotyped on the Illumina HAP300 SNP chip (~317,000 single-nucleotide polymorphisms [SNPs]) from the Consortium on Systemic Lupus Erythematosus (SLE) Genetics (21).

Genotyping. Genomic DNA samples (750 ng) isolated from RRNL cases (described above) were genotyped on the Illumina BeadStation 500GX following standard manufacturing protocols. Initially, 77% of the samples were genotyped on the Illumina HumanCNV370-Duo_v1 array (370,404 SNPs), and 23% of the samples on the Illumina HumanCNV370-Duo_v3 array (373,398 SNPs). There were 346,110 SNPs common to the HumanCNV370-Duo_v1 and HumanCNV370-Duo_v3 arrays (92.7% overlap). Genotypes were called using the Illumina BeadStudio 3.1 software package using previously generated cluster positions generated by Illumina. Only samples with genotype call rates >93% were included in the genome-wide association analysis. Cluster plots of the strongest associated SNPs were examined for genotype calling quality.

Autoimmune disease loci. A set of 374 SNPs on the Illumina 317K SNP chip that had previously been implicated as being associated with autoimmune diseases were identified from published genome-wide association studies; 207 of these passed our statistical quality control standards. The published genome-wide scans that were reviewed had data on $\geq 100,000$ SNPs and are available from the National Human Genome Research Institute's Catalog of Published Genome-Wide Association Studies (www.genome.gov/gwastudies) (22). All SNPs considered were reported to have a P for association value of less than 1.0×10^{-5} or were explicitly highlighted by the authors.

Statistical analysis. Admixture and SNP statistical quality control. To account for the potential confounding influences of population substructure, a principal components analysis (PCA) was computed using all SNPs with <5% missing data, no differential missingness between cases and controls ($P < 0.05$), no significant departures from Hardy-Weinberg equilibrium expectations ($P < 0.0001$ for cases and $P < 0.01$ for controls), and minor allele frequency >0.05. Due to the complexity of performing a PCA on >300K SNPs in ~3,500 individuals, recent advanced numerical algebraic techniques were used to reduce computation time (23,24). Velicer's algorithm (25) and the Tracy-Widom test (26,27) were used to identify the number of principal components. The association analyses (described below) were computed adjusting for the first 3 principal components from this analysis. At the outset of our study, genome-wide analysis was performed on 120 cardiac

neonatal lupus cases. Four individuals were removed due to relatedness (1 of each of 4 pairs of siblings).

Association analysis. Testing for association was completed using the program SNPGWA (www.phs.wfubmc.edu). For each SNP, missing data proportions for cases and controls, minor allele frequency, and exact tests for departures from Hardy-Weinberg equilibrium were calculated. The following 5 tests of genotypic association were computed: 2df overall test for 2×3 tables, dominant model, additive model (Cochran-Armitage trend test), recessive model, and lack-of-fit to an additive model. The genetic model and odds ratios (ORs) are defined relative to the minor allele. At least 10 individuals who were homozygous for the minor allele were required before computing the recessive model. The primary inference for this study was based on the additive genetic model, unless the lack-of-fit to an additive model was statistically significant ($P < 0.05$). When the lack-of-fit test was significant, then the minimum P value from the dominant, additive, or recessive models was used. ORs, 95% confidence intervals (95% CIs), and P values were calculated for all models.

RESULTS

The 116 cardiac neonatal lupus cases and 3,351 out-of-study controls were all of self-reported Caucasian ancestry (Table 1), which was consistent with the PCA. The case group was 51.7% female, and the control group was 65.7% female. Advanced heart block was present in 96% of the cases. PCA suggested adjusting for 3 principal components. Adjusting for these 3 principal components accounted for 9.8% of the genetic variation. To examine the inflation of the test statistics due to potential sources of bias (e.g., population substructure), the chi-square value from the additive genetic model adjusting for these 3 principal components via logistic regression was compared with its theoretical mean of 1. An inflation factor of 1.026 was observed, suggesting little evidence of inflation in the test statistic. Similarly, the Q-Q plot provided no evidence of systematic bias (Figure 1).

Combined association results for the entire ge-

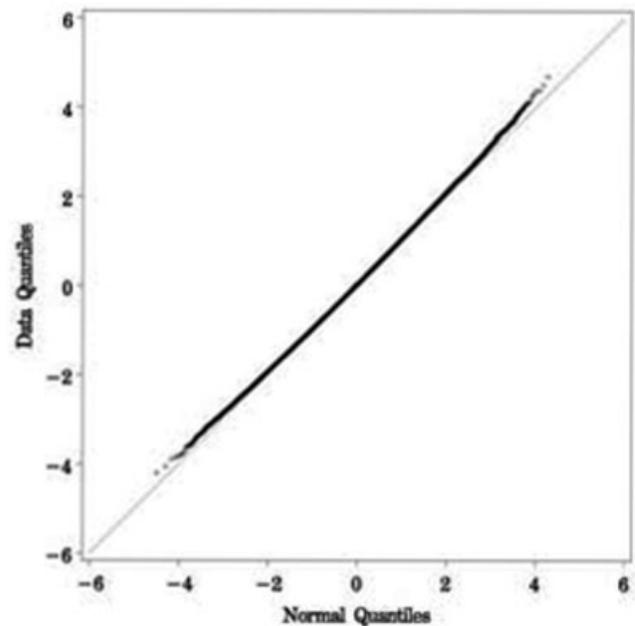


Figure 1. Q-Q plot of the genome-wide association study of cardiac neonatal lupus. The number and magnitude of observed associations (data quantile) were compared with expectations under no association (normal quantile). Each point represents 1 single-nucleotide polymorphism (of 346,110).

nome are shown in Figure 2. The 17 SNPs with the most significant associations resided in the HLA region at 6p21.3. As indicated by the farthest outlier in the Q-Q plot (Figure 1), the strongest association across the entire genome was found at rs3099844 ($P_{\text{dom}} = 4.52 \times 10^{-10}$, OR 3.34 [95% CI 2.29–4.89]), which lies in a region of copy number variation (<http://projects.tcag.ca/variation>). Interestingly, rs3099844 is near the class III major histocompatibility complex (MHC) region and 94 kb from the tumor necrosis factor α (TNF α) gene, which contains a polymorphism rs1800629 (SNP not genotyped) that is associated with cardiac neonatal lupus (Figure 3). (See Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at <http://www3.interscience.wiley.com/journal/76509746/home>, for a list of the most significant associations with cardiac neonatal lupus in the HLA region.)

The rs3099844 polymorphism is also near several flanking variants in the NFKBIL1-LTA-TNF-LTB-AIF1 region, including a signal that is located between the NCR3 and AIF1 genes (rs2857595; $P_{\text{add}} = 1.96 \times 10^{-9}$, OR 2.37 [95% CI 1.79–3.14]). In addition, within this region, there is evidence suggestive of association (rs2230365; $P = 1.00 \times 10^{-3}$, OR 0.46 [95% CI 0.29–

Table 1. Demographic characteristics of the 116 patients from the Research Registry for Neonatal Lupus*

Caucasian	116 (100)
Female†	60 (51.7)
Second-degree or third-degree heart block	107 (92)
Second-degree or third-degree heart block and cardiomyopathy	2 (1.7)
Second-degree heart block	2 (1.7)
First-degree heart block	2 (1.7)
Isolated cardiomyopathy	1 (0.8)
First-degree heart block and cardiomyopathy	2 (1.7)

* Values are the number (%) of patients.

† The control group was 65.7% female.

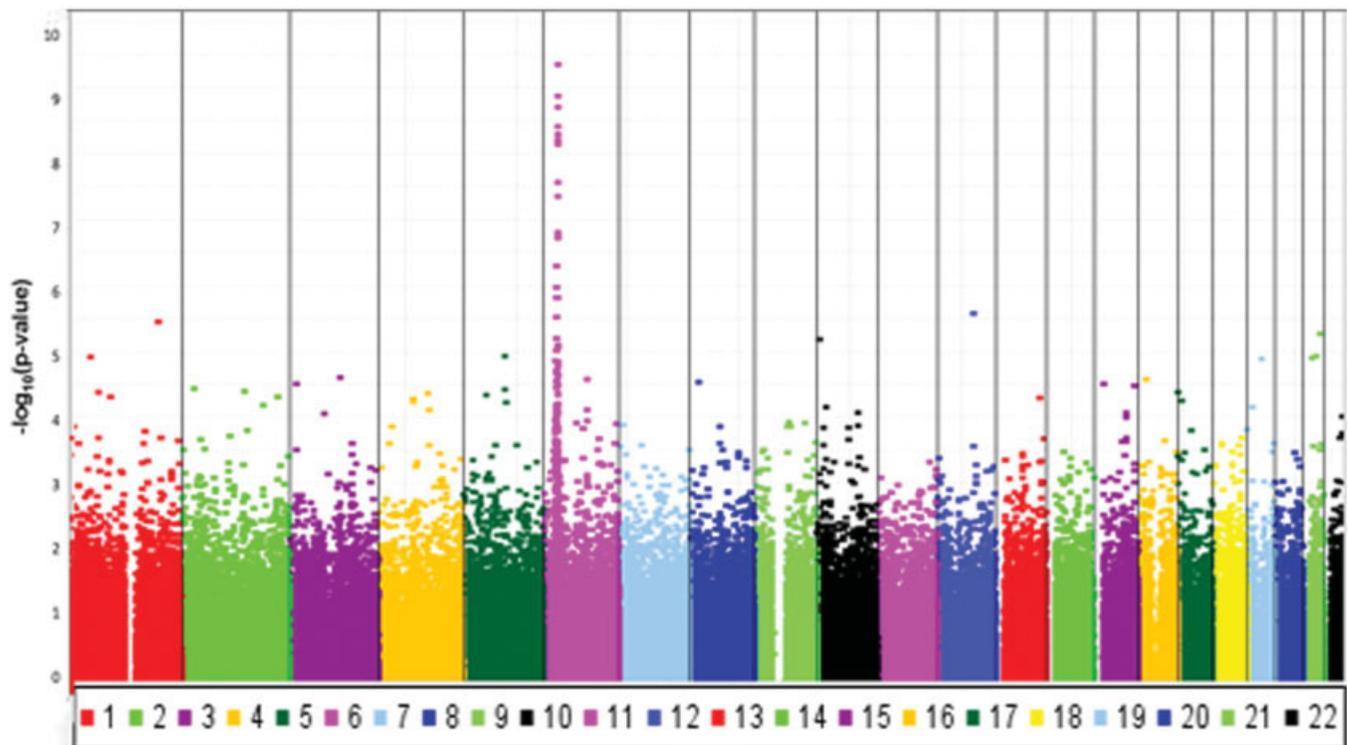


Figure 2. Combined association results for the entire genome. Each point represents 1 single-nucleotide polymorphism (of 370,000) versus the P value.

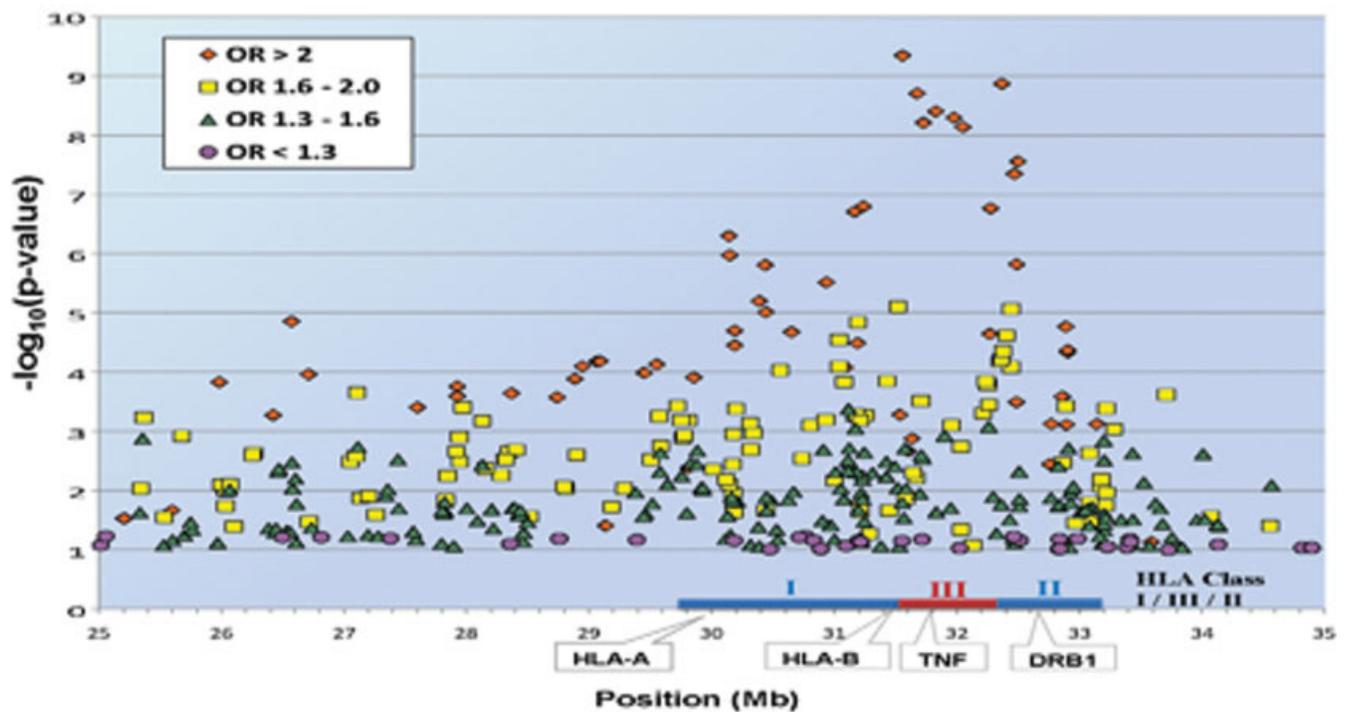


Figure 3. Graphic analysis of the HLA-A and HLA-B single-nucleotide polymorphisms with $P < 0.1$. OR = odds ratio.

Table 2. Strongest statistical associations with cardiac neonatal lupus in non-HLA regions*

Marker	Chr.	Position (kb)	Nearest genes	MAF in cases	MAF in controls	OR (95% CI)	Model	<i>P</i>
rs743446	21	38978	54 kb from NCRNA00114; 22 kb from ERG	0.64	0.49	2.40 (1.64–3.49)	Recessive	5.45×10^{-6}
rs2403106	12	82532	480 kb from TMTC2	0.15	0.07	2.48 (1.70–3.61)	Additive	2.62×10^{-6}
rs1890645	1	193798	None within 500 kb	0.38	0.30	2.98 (1.88–4.73)	Recessive	3.52×10^{-6}
rs1391511	10	4709	149 kb from AKR1E2; 15 kb from LOC338588	0.50	0.36	1.84 (1.41–2.40)	Additive	6.62×10^{-6}

* Chr. = chromosome; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval.

0.74] and rs3128982; $P = 6.40 \times 10^{-6}$, OR 1.86 [95% CI 1.41–2.43]). Outside of the NFKBIL1–LTA–TNF–LTB–AIF1 region, there is an intronic variant in HLA class III in the MSH5 gene, which is the next strongest association in this region (rs3131379; $P_{\text{add}} = 3.95 \times 10^{-9}$, OR 2.59 [95% CI 1.89–3.56]).

Within HLA class I, associated regions included PSORS1C1 (rs3130544; $P = 1.94 \times 10^{-7}$, OR 2.77 [95% CI 1.89–4.07]) and missense mutations within transcription factor 19 (TCF19) (rs7750641; $P_{\text{dom}} = 1.58 \times 10^{-7}$, OR 2.79 [95% CI 1.90–4.09]). The most centromeric side of the class I region contains a variant in proximity to the RING-finger protein 39 (RNF39) (rs9261290; $P_{\text{dom}} = 1.58 \times 10^{-7}$, OR 2.65 [95% CI 1.79–3.93]). Within HLA class II, there was a significant association between cardiac neonatal lupus and a missense variant within C6orf10 (rs7775397; $P_{\text{dom}} = 1.35 \times 10^{-9}$, OR 3.30 [95% CI 2.24–4.86]), which lies between *NOTCH4* and butyrophilin-like protein 2 (BTNL2) in the class III–class II boundary.

Outside the HLA region, an interesting association was observed between rs743446 (21q22) and cardiac neonatal lupus, near ets-related isoform 1 (ERG) ($P_{\text{rec}} = 5.45 \times 10^{-6}$, OR 2.4 [95% CI 1.64–3.49]; minor allele frequency 0.64 in cases and 0.49 in controls) (Table 2). Importantly, flanking SNPs were noted. Although they were not within the top 200 statistical associations, the multiple SNPs in proximity to rs743446 were within an extensive linkage disequilibrium block providing weak evidence of association ($P < 0.005$) (see Supplementary Table 2, available on the *Arthritis & Rheumatism* Web site at <http://www3.interscience.wiley.com/journal/76509746/home>).

Associations outside the HLA region were also noted at rs2403106 (12q21; $P_{\text{add}} = 2.62 \times 10^{-6}$, OR 2.48 [95% CI 1.70–3.61]) and rs1391511 (10p15; $P_{\text{add}} = 6.6 \times 10^{-6}$, OR 1.84 [95% CI 1.41–2.4]). Neither of these polymorphisms was in proximity to any known genes or structural variation. Also, rs1890645, in a region of a known copy number polymorphism, was associated with

cardiac neonatal lupus (1q31; $P_{\text{rec}} = 3.52 \times 10^{-6}$, OR 2.98 [95% CI 1.88–4.73]).

Given that this sample is the world's largest for any genetic study of cardiac neonatal lupus and the only genome-wide association study, it is important to highlight some suggestive associations that others might use to attempt more focused association studies (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at <http://www3.interscience.wiley.com/journal/76509746/home>). In the context of the functional category of cell adhesion, SNP rs2432143 is within an intron of ITGA1 at 5q11 ($P_{\text{dom}} = 4.5 \times 10^{-5}$, OR 2.31 [95% CI 1.54–3.45]). Within 1p34.2, rs9960 ($P_{\text{add}} = 1.2 \times 10^{-5}$, OR 1.94 [1.44–2.62]) is in the region containing the erythroblast membrane-associated protein (ERMAP), a gene that also plays a role in cell adhesion. In proximity to the CD200 cell surface glycoprotein receptor 2 (CD200R1L) resides rs6438101 (3q13.2; $P_{\text{rec}} = 2.5 \times 10^{-5}$, OR 2.29 [95% CI 1.56–3.36]). In a group related to membrane potential, at 1q43, rs1072319 and rs1578180 are 2 SNPs at an intron of the cholinergic receptor, muscarinic 3 (CHRM3), with suggestive associations at $P_{\text{rec}} = 7.4 \times 10^{-4}$ (OR 3.2 [1.63–6.4]) and $P_{\text{rec}} = 7.8 \times 10^{-4}$ (OR 3.22 [1.63–6.37]), respectively.

SNP rs6767890, a candidate involving lipid metabolism, was associated with cardiac neonatal lupus (3p24; $P_{\text{dom}} = 1.2 \times 10^{-5}$, OR 2.25 [95% CI 1.54–2.30]) and is within the raft-linking protein (RFTN1). RFTN1 is expressed in B cells, may regulate B cell antigen receptor-mediated signaling, and may be important in the formation and maintenance of lipid rafts. With regard to a function of signal transduction, an example is rs1913342, which is located at locus 1q42, within intron 1 of CDC42BPA ($P_{\text{rec}} = 6.55 \times 10^{-4}$, OR 4.12 [95% CI 1.81–9.23]). Also within this group, SNP rs3746314 (19q12; $P_{\text{dom}} = 1.3 \times 10^{-5}$, OR 2.37 [95% CI 1.61–3.48]) is near the Pleckstrin homology domain-containing family F member 1 (PLEKHF1) gene, an apoptosis-inducing protein gene that is expressed in the heart and placenta.

Table 3. Screening of SNPs previously found to be associated with autoimmune diseases for associations with cardiac neonatal lupus*

Marker	Chr.	Position (kb)	Locus	MAF in cases	MAF in controls	OR (95% CI)	P	Associated diseases
rs7517847	1	67454	IL23R	0.42	0.42	0.56 (0.31–1.01)	$5.47 \times 10^{-2}\dagger$	CD, IBD
rs1801274	1	159746	FCGR2A	0.57	0.49	1.36 (1.04–1.78)	$2.69 \times 10^{-2}\ddagger$	UC
rs2022013	1	181620	NMNAT2	0.34	0.42	0.72 (0.55–0.95)	$2.19 \times 10^{-2}\ddagger$	SLE
rs13017599	2	61018	REL	0.27	0.34	0.71 (0.53–0.96)	$2.35 \times 10^{-2}\ddagger$	RA
rs3197999	3	49697	MST1	0.23	0.30	0.63 (0.43–0.93)	$1. \times 10^{-2}\S$	CD
rs6439924	3	141652	CLSTN2	0.23	0.18	1.46 (1.07–1.99)	$1.78 \times 10^{-2}\ddagger$	CD
rs6897932	5	35910	IL7R	0.21	0.25	0.67 (0.46–0.99)	$4.42 \times 10^{-2}\S$	MS, type 1 DM
rs4613763	5	40428	PTGER4	0.17	0.11	1.59 (1.11–2.27)	$1.07 \times 10^{-2}\ddagger$	CD
rs20541	5	132024	IL13	0.15	0.19	0.67 (0.44–1.03)	$6.81 \times 10^{-2}\S$	Psoriasis
rs729302	7	128356	IRF5-TNPO3	0.24	0.31	0.59 (0.41–0.87)	$6.95 \times 10^{-3}\S$	SLE
rs10239340	7	128456	IRF5-TNPO3	0.28	0.37	0.64 (0.48–0.85)	$2.40 \times 10^{-3}\ddagger$	SLE
rs12537284	7	128505	IRF5-TNPO3	0.18	0.13	1.45 (1.03–2.05)	$3.24 \times 10^{-2}\ddagger$	SLE
rs7111341	11	2170	INS	0.31	0.25	1.32 (1.00–1.75)	$4.76 \times 10^{-2}\ddagger$	Type 1 DM
rs4788084	16	28447	IL27	0.34	0.39	0.76 (0.58–1.01)	$6.07 \times 10^{-2}\ddagger$	Type 1 DM
rs8049439	16	28745	IL27	0.31	0.38	0.73 (0.55–0.97)	$3.02 \times 10^{-2}\ddagger$	IBD

* SNPs = single-nucleotide polymorphisms; CD = Crohn's disease; IBD = inflammatory bowel disease; UC = ulcerative colitis; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; MS = multiple sclerosis; type 1 DM = type 1 diabetes mellitus (see Table 2 for other definitions).

† Recessive model.

‡ Additive model.

§ Dominant model.

In a category of candidates with unknown function, the SNP rs29911 (5q15; $P_{\text{add}} = 1.2 \times 10^{-5}$, OR 2.17 [95% CI 1.53–3.07]) is in the region containing KIAA0825 and is located in a known copy number region. At 21q22.3, a variant is in proximity to WD repeat domain 4 protein (WDR4) (rs2839586; $P_{\text{rec}} = 2.0 \times 10^{-4}$, OR 4.63 [95% CI 2.04–10.53]) and rs2839597 (11 kb downstream from rs2839586, in the promoter region of WDR4) ($P_{\text{rec}} = 9.5 \times 10^{-4}$, OR 3.94 [95% CI 2.08–9.68]).

To test whether polymorphisms implicated in other autoimmune diseases might influence the risk for cardiac neonatal lupus, 204 SNPs reported in genome-wide association studies of autoimmune diseases that passed our quality control filters were screened for evidence of association (Table 3). (For additional data, see Supplementary Table 3, available on the *Arthritis & Rheumatism* Web site at <http://www3.interscience.wiley.com/journal/76509746/home>.) Not surprisingly given the overall sample size, the data did not support a significant association of any previously described autoimmune-associated polymorphisms with cardiac neonatal lupus. The highest association was displayed by 3 SNPs in the IRF5 region, which is one of the genes with a most robustly established association with SLE (21) (rs10239340; $P_{\text{add}} = 2.40 \times 10^{-3}$, OR 0.64 [95% CI 0.48–0.85]). It is interesting to note that if the autoimmune disease loci do not correlate with cardiac neonatal lupus, then the expected inflation factor for these loci should coincide with that of the rest of the genome (i.e., 1.01). However, the observed inflation factor for

the autoimmune-associated SNPs was significantly larger (i.e., 1.22; $P < 0.03$), suggesting collectively an enrichment of association with these loci. Whether this pattern reflects maternal enrichment of these risk variants and/or cardiac neonatal lupus loci requires further study. As noted above and consistent with many autoimmune diseases, the HLA region contained several strong associations (Figure 3 and Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at <http://www3.interscience.wiley.com/journal/76509746/home>).

DISCUSSION

Through the application of genome-wide association methods and subsequent replication studies, it is anticipated that validated markers will emerge that can be used as novel diagnostic and prognostic tools for risk stratification in counseling mothers with anti-Ro/SSA antibodies. In this first genome-wide association study of 116 Caucasian children with cardiac neonatal lupus, the most strongly associated variants (MICB region at class I, NFKBIL1–LTA–TNF–LTB–AIF1 at class III, and C6orf10 at class II) were found in the MHC region, a locus with extensive linkage disequilibrium. Outside the HLA locus, an association was identified at locus 21q22, 22 kb upstream of ERG. With the exception of the HLA region, no locus previously implicated in autoimmune diseases was found to have genome-wide significance in children with cardiac neonatal lupus.

In recent genome-wide association studies of SLE patients, the most significant association was found in the HLA region at 6p21.3. In this 7.05-Mb region, 93 SNPs had a P value of less than 10^{-6} , a result which represents the long-range linkage disequilibrium related to the extended HLA-A1;B8;DR3 haplotype (28). The association of this extended haplotype with the generation of antibodies to Ro/SSA and La/SSB in the context of Sjögren's syndrome and SLE has been consistently demonstrated (29,30). The class III TNF α polymorphism at position -308 (TNF α , rs1800629, TNF α -308A polymorphism-TNF2 allele, proinflammatory), which is also part of the extended haplotype, is associated with a number of autoimmune diseases, including Sjögren's syndrome (31), SLE (32), subacute cutaneous lupus erythematosus (33), rheumatoid arthritis (34), and ulcerative colitis (35). In earlier limited studies of families enrolled in the RRNL (36), the TNF2 allele was significantly overrepresented in the mothers as well as the affected and unaffected siblings compared with healthy controls. The present study corroborated these findings in children with cardiac neonatal lupus. Specifically, 2 SNPs, which are in proximity to rs1800629, reached genome wide-significance (rs3099844 [$P_{\text{dom}} = 4.5 \times 10^{-10}$] and rs2857595 [$P_{\text{add}} = 1.96 \times 10^{-9}$]). Notably, there is strong linkage disequilibrium between rs3099844 and rs1800629 ($r^2 = 0.64$) and between rs2857595 and rs1800629 ($r^2 = 0.86$). What is yet to be established is whether the HLA candidates reflect the inheritance of maternal associations expected in women with anti-Ro/SSA and anti-La/SSB antibodies. Association analysis in these families using the transmission disequilibrium test (TDT) will help to elucidate this relationship.

The genetic factors may represent a dual hit by differentially influencing disease in this maternal/fetal dyad. On the maternal side the genes promote the necessary autoantibodies, and on the fetal side the genes promote tissue inflammation in a permissive in utero environment, e.g., hypoxia. The functional biology is supported by an in vitro model of cardiac neonatal lupus in which TNF α was secreted by macrophages cocultured with anti-Ro/SSA-bound human fetal cardiocytes (37).

The identification of associations with HLA class I genes in the present study is consistent with the results of a prior study of 40 children with cardiac neonatal lupus that revealed an enrichment of HLA-Cw7 (38). HLA-Cw7 is inclusive of the PSORS1 region (which is associated with psoriasis [39]). In the present study, associations in this region were found for rs3130544 and rs7750641. The functional effects of these candidate genes within PSORS1 may relate to stimulation of

receptors for class I MHC and dysfunction of natural killer (NK) or T cell self tolerance. A combination of class I MHC and ligand (on NK or T cells) may augment susceptibility to autoimmune injury during pregnancy.

Cardiac neonatal lupus was also associated with non-HLA regions, including loci at 21q22, 12q21,1q31, and 10p15. A cluster of associated SNPs at 21q22 is in proximity to ERG-ETS2/WDR4. ERG is a transcription factor that serves as a "brake" to both apoptosis and inflammation, components previously described in the cascade to injury and replacement of the atrioventricular node by fibrosis (36,40,41). Yi and coworkers demonstrated that ERG protects fibroblasts against apoptosis induced by serum deprivation (42). Recently, Yuan and coworkers demonstrated that ERG plays a role in repressing the expression of interleukin-8 (IL-8) (43), a mediator of inflammatory cell accumulation produced by numerous cell types, including macrophages and fibroblasts. In addition, it has been demonstrated that ERG plays a role in augmenting the expression of transforming growth factor β (TGF β) receptor type II (44). Thus, a polymorphism associated with low expression or diminished function of the encoded protein may represent the absence of a needed protective factor for fetuses exposed to maternal anti-Ro/SSA antibodies.

In psoriasis, risk alleles also reside at non-HLA genes such as chromosomes 1 and 5 (45), which include IL23R and IL13, respectively. Although they did not reach genome-wide significance, SNPs associated with each of these genes were associated with cardiac neonatal lupus. IL-13 is a prototypic Th2 cytokine that is also strongly profibrotic. Mice deficient in IL-13 are protected against a fluorescein isothiocyanate-induced model of lung fibrosis, and IL-13 can stimulate fibroblast collagen production independently of TGF β (46). However, it has recently been demonstrated that signaling through IL-13R α 2, initially thought to be a decoy receptor for IL-13, results in the production of TGF β (47). Moreover, in vivo gene silencing of IL-13R α 2 using small interfering RNA attenuates bleomycin-induced lung fibrosis, with decreases in TGF β 1 secretion and collagen formation (48).

In this passively acquired autoimmune disease, with the exception of HLA, there were no significant genome-wide associations with identified polymorphisms that have been implicated in other autoimmune diseases, such as SLE, in which there are genetic contributions relating to dysfunction of the acquired immune system. Overall, however, there was an inflation factor of 1.22 for these autoimmune SNPs in cardiac neonatal lupus, compared with an inflation factor of 1.01 for the

entire genome. Specifically, the enrichment of a variant of IRF5 is intriguing, and the variation at 7q32 has attracted a substantial amount of attention (21). However, it is acknowledged that maternal inheritance may account for some of these genes for which the associations are just below the level of significance. There are several possible and non-mutually exclusive interpretations. First, the associations may simply reflect the maternal enrichment of the genome for autoimmunity risk alleles, given the autoantibody state of the mother. Second, there may be an enrichment in autoimmunity-predisposing risk alleles in children with cardiac neonatal lupus independent of the maternal state. Third, there may be no significant enrichment, consistent with the relative immaturity of the human fetal immune system, which relies almost exclusively on adaptive maternal immunity, and the genetic contribution to cardiac injury may be restricted to the innate immune system and tissue reactivity.

These data require careful consideration and recognition of their inferential limits. As discussed above, maternal inheritance may be a limitation. Specifically, at 6p21, it is difficult to distinguish whether inheritance reflects transmission of genes related to maternal autoimmunity or whether these genes represent enrichment of genes that are biologically important. TDT will be informative. The limited power of the study is another limitation. However, the strength of the study is in the precise clinical phenotyping of the cases, with 96% having advanced atrioventricular block, the most characteristic cardiac manifestation associated with maternal anti-Ro/SSA antibodies. Given the rarity of the disease, the study included 4 cases of first-degree block, persisting after birth in 3 as shown by EKG, suggesting sustained injury, and 1 case of isolated cardiomyopathy. Arguably, combining cases expressing full progression of disease with those having less advanced disease may also represent a limitation.

Given the current interest in identifying a biomarker to predict more serious lifelong injury, isolated in utero prolongation of the PR interval has become an important focus of attention (10,49,50). As such, these cases could provide potential candidates to increase the power of the study of a rare disease. However, given the uncertainty as to the pathologic significance of transient first-degree block, we did not include these children in the analysis due to concerns of underestimating the genetic influence. Thus, unlike SLE, in which the manifestations are heterogeneous with regard to organ system, severity, and temporal course, cardiac neonatal lupus represents a more homogeneous phenotype.

This study represents the first large-scale investigation of genes associated with cardiac neonatal lupus. Identification of risk alleles is an incremental step toward the discovery of a fetal genetic component that contributes to the development of lifelong cardiac damage in newborns exposed to maternal anti-Ro/SSA antibodies. These analyses support the potential of this first cohort to provide clues that are immediate and of high impact in the study of the genetics of cardiac neonatal lupus, with candidates identified in several pathways relevant to the pathogenesis of disease, antigen presentation, apoptosis, and inflammation. The absence of a significant association with genes previously identified as being associated with autoimmune diseases emphasizes the passive nature of this unique disease.

ACKNOWLEDGMENTS

We gratefully acknowledge the International Consortium on Systemic Lupus Erythematosus Genetics (SLEGEN) and Drs. Kathy L. Moser, Lindsey A. Criswell, Marta E. Alarcon-Riquelme, Chaim O. Jacob, Robert P. Kimberly, Betty P. Tsao, and Timothy J. Vyse for the use of the SLEGEN control data.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Clancy had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Clancy, Langefeld.

Acquisition of data. Kaufman, Adler, Harley, Buyon.

Analysis and interpretation of data. Marion, Ramos, Langefeld.

REFERENCES

1. Chameides L, Truex RC, Vetter V, Rashkind WJ, Galioto FM Jr, Noonan JA. Association of maternal systemic lupus erythematosus with congenital complete heart block. *N Engl J Med* 1977;297:1204-7.
2. 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.
3. Reed BR, Lee LA, Harmon C, Wolfe R, Wiggins J, Peebles C, et al. Autoantibodies to SS-A/Ro in infants with congenital heart block. *J Pediatr* 1983;103:889-91.
4. Buyon JP, Clancy RM, Friedman 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.
5. Buyon J, Roubey R, Swersky S, Pompeo L, Parke A, Baxi L, et al. Complete congenital heart block: risk of occurrence and therapeutic approach to prevention. *J Rheumatol* 1988;15:1104-8.
6. Rivera TL, Izmirly PM, Birnbaum BK, Byrne P, Brauth JB, Katholi M, et al. Disease progression in mothers of children enrolled in the Research Registry for Neonatal Lupus. *Ann Rheum Dis* 2009;68:828-35.

7. Nield LE, Silverman ED, Smallhorn JF, Taylor GP, Mullen JB, Benson LN, et al. Endocardial fibroelastosis associated with maternal anti-Ro and anti-La antibodies in the absence of atrioventricular block. *J Am Coll Cardiol* 2002;40:796–802.
8. Moak JP, Barron KS, Hougen TJ, Wiles HB, Balaji S, Sreeram N, et al. Congenital heart block: development of late-onset cardiomyopathy, a previously underappreciated sequela. *J Am Coll Cardiol* 2001;37:238–42.
9. Buyon JP, Hiebert R, Copel J, Craft J, Friedman D, Katholi M, 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;31:1658–66.
10. Friedman DM, Kim MY, Copel JA, Davis C, Phoon CK, Glickstein JS, et al. Utility of cardiac monitoring in fetuses at risk for congenital heart block: the PR Interval and Dexamethasone Evaluation (PRIDE) prospective study. *Circulation* 2008;117:485–93.
11. Cimaz R, Spence DL, Hornberger L, Silverman ED. Incidence and spectrum of neonatal lupus erythematosus: a prospective study of infants born to mothers with anti-Ro autoantibodies. *J Pediatr* 2003;142:678–83.
12. Brucato A, Frassi M, Franceschini F, Cimaz R, Faden D, Pisoni MP, 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;44:1832–5.
13. Llanos C, Izmirly PM, Katholi M, Clancy RM, Friedman DM, Kim MY, et al. Recurrence rates of cardiac manifestations associated with neonatal lupus and maternal/fetal risk factors. *Arthritis Rheum* 2009;60:3091–7.
14. Fritzler MJ, Pauls JD, Kinsella TD, Bowen TJ. Antinuclear, anticytoplasmic, and anti-Sjögren's syndrome antigen A (SS-A/Ro) antibodies in female blood donors. *Clin Immunol Immunopathol* 1985;36:120–8.
15. Harmon CE, Lee LA, Huff JC, Norris DA, Weston WL. The frequency of autoantibodies to the SS-A/Ro antigen in pregnancy sera [abstract]. *Arthritis Rheum* 1984;27 Suppl 4:S20.
16. Siren MK, Julkunen H, Kaaja R. The increasing incidence of isolated congenital heart block in Finland. *J Rheumatol* 1998;25:1862–4.
17. Gawkrödger DJ, Beveridge GW. Neonatal lupus erythematosus in four successive siblings born to a mother with discoid lupus erythematosus. *Br J Dermatol* 1984;111:683–7.
18. Cooley HM, Keech CL, Melny BJ, Menahem S, Morahan G, Kay TW. Monozygotic twins discordant for congenital complete heart block. *Arthritis Rheum* 1997;40:381–4.
19. 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.
20. Barquero Genoves IM, Figueras Aloy J, Guerola Serret M, Jimenez Gonzalez R. A complete congenital heart block in twins. *An Esp Pediatr* 1996;44:164–6. In Spanish.
21. Harley JB, Alarcon-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, Moser KL, et al. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXX, KIAA1542 and other loci. *Nat Genet* 2008;40:204–10.
22. Hindorf LA, Junkins HA, Mehta JP, Manolio TA. A catalog of published genome-wide association studies. Washington: NIH/National Human Genome Research Institute. URL: <http://www.genome.gov/gwastudies/>.
23. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* 2006;38:904–9.
24. Narayanaswamy C, Raghavarao D. Principal component analysis of large dispersion matrices. *Appl Stat* 1991;40:309–16.
25. Velicer WF. Determining the number of components from the matrix of partial correlations. *Psychometrika* 1976;41:321–7.
26. Tracy C, Widom H. Level-spacing distributions and the Airy kernel. *Commun Math Phys* 1994;159:151–74.
27. Patterson N, Price AL, Reich D. Population structure and eigenanalysis. *PLoS Genet* 2006;2:e190.
28. Graham RR, Ortmann W, Rodine P, Espe K, Langefeld C, Lange E, et al. Specific combinations of HLA-DR2 and DR3 class II haplotypes contribute graded risk for disease susceptibility and autoantibodies in human SLE. *Eur J Hum Genet* 2007;15:823–30.
29. Harley JB, Reichlin M, Arnett FC, Alexander EL, Bias WB, Provost TT. Gene interaction at HLA-DQ enhances autoantibody production in primary Sjögren's syndrome. *Science* 1986;232:1145–7.
30. Scofield RH, Frank MB, Neas BR, Horowitz RM, Hardgrave KL, Fujisaku A, et al. Cooperative association of T cell β receptor and HLA-DQ alleles in production of anti-Ro in systemic lupus erythematosus. *Clin Immunol Immunopathol* 1994;72:335–41.
31. Guggenbuhl P, Veillard E, Quelvenec E, Jégo P, Semana G, Jean S, et al. Analysis of TNF α microsatellites in 35 patients with primary Sjögren's syndrome. *Joint Bone Spine* 2000;67:290–5.
32. Rood MJ, van Krugten MV, Zanelli E, van der Linden MW, Keijsers V, Schreuder GM, et al. TNF-308A and HLA-DR3 alleles contribute independently to susceptibility to systemic lupus erythematosus. *Arthritis Rheum* 2000;43:129–34.
33. Werth VP, Zhang W, Dortzbach K, Sullivan K. Association of a promoter polymorphism of tumor necrosis factor- α with subacute cutaneous lupus erythematosus and distinct photoregulation of transcription. *J Invest Dermatol* 2000;115:726–30.
34. Martínez A, Fernández-Arquero M, Pascual-Salcedo D, Conejero L, Alves H, Balsa A, et al. Primary association of tumor necrosis factor-region genetic markers with susceptibility to rheumatoid arthritis. *Arthritis Rheum* 2000;43:1366–70.
35. Sashio H, Tamura K, Ito R, Yamamoto Y, Bamba H, Kosaka T, et al. Polymorphisms of the TNF gene and the TNF receptor superfamily member 1B gene are associated with susceptibility to ulcerative colitis and Crohn's disease, respectively. *Immunogenetics* 2002;53:1020–7.
36. Clancy RM, Backer CB, Yin X, Kapur RP, Molad Y, Buyon JP. Cytokine polymorphisms and histologic expression in autopsy studies: contribution of TNF- α and TGF- β 1 to the pathogenesis of autoimmune-associated congenital heart block. *J Immunol* 2003;171:3253–61.
37. Miranda-Carus ME, Askanase AD, Clancy RM, Di Donato F, Chou TM, Libera MR, et al. 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.
38. Clancy R, Yin X, Askanase A, Miranda-Carus E, Nelson JL, Sestak A, et al. HLA-DRB1 relationships in neonatal lupus families [abstract]. *Arthritis Rheum* 2003;48 Suppl 9:S410–1.
39. Nair RP, Stuart PE, Nistor I, Hiremagalore R, Chia NV, Jenisch S, et al. Sequence and haplotype analysis supports HLA-C as the psoriasis susceptibility 1 gene. *Am J Hum Genet* 2006;78:827–51.
40. 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;50:173–82.
41. Clancy RM, Neufing PJ, Zheng P, O'Mahony M, Nimmerjahn F, Gordon TP, et al. Impaired clearance of apoptotic cardiocytes is linked to anti-SSA/Ro and-SSB/La antibodies in the pathogenesis of congenital heart block. *J Clin Invest* 2006;116:2413–22.
42. Yi H, Fujimura Y, Ouchida M, Prasad DD, Rao VN, Reddy ES. Inhibition of apoptosis by normal and aberrant Fli-1 and erg proteins involved in human solid tumors and leukemias. *Oncogene* 1997;14:1259–68.

43. Yuan L, Nikolova-Krsteovski V, Zhan Y, Kondo M, Bhasin M, Varghese L, et al. Antiinflammatory effects of the ETS factor ERG in endothelial cells are mediated through transcriptional repression of the interleukin-8 gene. *Circ Res* 2009;104:1049–57.
44. Im YH, Kim HT, Lee C, Poulin D, Welford S, Sorensen PH, et al. EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor β type II receptor gene. *Cancer Res* 2000;60:1536–40.
45. Cargill M, Schrodi SJ, Chang M, Garcia VE, Brandon R, Callis KP, et al. A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes. *Am J Hum Genet* 2007;80:273–90.
46. Kolodsick JE, Toews GB, Jakubzick C, Hogaboam C, Moore TA, McKenzie A, et al. Protection from fluorescein isothiocyanate-induced fibrosis in IL-13-deficient, but not IL-4-deficient, mice results from impaired collagen synthesis by fibroblasts. *J Immunol* 2004;172:4068–76.
47. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13 α 2 receptor is involved in induction of TGF- β 1 production and fibrosis. *Nat Med* 2006;12:99–106.
48. Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis: the myofibroblast in focus. *Chest* 2007;132:1311–21.
49. 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.
50. Rein AJ, Mevorach D, Perles Z, Gavri S, Nadjari M, Nir A, et al. Early diagnosis and treatment of atrioventricular block in the fetus exposed to maternal anti-SSA/Ro-SSB/La antibodies: a prospective, observational, fetal kinetocardiogram-based study. *Circulation* 2009;119:1867–72.