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Congenital Heart Disease Linked to Maternal Autoimmunity against Cardiac Myosin

Charles R. Cole,* Katherine E. Yutzey,* Anoop K. Brar,† Lisa S. Goessling,† Sarah J. VanVickle-Chavez,† Madeleine W. Cunningham,‡,1 and Pirooz Eghtesady †,1

Structural congenital heart disease (CHD) has not previously been linked to autoimmunity. In our study, we developed an autoimmune model of structural CHD that resembles hypoplastic left heart syndrome (HLHS), a life-threatening CHD primarily affecting the left ventricle. Because cardiac myosin (CM) is a dominant autoantigen in autoimmune heart disease, we hypothesized that immunization with CM might lead to transplacental passage of maternal autoantibodies and a prenatal HLHS phenotype in exposed fetuses. Elevated anti-CM autoantibodies in maternal and fetal sera, as well as IgG reactivity in fetal myocardium, were correlated with structural CHD that included diminished left ventricular cavity dimensions in the affected progeny. Further, fetuses that developed a marked HLHS phenotype had elevated serum titers of anti-β-adrenergic receptor Abs, as well as increased protein kinase A activity, suggesting a potential mechanism for the observed pathological changes. Our maternal–fetal model presents a new concept linking autoimmunity against CM and cardiomyocyte proliferation with cardinal features of HLHS. To our knowledge, this report shows the first evidence in support of a novel immune-mediated mechanism for pathogenesis of structural CHD that may have implications in its future diagnosis and treatment. The Journal of Immunology, 2014, 192: 4074–4082.

C
ongenital heart disease (CHD) is the most common cause of infant death resulting from birth defects (1). Hypoplastic left heart syndrome (HLHS), a severe and devastating congenital heart malformation, accounts for nearly 25% of all neonatal deaths from CHD (1–3). HLHS is uniformly fatal without intervention, and despite aggressive medical and surgical palliation, many affected children experience a significant developmental delay and decreased quality of life (4, 5). Although etiological mechanisms leading to HLHS are largely unknown, both genetic and environmental insults are potential contributors (6–10). About one fourth of HLHS cases occur in the context of recognized genetic disorders or syndromes; studies involving nonsyndromic family members suggest that heritability is complex (9, 11) and environmental influences such as infection and autoimmunity might contribute to the phenotypic expression of certain subsets of HLHS (3, 6, 12, 13).

In some cases of CHD, transplacental passage of maternal IgG has been reported to affect the fetus. For instance, in congenital heart block, maternal autoantibodies in patients with systemic lupus erythematosus cause injury to the conduction system of the fetal heart (14–16). We had previously hypothesized that autoimmunity might play a role in a maternal–fetal model of structural left-sided CHD (12). Our hypothesis has been supported by the observation of high titers of anti–human cardiac myosin (CM) IgG autoantibodies in sera from mothers of babies with HLHS, but not other CHD or healthy controls, in an ongoing clinical study (Clinicaltrial.gov 201102410). Anti-CM autoantibodies are linked to several autoimmune diseases of the heart, including autoimmune myocarditis (17–22) and rheumatic carditis, the most serious manifestation of group A streptococcus–induced rheumatic fever (23–25). In this study we determined whether maternal immunization with CM, a major autoantigen in human heart (22), could produce an HLHS-like phenotype in susceptible offspring following transplacental passage of anti–heart Abs. Experiments conducted in the Lewis rat, an established model of CM-induced autoimmune heart disease (19, 20), led to an HLHS-like phenotype seen in human infants. Autoimmunity against the heart is a new concept in the pathogenesis of HLHS.

Materials and Methods

Ag preparation

Rat CM was purified from rat heart tissue according to previously described techniques, with slight modifications (25, 26). Heart tissue was homogenized in a low-salt buffer [40 mM KCl, 20 mM imidazole, 5 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid, 5 mM DTT, 0.5 mM PMFS, 1 μg leupeptin per milliliter for 15 s on ice. The washed myofibrils were collected by centrifugation at 16,000 × g for 10 min. The pellets were then resuspended in high-salt buffer [0.3 M KCl, 0.15 M K2HPO4, 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid, 5 mM DTT, 0.5 mM PMFS, 1 μg leupeptin per milliliter] and homogenized for three 30-s bursts on ice. The homogenized tissue was allowed to proceed for 30 min. After clarification by centrifugation, actomyosin was collected by centrifugation at 16,000 g for 10 min. The actomyosin pellet was then resuspended...
in high-salt buffer, ammonium sulfate was increased to 33%, and the KCl concentration was increased to 0.5 M. After the actomyosin pellet and salts were dissolved, ATP was added to 10 mM and MgCl₂ was added to 5 mM, and then the solution was centrifuged at 20,000 × g for 15 min to remove actin filaments. The supernatant was removed and stored at 4°C in the presence of the following inhibitors: 0.5 mM PMSF; 5 μg/ml N-tosyl-L-lysine chloromethyl ketone, and 1 μg leupeptin per milliliter. The presence of CM was verified and quantitated by ELISA and Western immunoblot using mAb specific for CM protein.

**Immunization protocol**

Specific pathogen–free female Lewis rats (LEW-R1) (~8 wk old) were purchased from Charles River Laboratories (Raleigh, NC) and were maintained in a pathogen-free environment at Cincinnati Children’s Hospital animal facility. Rats were acclimated for 3 d prior to the immunization protocol. All animals were treated according to institutional guidelines and Institutional Animal Care and Use Committee–approved protocols. Females rats were immunized with either rat cardiac CM (n = 8) or saline (controls; n = 5). A schematic of the immunization protocol is shown in Supplemental Fig. 1. The rats treated with CM were immunized on day 0 by s.c. injection of 1 mg rat CM extract emulsified in CFA at a 1:1 ratio (v/v) in a total volume of 400 μl. On days 14, 28, and 42 after the initial immunization, all the rats were boosted i.p. with 500 μg extract emulsified in a 1:1 ratio (v/v) of CFA and saline in a total volume of 200 μl. Serum titers of CM Abs were determined by ELISA assays every 7–14 d. Rats with no response (<1:100) exited the protocol, and rats with medium titers (<1:6400) were given up to a total of three additional boosters. In the control group, CM extract was exchanged for saline in the presence of adjuvant. Control animals all received three booster injections. Beginning d7 after the final booster. No boosters were administered during gestation, which in the Lewis rat is 22.6400) were given up to a total of three additional boosters. In the control group, CM extract was exchanged for saline in the presence of adjuvant. Control animals all received three booster injections. Beginning d7 after the final booster. No boosters were administered during gestation, which in the Lewis rat is 22.6400) were given up to a total of three additional boosters. In the control group, CM extract was exchanged for saline in the presence of adjuvant. Control animals all received three booster injections. Beginning d7 after the final booster. No boosters were administered during gestation, which in the Lewis rat is 22.

Ab quantification by direct ELISA

The assays were conducted as described in previous publications (21, 22, 25). Immunol 4 (Dynatech) microtiter plates were coated at 4°C overnight with rat CM at 10 μg/ml in 0.1 M carbonate–bicarbonate coating buffer (pH 9.6). Plates were washed with PBS containing 0.05% Tween 20 and blocked for 1 h at 37°C with 1% BSA (Fisher Scientific, Hanover Park, IL). Plates were washed once again with PBS/Tween 20. To determine the rat anti-CM ELISA Ab titer, rat sera were titrated at an initial dilution of 1:500 in 1% BSA in PBS buffer and thereafter diluted 2-fold, up to a final dilution of 1:12,800. A total of 50 μl diluted serum was loaded into microtiter wells in duplicates and incubated overnight at 4°C. Plates were washed 5× with PBS/Tween 20, and 50 μl goat anti-rat IgG (Sigma-Aldrich, St Louis, MO) conjugated with alkaline phosphatase (1/500 dilution) was added and incubated at 37°C for 1 h. Plates were washed with PBS/Tween 20, and 50 μl substrate para-nitrophenyl phosphate (Sigma-Aldrich) in 0.1 M diethanolamine buffer (pH 9.8) was added to each well. OD was measured at 405 nm in an ELISA plate reader (ELx800, BioTek Instruments, Winooski, VT). Titters were determined with an OD value of 0.10 ± 0.60. Anti-rat CM Ab titers in the ELISA were standardized and controlled using negative and positive control sera, obtained from previous experiments. β-adrenergic receptors (β-AR) 1 (β₁-AR) and 2 (β₂-AR) (PerkinElmer) were coated at 10 μg/ml onto microtiter plates for testing for rat IgG Abs against the anti-β₁-AR and anti-β₂-AR Abs in the serum. ELISA was performed according to the same procedure stated above. Activation of serum protein kinase A (PKA) by the β-AR was performed as previously described (21, 22).

**Histology and morphometry studies**

Sections from each fetal heart were stained with Movat’s pentachrome or Masson’s trichrome (both from American Mastertech Scientific). All morphometric measurements were obtained using ImageJ software. Comparable apical four-chambered sections from each fetal heart were photographed (Nikon DS R1) and coded to eliminate bias. Two blinded observers obtained measurements. At the leftventricular (LV) and right ventricular (RV) lateral and apical free walls, three measurements were taken in 100-μm intervals. Three area measurements of the LV and RV were also obtained using comparable apical four-chambered sections. Measurements were then averaged from each location for statistical analysis. Owing to some cardiac damage during harvest that altered heart chamber dimensions, two affected and two unaffected fetal hearts were excluded from LV/RV lumen area measurements. Maternal hearts were processed as previously described (20). A veterinary pathologist evaluated maternal heart sections for the presence of myocarditis and valvulitis.

**Western blot analysis**

Binding of maternal and fetal serum to lysates (10 μg total protein) of adult rat heart, liver, lung, and spleen was determined by Western blot analysis, as previously described (22). Rats from CM-injected maternal rats and affected fetal offspring, along with sera from control maternal rats and fetal offspring, were analyzed at a dilution of 1:1000. Preincubation of the sera with porcine CM (20 μg; Sigma-Aldrich) prior to incubation of the blots was performed to determine specificity.

**Immunohistochemistry studies**

The anti-rat IgG staining was performed as previously described, with slight modifications, (21) and was γ-chain specific for rat IgG. Briefly, mounted tissues were baked at 60°C for 20 min and deparaffinized using a 3:1 ratio of Hemo-D (Fisher) to xylene. After rehydration in graded ethanol washes, tissues were washed twice with PBS, blocked with Protein Block (Bio-Genex, San Ramon, CA) for 30 min at room temperature, and washed twice with PBS; Isotype control goat IgG biotin or biotin-conjugated goat anti-rat IgG Abs (diluted 1:500; Jackson ImmunoResearch, West Grove, PA) were incubated on tissues overnight at 4°C in a humidity chamber, followed by three washes in PBS. Alkaline phosphatase–conjugated streptavidin was incubated with the tissues at 1 mg/ml for 30 min at room temperature. After three washes in PBS, Ab binding was detected with Fast Red substrate (Biogenex) against a counterstain of Mayer’s hematoxylin (BioGenex). Stained slides were mounted with crystal mount (Fisher), dried, and coverslipped using Permount (Sigma Chemical) The amount of IgG bound is indicated by scoring the intensity of visual Fast Red staining in the heart tissue. Similar to the previously described process, sections were deparaffinized and rehydrated and then had antigenic sites unmasked using the citrate-based solution (#H-3300; Sigma-Aldrich) and high temperature-pressure protocol of Vector Laboratories. Sections were blocked for 1 h at room temperature with 8% normal goat serum (#G9023; Sigma-Aldrich), then incubated overnight at 40°C with primary Abs PHH3 (#06-570 [1:350]; Millipore), to identify mitotic cells, and MF20 (#MF 201 [2000]; Developmental Studies Hybridoma Bank), to identify myocytes. For immunofluorescent detection, sections were incubated with Alexa Fluor–conjugated secondary Abs (goat anti-rabbit IgG #A11011 and goat anti-mouse IgG #A11001 [1:100]; Invitrogen) for 1 h at room temperature, followed by a 30-min incubation with the nuclear stain TO-PRO-3 (#T3605 [1:1000]; Invitrogen). Samples were imaged using a Zeiss LSM 510 confocal microscope. The total number of nuclei, the number of nuclei in myocytes, the total number of phH3-positive nuclei, and the number of phH3-positive nuclei in myocytes were counted for each image, using ImageJ software. The total proliferative rate was calculated using total phH3-positive nuclei divided by total cardiomyocyte nuclei. To determine the mitotic index, counts from four areas of the LV, using comparable areas from each heart, were totaled and used to calculate the percentage of phH3-positive nuclei. TUNEL staining (cat. no. 11 684 809 910; Roche Applied Science) was performed according to the manufacturer’s instruction. Cardiomyocytes were identified using MF20 staining, and the cardiomyocyte-specific proliferative rate was calculated using cardiomyocyte phH3-positive nuclei divided by total cardiomyocyte nuclei.

**Statistical analysis**

All statistical analyses were performed using SAS version 9.2. Two variables were analyzed using two-sided, independent sample t tests, and three variables were analyzed with two-way ANOVA with Tukey–Kramer adjustment for multiple comparisons.

**Results**

Heart defects correlated with elevated anti-CM titers

Female Lewis rats immunized with CM developed peak anti-CM autoantibody titers ranging from 1:6000 to >1:12,800, prior to pregnancy (Fig. 1A). Most importantly, all maternal rats (n = 8 mothers with 47 fetuses) with elevated anti-CM Ab titers had at
Immunization with CM induces elevated anti-CM Ab titers in adult rats and their fetal offspring. (A) Serum titers measured in individual female Lewis rats (8 wk old) immunized with purified rat CM (n = 8 mothers with 47 fetuses), followed by three to four booster injections administered at 2-wk intervals, are shown. Adjuvant was injected in control rats (data not shown). (B) Average fetal (harvested at estimated gestational day 20) anti-CM Ab titers in litters of individual adult animals with positive anti-CM Ab response (n = 5 mothers) prior to mating, during pregnancy, and up to time of harvest. Bars shown are the mean ± SEM.

The HLHS-like pathology observed in fetuses from CM-immunized mother rats is illustrated in Fig. 3. We observed that 32% (15 of 47) of fetuses in the CM treatment group developed a left-sided structural CHD and 28% (13 of 47) of the fetuses had reduced or hypoplastic LV cavities (Fig. 3B, 3D), whereas none of the control fetuses (n = 19) had cardiac abnormalities (Fig. 3A, 3C). The congenitally malformed, affected fetal hearts with an HLHS phenotype had a thickened LV myocardium (30%; 14 of 47) and/or loss of normal mitral and aortic valve structure (26%; 12 of 47) (Fig. 3D). The left-sided valve structures displayed loss of smooth rounded edges and were foreshortened. The affected fetal hearts that were severely malformed (23%; 11 of 47) showed a 50–160% increase in LV myocardial wall thickness, whereas the moderately malformed hearts (6%; 3 of 47) displayed a 15–50% increase in LV wall thickness. In adjuvant controls, the fetal LV free wall myocardium displays normal compact myocardium (Fig. 3E). The severely malformed hearts also had a “spongy” LV myocardium (Fig. 3F). Examination of H&E-stained maternal heart sections from all CM-immunized adult maternal test rats and adult maternal control rats receiving only adjuvant appeared normal and showed no evidence of myocarditis or any abnormal histological features in either maternal group. In addition, in the fetal hearts, no myocarditis or cellular infiltration of the myocardium was found.

LV primarily affected in rat model with HLHS phenotype

HLHS is characterized by a reduced, or hypoplastic, LV cavity that is unable to support the systemic circulation, although anatomic variation within the classification of HLHS yields a continuum of phenotypic heterogeneity (27, 28). To evaluate chamber-specific structural differences, comparable apical four-chamber sections of each heart were studied for cardiac morphometric measurements of the total CM treatment group (n = 47) compared with the adjuvant control group (n = 19). RV myocardial thickness of the total CM treatment group was not significantly different from that in the adjuvant control group. In contrast, the total CM treatment group had significantly increased myocardial thickness of the LV

Table 1. Maternal immune response against CM and associated left-sided structural congenital cardiac abnormalities in the progeny

<table>
<thead>
<tr>
<th>Fetal Litter</th>
<th>Litter Size</th>
<th>Total Maternal Ab Burden</th>
<th>Progeny with Heart Defects, % (No. Affected)</th>
<th>LV Cavity Hypoplasia, % (No. Affected)</th>
<th>Loss of Normal Valve Structure, % (No. Affected)</th>
<th>Severely Increased LV Myocardial Wall Thickness, % (No. Affected)</th>
<th>Moderately Increased LV Myocardial Wall Thickness, % (No. Affected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM4</td>
<td>11</td>
<td>$3.2 \times 10^5$</td>
<td>36 (4)</td>
<td>27 (3)</td>
<td>36 (4)</td>
<td>9 (1)</td>
<td>18 (2)</td>
</tr>
<tr>
<td>CM5</td>
<td>12</td>
<td>$4.0 \times 10^5$</td>
<td>8 (1)</td>
<td>8 (1)</td>
<td>8 (1)</td>
<td>8 (1)</td>
<td>0</td>
</tr>
<tr>
<td>CM7</td>
<td>8</td>
<td>$2.6 \times 10^5$</td>
<td>25 (2)</td>
<td>25 (2)</td>
<td>25 (2)</td>
<td>25 (2)</td>
<td>0</td>
</tr>
<tr>
<td>CM8</td>
<td>9</td>
<td>$7.3 \times 10^5$</td>
<td>67 (6)</td>
<td>55 (5)</td>
<td>44 (4)</td>
<td>55 (5)</td>
<td>11 (1)</td>
</tr>
<tr>
<td>CM10</td>
<td>7</td>
<td>$2.3 \times 10^5$</td>
<td>29 (2)</td>
<td>29 (2)</td>
<td>14 (1)</td>
<td>29 (2)</td>
<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>13</td>
<td>&lt;100</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C2</td>
<td>3</td>
<td>&lt;100</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3</td>
<td>3</td>
<td>&lt;100</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The total maternal Ab burden was calculated using area under the curve of the maternal Ab titer graph (Fig. 1). The values given are the mean ± SEM of each litter. Neither maternal nor fetal adjuvant-injected control animals (C1–3) had elevated anti-CM Ab titers.

C, adjuvant injected controls; CM, CM-immunized; D, day.
lateral free wall ($p < 0.001$) and LV apical free wall ($p < 0.05$), compared with the adjuvant control group (Fig. 4A). Statistical analysis of the affected fetal hearts with the HLHS phenotype ($n = 15$) versus the unaffected fetal hearts ($n = 32$) and adjuvant controls (Fig. 4B) demonstrated no difference in RV myocardial thickness between the three groups. Further, the HLHS-like phenotype in fetal hearts had increased LV lateral free wall thickness, compared with the unaffected fetal hearts ($p < 0.0001$) and adjuvant controls ($p < 0.0001$). Affected fetal hearts with the HLHS-like phenotype also demonstrated increased LV apical free wall thickness, compared with the unaffected fetal hearts ($p = 0.0009$) and adjuvant controls ($p = 0.0001$). No significant difference was observed in the LV wall thickness of the unaffected compared with adjuvant control fetal hearts. These findings indicate that the maternal immune response against CM was associated with increased LV, but not RV, myocardial wall thickness.

The significant impact of HLHS results from altered development of the LV and left-sided valve structures, characterized by a reduced, or hypoplastic, LV cavity, rendering the heart unable to support the systemic circulation (29, 30). To determine ventricular chamber size in our model, the LV lumen area and RV lumen area of each specimen were measured, and an LV/RV lumen area ratio was used to determine relative LV chamber size. The RV served as an internal control for this comparison. The affected HLHS-like phenotype had a significantly decreased LV/RV lumen area ratio when compared with the unaffected normal fetal hearts ($p = 0.002$) and adjuvant controls ($p = 0.0007$) (Fig. 4C). No significant difference in the LV/RV lumen area ratio was noted between unaffected and adjuvant control fetal hearts. These findings indicate that the affected fetal hearts had a hypoplastic LV cavity reminiscent of HLHS in human infants.

**Increased Ab binding in affected hearts**

Because maternally acquired IgG is essential in newborn immunity, and maternally transferred Abs can mediate tissue injury (31, 32), we examined IgG binding to the fetal hearts in our study. The myocardium of affected fetal hearts with the HLHS phenotype had increased IgG deposition, compared with unaffected fetal hearts ($p = 0.03$) and adjuvant controls ($p = 0.002$) (Fig. 5). Moreover, IgG deposition in the hearts of offspring of CM-immunized mothers correlated with the observed cardiac malformations. The IgG deposition was principally found in the fetal myocardium, with minimal staining on valve structures or atrioventricular cushions. There was no IgG deposition in maternal heart sections from CM-treated or control groups.

**Cardiomyocyte proliferation increased in the HLHS phenotype**

Although the causes of certain subtypes of HLHS may originate through primary valve defects (7, 28, 33), there is evidence that HLHS may result as a consequence of abnormal myocyte proliferation during development (34, 35). Further, to determine whether increased cardiomyocyte proliferation contributed to the thickening of the LV myocardium in our model, both compact and trabeculated myocardium were examined, as the pathological specimens had increased thickness of both. Affected fetal hearts with the HLHS phenotype had an increased total LV proliferative rate, compared with the unaffected fetal hearts ($p = 0.05$) and adjuvant controls ($p = 0.003$) (Fig. 6A, 6B). The affected fetal hearts with the HLHS phenotype had an increased cardiomyocyte-specific proliferative rate, compared with the unaffected fetal hearts ($p = 0.05$) and adjuvant controls ($p = 0.002$) (Fig. 6C). In contrast, no significant differences were found between groups in the proliferative rate of nonmyocyte nuclei or in apoptosis of the myocardinium or valve structures (data not shown). Further, maternal hearts from CM-treated groups did not display any abnormal histopathological features or changes in cardiomyocyte proliferation.

**Increased anti–β-AR titers in affected fetuses**

Our previous work has shown that anti-CM Abs cross-react with the β-AR on the cardiomyocyte surface and induce cAMP-dependent PKA activity in heart cells (21). Because the β-AR also plays a regulatory role in cardiomyocyte proliferation in early life (36), we measured anti–β-AR titers in affected fetuses that had elevated anti-CM titers. We found increased anti–β1-AR ($p = 0.007$)
(Fig. 7A) and anti-β2-AR (p < 0.0001) (Fig. 7B) Ab titers in fetal sera from the CM treatment group, compared with adjuvant control sera. Because β-ARs on the heart cell surface stimulate cAMP-dependent PKA activity, we next incubated fetal sera with cultured rat heart cells (H9c2 primary cells) to determine if sera from CM-treated animals could modulate PKA activity. We found a significant increase in PKA activity above basal levels only in fetal sera from the CM treatment group that developed heart disease (affected group) compared with fetuses from the CM treatment group that were unaffected (p = 0.00023) or controls (p = 0.0005) (Fig. 8). No significant difference was noted in PKA activity in sera from unaffected fetuses from the CM group or controls (p = 0.1849).

Discussion
This study presents data supporting a novel concept that defines an HLHS-like phenotype caused by a maternal autoimmune response against CM. Observations in our fetal rat model of elevated autoantibodies against CM, including heart-specific binding of CM-immunized maternal and affected fetal serum, IgG deposition in fetal rat hearts, and the appearance of an HLHS-like phenotype,
The development of the congenital HLHS-like phenotype in our model in association with elevated titers against CM occurred in ∼32% of fetal rats. This rate is comparable to that in other autoimmune animal models, including experimental models of neonatal lupus, in which congenital heart block phenotype was observed in 20–30% of immunized pups (39). Although it is not clear why the disease process is primarily localized to left-sided heart structures, it is well known that in fetal circulation oxygen- and Ab-rich blood returning from the placenta will preferentially pass through the foramen ovale into the left side of the heart. Thus, fetal left-sided heart structures that are exposed to higher maternal Ab concentrations could be more susceptible to damage than right-sided structures. Further, maternal hearts in animals immunized against CM did not demonstrate any structural or inflammatory cardiac defects of the myocardium or valves, when compared with adjuvant injected controls. Thus, our observations support the hypothesis of an immune-mediated pathogenic mechanism in the development of congenital HLHS-like lesions in the fetal rat heart.

Of human cases of HLHS, ≤70% show a reduced LV cavity surrounded by a thickened LV myocardium (4, 28). Affected fetal rat hearts from CM-immunized mothers displayed an HLHS phenotype similar to that of human infants, including the characteristic hypoplastic, or decreased, LV cavity dimensions, although the RV dimensions were preserved. The affected hearts also had increased LV myocardial thickness, loss of normal structure of the mitral and aortic valves, and a disorganized myocardium, as seen in HLHS on histopathological assessment (3, 35, 37, 38). Moreover, cardiomyocyte proliferation was increased in the hearts of affected animals, which could contribute to the reduction of LV cavity size, as in HLHS. The severely malformed rat hearts also displayed a “spongy” LV myocardium, which has also been described in histopathology reports of HLHS (38).

The development of the congenital HLHS-like phenotype in our model in association with elevated titers against CM occurred in ∼32% of fetal rats. This rate is comparable to that in other autoimmune animal models, including experimental models of neonatal lupus, in which congenital heart block phenotype was observed in 20–30% of immunized pups (39). Although it is not clear why the disease process is primarily localized to left-sided heart structures, it is well known that in fetal circulation oxygen- and Ab-rich blood returning from the placenta will preferentially pass through the foramen ovale into the left side of the heart. Thus, fetal left-sided heart structures that are exposed to higher maternal Ab concentrations could be more susceptible to damage than right-sided structures. Further, maternal hearts in animals immunized against CM did not demonstrate any structural or inflammatory cardiac defects of the myocardium or valves, when compared with adjuvant injected controls. Thus, our observations
immunized group (CM-immunized rats with the HLHS phenotype. Fetal serum from the CM-immunized rats with HLHS were noted via prenatal echocardiography, are appreciated between 14 gestation in humans (14, 32). Early findings of HLHS, as diagnosed via prenatal echocardiography, are appreciated between 14 and 24 wk of gestation in nearly all cases (41, 42). This gestational period correlates with the chronology of transplacental transfer of maternal IgG when maternally transferred Abs can mediate tissue injury (14). Recent work has demonstrated that myocytes in HLHS are well differentiated (37), suggesting that HLHS results from an in utero insult to the fetus after the completion of primary cardiac morphogenesis (i.e., after the first 8 wk of human pregnancy) and corresponding to about gestation day 15.5 in the rat (34, 37, 43). IgG Ab distribution in our model suggests that the heart defects in affected animals were primarily myocardial in origin and that the valve abnormalities may be secondary in nature.

We examined a possible mechanism by which the observed anti-CM IgG response in the CM group could lead to the fetal heart disease in our model. Passive transfer of cross-reactive anti-CM/anti-β-AR IgG autoantibodies into adult rats can cause myocardial injury (21). Further, immune absorption of circulating autoantibodies improves cardiac function of patients with cardiomyopathy (44, 45). In animal models, Ab-induced cardiomyopathy induced by stimulation of the β1-AR agonist can be prevented by pharmacological neutralization of functionally active anti-β1-AR Abs or by the elimination of Abs by anti-β1-AR–selective immune absorption (46). Moreover, blocking the β-AR inhibits cardiomyocyte proliferation (36), suggesting a key role for the β-AR in the heart. In addition, studies in rats and humans have shown that removal of IgG or of specific anti-CM and anti-β-AR Abs from the sera depletes the PKA activation properties of the sera (21, 22).

Studies in our rat model showed that fetal sera contained elevated IgG autoantibody titers against CM as well as the β-AR, and furthermore, only sera from affected fetuses stimulated PKA activity in rat heart cells in culture. These data strengthen our hypothesis that functional signaling autoantibodies reactive against both the CM and the β-AR are associated with the observed HLHS-like phenotype in the Lewis rat. Further, the known cross-reactivity between CM and the β-AR may mediate the increased cardiomyocyte proliferation contributing to the thickening of the LV myocardium and, subsequently, to a reduction in LV cavity size. Other antigenic targets are also plausible as etiological factors in abnormal fetal cardiomyocyte development resulting in an HLHS-like phenotype. Pathogenesis of certain kinds of CHD such as HLHS may be influenced, either wholly or in part, by alterations in the developing fetal heart by autoantibodies leads to destruction of normal cardiac tissue of the newborn, including erythroblastosis fetalis (or hemolytic disease of the newborn), hypothyroidism, lupus erythematosus, pemphigus vulgaris, and thyrotoxicosis (40). There is also precedence for such a mechanism leading to fetal heart disease, in congenital heart block. Cardiac injury in congenital heart block is presumed to arise from the active transplacental transport of maternal IgG Abs into the fetal circulation. In this condition, injury to conduction tissue of the fetal heart by autoantibodies leads to destruction of normal pacing mechanisms (14–16).

Intrauterine and perinatal exposure of the fetus to maternal IgG during pregnancy takes place when the IgG is transported from mother to fetus across the placenta, beginning at ∼12 wk of gestation in humans (14, 32). Early findings of HLHS, as diagnosed via prenatal echocardiography, are appreciated between 14 and 24 wk of gestation in nearly all cases (41, 42). This gestational period correlates with the chronology of transplacental transfer of

![FIGURE 7.](http://www.jimmunol.org/) Increased anti–β-AR Abs and PKA activity in fetal serum of CM-immunized rats with the HLHS phenotype. Fetal serum from the CM-immunized group (n = 29) had significantly increased (A) anti–β1-AR Abs (**p = 0.007) and (B) anti–β2-AR Abs (**p < 0.0001), compared with adjuvant controls (n = 15).
in fetal cardiomyocyte proliferation or differentiation caused by autoantibodies directed to CM or related Ags.

In conclusion, our evidence suggests a potential novel autoimmune mechanism that may contribute in part to the pathogenesis of CHD such as HLHS. However, the immune response genes and factors that can have an impact on such a response—in particular, as seen in other autoimmune and cardiac diseases (47), including autoimmune myocarditis (17), remain to be elucidated. The presence of abundant IgG deposition primarily in the myocardium of affected fetuses, in addition to the absence of cellular inflammatory changes in the CM-immunized mothers and their offspring, may suggest an Ab-mediated alteration in susceptible individuals. The binding of IgG to the β-AR and activation of signaling by increasing PKA activity could be a potential plausible mechanism that stimulates myocyte proliferation, leading to the downstream increasing PKA activity could be a potential plausible mechanism that stimulates myocyte proliferation, leading to the downstream changes in CM myosin.

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Disclosures

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