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Tolerance Induction Or Sensitization In Mice Exposed To Non-Inherited Maternal Antigens (NIMA): Significant Differences In Heart Allograft Survival Between Different F1 Backcrosses^{1,2}

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Abstract

Developmental exposure to non-inherited maternal antigens (NIMA) exerts a tolerizing influence on clinical transplantation in humans and experimental animals. The aim of this study was to determine if strain and gender differences influence the NIMA effect. Six different mouse strain backcross matings of F₁ females with homozygous males (“NIMA backcross”) and corresponding control breedings of F₁ males with homozygous females were performed. H-2 homozygous offspring underwent heterotopic heart transplantation from fully allogeneic donors expressing non-inherited H-2 antigens. A NIMA tolerizing effect on heart allograft outcome was found in three of six breeding models. In all three cases the tolerizing antigens were from an H-2^{d+} strain. The tolerogenic effect was greatest in male as compared with female recipients. Offspring from the three breeding models in which no tolerance was seen appeared to be sensitized based on poorer graft survival, or enhanced T or B cell responses to the non-inherited H-2^b or ^k antigens. Significantly higher percentages of maternal antigen⁺ cells were found in the peripheral blood of tolerant versus non-tolerant strains of backcross mice prior to transplant. Our findings imply that transplants are predisposed to tolerance or rejection due to recipient developmental history and immunogenetic background.

Introduction

In the search for natural mechanisms of allotolerance, Owen et al (1) discovered almost fifty years ago that Rh-negative mothers of Rh⁺ babies had a significantly reduced likelihood of forming anti-Rh antibodies if their mothers had been Rh⁺. However, this B cell tolerance to non-inherited maternal antigens (NIMA) did not occur in every individual, and in a small subset of females, prior exposure to NIMA-Rh resulted in humoral sensitization to fetal Rh antigens and more severe erythroblastosis fetalis (1). Claas et al (2) repeated this observation in the HLA system while analyzing anti-HLA antibodies in multiply transfused, highly sensitized patients awaiting renal transplant. Although most allogeneic target cells were lysed in the presence of their anti-HLA antibodies and complement, such individuals frequently failed to make antibody against cells expressing the NIMA HLA class I. No such “immune privilege” was afforded to the HLA not inherited from the father. Upon further analysis, they found that some patients produced antibody to one of the two major class I NIMAs (either HLA-A or –

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B), suggesting that not all HLA antigens have the same ability to induce non-responsiveness. In particular, HLA-A2 and -B8 were among the NIMAs that failed to tolerize B cells of the offspring; both known to have strong immunogenicity compared to other class I HLA antigens (2). Although in both Rh and HLA studies a beneficial outcome resulting from exposure to NIMA was found, the fact that tolerance to NIMA was not universal indicated that other factors must influence the development of tolerance versus sensitization.

A similar heterogeneity has been noted in the beneficial NIMA effect on clinical transplantation. For example, Campbell et al (3) reported an improvement in maternal live donor transplants in recipients that had breast-fed as neonates. However, maternal transplants overall do not enjoy better graft survival than paternal ones (4) except in infants (5). This observation, although inherently gender-biased (since NIPA grafts were always male, the NIMAs' female), led many to doubt the existence of a clinical NIMA effect. Burlingham et al (6) subsequently found in a multi-center study that recipients of a one-HLA haplotype-mismatched renal allograft from a sibling expressing the NIMA HLA experienced significantly better long-term survival than did recipients of a sibling transplant expressing non-inherited paternal antigens (NIPA). The benefit in long-term survival was observed despite an increased incidence of early acute rejection in NIMA versus NIPA sibling recipients, suggesting a duality of sensitization along with tolerization in the NIMA subgroup (6). Smits et al (7) reported that cadaveric renal transplants, when mismatched for NIMA HLA-A, displayed significantly better graft survival rates compared to non-NIMA HLA-A mismatched grafts. Interestingly, no beneficial NIMA effect on graft survival for HLA-B or -DR was seen by this analysis.

We have previously described in a mouse heart transplant model a form of maternally induced organ allograft tolerance that closely parallels the human clinical findings (8). In this model, B6 male (H-2^{b/b}) mice were crossed with a (B6 × DBA/2)F₁ (H-2^{b/d}) female, resulting in 50% H-2^{b/b} homozygous offspring, all of which have been intimately exposed to the NIMA^d Ags in utero and orally via nursing. To control for non-MHC genes that re-assort in the F₁ backcross, the parental haplotypes were switched (B6 female × B6D2F₁ male) resulting in H-2^{b/b} offspring with similar heterogeneity in non-MHC background genes that did not have the neonatal exposure to the H-2^d haplotype. Following a fully allogeneic DBA/2 (H-2^{d/d}) heterotopic heart transplant, 57% of NIMA^d-exposed mice experienced allograft acceptance (graft survival >180 days) without any drug or conditioning treatment, whereas the NIPA^d controls uniformly rejected around day 11 post-transplant (8). We recently have shown that this beneficial NIMA effect is due to induction of NIMA-specific T regulatory cells (T_R) during ontogeny (9). While all NIMA-exposed mice showed diminished T effector responses to NIMA, the 50% of the NIMA-exposed offspring that achieved heart allograft tolerance were able to induce NIMA-specific TGF-β and IL-10 producing CD4⁺CD25⁺ T_R cells and mobilize them to the allograft.

Maternal microchimerism is present in cord blood (10) and was found to persist into adulthood in at least half of all humans tested (11,12). Most recently, Japanese transplant centers have successfully used NIMA-mismatched sibling and maternal donors in stem cell transplantation introducing the parameter of mutual fetomaternal microchimerism in donor and recipients in the selection of donors (13). There have been several studies demonstrating the frequent occurrence in fetal, neonatal and adult lymphoid, and hematopoietic tissues of maternal cells that have been transferred *in utero* and/or through nursing (14–16). Yet, maternal microchimerism is not always beneficial: it has also been associated with autoimmunity (17) as well as tolerance (8) and tissue regeneration (18,19). In this study, we investigated the importance of strain differences on the tolerizing versus sensitizing effects of NIMA-exposure using a mouse model of heterotopic cardiac transplantation and *in vitro* immunologic assays.

Materials and Methods

Source of Mice and Typing Reagents

C57BL/6 (H-2^{b/b}), DBA/2 (H-2^{d/d}), C3H (H-2^{k/k}), BALB/c (H-2^{d/d}), B6D2F₁ (H-2^{b/d}), C3D2F₁ (H-2^{k/d}), B6C3F₁ (H-2^{b/k}), and (BALB/c × B6)F₁ (H-2^{d/b}) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Offspring of F₁ backcross breeding pairs (NIMA and NIPA offspring) were weaned after 21 days, and typed for H-2 locus encoded antigens by flow cytometry using antibodies specific for H-2K^d and H-2K^k (BD Pharmingen, San Jose, CA), and H-2K^bD^b (Cedarlane, Westbury, NY). Six-twelve week old H-2 homozygous offspring were used as the graft recipients. Table 1 shows all of the F₁ backcross NIMA models studied. Experiments were performed in accordance with the National Institutes of Health and U.S. Department of Agriculture guidelines, after approval by University of Wisconsin Institutional Animal Care and Use Committee.

Heart Transplantation

Heterotopic heart transplantation was performed using an abdominal microsurgical technique (20). Grafts were monitored by daily palpation and graded from 4+ (strongest beat) to 0 (no beat). Graft rejection was determined by complete cessation of heart beat (grade 0) and was confirmed by laparotomy. In all cases, a fully allogeneic, H-2 homozygous donor was used to assess tolerance induced by a semi-allogeneic, H-2 heterozygous mother.

Post Transplant Serum Antibody Production Measurements

Serum samples were obtained from mice post transplant and analyzed for production of anti-NIMA antibodies (anti-H-2^d for D1 model, and anti-H-2^b for B1 model). Varying concentrations of serum were incubated with 10⁶ B6D2F₁ or B6C3F₁ thymocytes for 30 minutes. After washing, anti-mouse IgG₁ FITC or anti-mouse IgG_{2b}-FITC (Southern Biotechnology Associates, Birmingham, AL) were added. After 30-minute incubation, cells were washed and the mean fluorescence intensity was determined by flow cytometry using a FACs Caliber (Becton Dickinson, Franklin Lakes, NJ).

ELISpot Assay

PVDF membrane ELISpot plates (Whatman Inc, Clifton, NJ) were coated with primary antibody and incubated overnight at 4°. Plates were blocked with 1% PBSA, washed in HL-1 serum free media (Cambrex BioScience, Walkersville, MD) supplemented with Pen/Strep and L-glut, and 10⁶ responder + 10⁶ irradiated stimulator cells were added. Plates were incubated at 37° with 5% CO₂. After 48 hours, plates were washed in .05% Tween-20 (5x) + PBS then in 1×PBS (5x). Secondary antibodies in 1% PBSA were added to plates and incubated at 4°C overnight. Plates were washed and spot development was performed using ELISpot Blue Development Module (R&D Systems, Minneapolis, MN) and analyzed using an AID ELISpot plate reader (AutoImmun Diagnostika, Germany). Antibodies include: IFN-γ coating Ab used at 4μg/mL and IFN-γ biotinylated detecting Ab used at 3μg/mL (BD Biosciences)

Flow Cytometric Analysis for Microchimerism

PBMC were obtained from mice 4–8 weeks of age and were stained with biotinylated antibodies for 30 minutes on ice. Cells were washed with FACS wash buffer, incubated with streptavidin APC, and washed again. Data was collected using a FACs Caliber (Becton Dickinson). Biotinylated antibodies included H-2K^d, H-2K^k, and H-2K^bD^b (Cedarlane Laboratories). A minimum of 100,000 live lymphocytes were collected per sample, and the machine was washed with bleach and FACS wash buffer between each sample to eliminate cross contamination. The sensitivity of the assay was estimated by diluting maternal splenocytes into corresponding homozygous splenocytes at ratios from 1/1 to 1:10⁵.

Maternal⁺ cells were still reproducibly detectable when they represented 1:10⁴ (0.01%), but not 1/10⁵.

Statistical Analysis

Statistical analysis of graft survival data was performed using Wilcoxon's log rank test. All other statistical analyses are performed using the Student's t Test.

Results

Table 1 summarizes the previously described F₁ × P backcross breeding scheme (8) termed the D1 model, as well as the five other F₁ backcross breedings in which the non-inherited maternal haplotype is presented to mice with different H-2 and background genotypes. To create controls for the NIMA-exposed mice, the parental female and male strains listed in Table 1 were switched, resulting in H-2 homozygous mothers giving birth to H-2 homozygous offspring that will not have had *in utero* and oral exposure to the H-2 alloantigens, since the non-inherited H-2 allo-antigens [termed non-inherited paternal antigens or NIPA] are carried by the heterozygous father. These non-exposed NIPA mice are the ideal controls for the NIMA-exposed mice and are used as controls throughout this study (referred to as non-exposed controls or NIPA-control). We will refer to the H-2 homozygous offspring from each F₁ backcross breeding combination by the model name designated in Table 1. Adult NIMA-exposed and non-exposed control H-2 homozygous offspring from each model received a fully allogeneic heterotopic heart transplant that corresponded to the source strain of the non-inherited haplotype (Table 1). For example, H-2 homozygous mice derived from the D1 and D2 breeding models each received a DBA/2 heart transplant. In the case of D3, where the H-2^d haplotype originated from the BALB/c strain, mice received a BALB/c heart allograft. Mice from the B1 and B2 models each received a B6 heart allograft, and mice derived from the K model received a C3H heart allograft.

Figure 1 compares fully allogeneic heart allograft survival in the six F₁ backcross models. As we previously reported (9), we observed a tolerance rate of 36% (22/61) for the NIMA-exposed mice from the D1 model, with a DBA/2 heart allograft mean survival time (MST) of 30 days versus 9.5 days for the corresponding non-exposed controls ($p < .001$). Only one out of twelve NIPA-control mice had a heart allograft that survived >60 days; this mouse rejected its graft on day 69 (Figure 1A). We have previously reported that NIMA-exposed recipients of third party C3H heart allografts uniformly reject around day 10 post-transplant demonstrating that this tolerance is specific for NIMA (8). A similar pattern of NIMA tolerance was found in mice from the D2 model in which 50% (6/12) of NIMA-exposed mice experienced long-term DBA/2 heart allograft survival; their overall MST was 60 days (Figure 1B). In this model, 17% (1/6) of non-exposed controls also accepted a DBA/2 heart allograft, with a group MST of 21 days (Figure 1B). In the third NIMA H-2^d model (D3, Table 1), the H-2^d haplotype originated from the BALB/c instead of DBA/2. While only 13% (1/8) of NIMA-exposed mice accepted a BALB/c heart, the difference in MST between NIMA and their non-exposed control group was statistically significant ($p = .05$) (Figure 1C).

The remaining three F₁ backcross models showed very different results compared to the three D models. In mice from the B1 (Figure 2D) and K (Figure 2F) models, maternal antigen exposure was provided by a C3B6F₁ mother. There were no differences in graft survival between NIMA-exposed and non-exposed controls in these two models. NIMA-exposed mice derived from the B2 model showed a trend towards worse B6 heart allograft survival than the corresponding non-exposed controls however, most B6 heart allografts were accepted in this model, so the impact of the H-2^b NIMA exposure, if any, was in the direction of sensitization (Figure 1E). Interestingly, the NIMA H-2^b in the B2 model was provided by the same

B6D2F₁ maternal strain as in the D1 model in which we observed a strong tolerance effect prolonging DBA/2 heart allograft survival.

The results shown in Figure 1 included both female and male heart transplant recipients. To see if gender of the offspring influences the NIMA effect we compared DBA/2 heart allograft survival in female and male mice from the D1 breeding model (Figure 2A vs 2B). Both males and females showed a beneficial NIMA effect on graft survival versus sex-matched non-exposed controls; however female mice had a lower incidence of long-term tolerance compared to male mice (18% vs 46%). When comparing the MST, males fared significantly better, with a MST of 41 days versus only 11 days for females ($p=.004$).

It is often difficult to assess sensitization based on graft survival alone. To further explore the mechanism of strain-specific differences in the NIMA-effect in heart-allografted mice, we analyzed serum samples for the production of anti-NIMA antibody production post-transplantation. As shown in Figure 3A, NIMA-exposed mice from the D1 model produced significantly less complement fixing IgG₂ alloantibody to H-2^d compared to their non-exposed controls ($p=.03$ for NIMA-exposed tolerant, and $p=.04$ for NIMA-exposed rejectors). No differences in the production of anti-H-2^d IgG₁ antibodies was found post-transplant in mice from the D1 model (Figure 3A). The opposite was true for the NIMA-exposed mice from the B1 model, which produced significantly more anti-H-2^b IgG₂ antibodies in response to a B6 heart allograft compared to the B1 non-exposed controls ($p=.004$) (Figure 3B). This trend was also seen in the production of IgG₁, although not statistically significant.

Besides humoral sensitization to NIMA, T cell sensitization or regulation may also account for strain differences in NIMA effect. To test this possibility, pre-transplant ELISpot analysis was performed to analyze the frequency of IFN- γ producing alloreactive T cells against maternal type F₁ NIMA-expressing cells. We found that there were no differences in the number of IFN- γ producing cells between NIMA-exposed and NIPACONTROL mice from the D1 and B1 models when stimulated with irradiated B6D2F₁ or B6C3F₁ splenocytes, respectively (Figure 4). This finding is consistent with our previously published study (9) which showed that differences in pre-transplant T cell status were not detectable in vitro, but were only manifested using in vivo assays. However, in the K model, pre-transplant NIMA-exposed mice produced significantly more IFN- γ compared to NIPA controls when stimulated with parental type B6C3F₁ cells (Figure 4). These results indicate that the NIMA-exposed mice from the K model were pre-sensitized by exposure to maternal antigens at the T cell precursor level.

The balance between immune regulation and sensitization may be correlated with the degree of chronic exposure to maternal antigens. One measure of chronic antigen exposure is the level of NIMA H-2 antigen expression found in the circulating leukocytes of backcross mice (15). To test for differences in the level of maternal antigen expression in the offspring from different models, we analyzed PBMC from NIMA-exposed and non-exposed control mice from the D1, K, and B1 models for the presence of cells positive for the non-inherited MHC class I antigen using anti-K^d, K^k, and K^{bD^b} antibodies. Figure 5A shows representative staining profiles for PBMC from a positive control (F₁ parent), a NIMA-exposed, and a non-exposed control from all three models. In the examples shown, the mice from the D1 model had a distinct population of H-2K^{d+} cells in their PBMC that was lacking in the corresponding non-exposed control mouse tested in parallel. In contrast, mice bred in the K and B1 backcross models showed no differences in PBMC staining for H-2K^b or H-2K^k between NIMA-exposed and non-exposed controls. When the mean values of percent positive staining cells were compared, the NIMA-exposed mice from the D1 model had significantly more circulating NIMA H-K^{d+} cells than their non-exposed controls ($p=.004$), whereas no differences were found in the percent of cells staining for non-inherited MHC class I antigen between the NIMA-exposed and non-exposed controls in the K (H-2K^{k+} cells) and B1 (H-2K^{bD^b} cells) models.

Discussion

Using different parental strains of mice to create several models of exposure to NIMA in offspring has revealed that strain differences play a significant role in determining whether exposure to NIMA results in tolerance or sensitization to fully allogeneic heart allografts. Interestingly, all three D models showed some degree of maternal tolerance effect to a fully allogeneic transplant, with mice from the D1 and D2 models (DBA/2 donor) faring the best, suggesting that the specific non-inherited maternal H-2 haplotype may be important. Strain “background” genes may also play a role in the tolerance to NIMA, since the D3 model (BALB/c donor) showed a weaker tolerizing effect compared to the other two NIMA D models. In this context, it is also important to remember that the numbers of mismatched minor histocompatibility antigens will differ among different donor-recipient pairs, ultimately affecting the amount of total NIMA mismatch, exposure, and response to those mismatches. One such minor H antigen could be the highly immunogenic H60 non-classical MHC class I antigen, which is expressed in BALB/c, but not in B6 (21) or DBA/2 (personal communication J. Kwun) strains. Importantly, H60 may serve not only as a target for adaptive immunity, but may also subject maternal (B6 × C3H)F₁ cells to NK-mediated lysis in H60-negative offspring (22).

Peugh et al (23) reported that the responsiveness of the DBA/2 recipient to heart allografts from other mouse inbred strain donors tended to be lower in all combinations tested than that of any other recipient strain responding to the same graft donor, a phenomenon that may be related to higher natural T_R CD4⁺CD25⁺ cell levels in this strain (24). The authors could also show that this weak response was not solely associated with H-2^d haplotype, as BALB/c mice (H-2^d) had rapid allograft rejection times (23).

It is possible that passenger T lymphocytes emerging from the donor allograft play a role in facilitating or disrupting tolerance; this might explain why a graft from a “low-responder” strain might favor tolerance, while a graft from a “high responder” strain (like B6) might favor rejection. In the present study, offspring from the D1 and B1 models had markedly different NIMA effects—tolerance to a DBA/2 heart vs humoral sensitization to a B6 heart despite having the same maternal antigen source [a (B6 × DBA/2)F₁ mother]. These results indicate that allograft survival in different strain combinations is likely to be caused by genetic differences in the immune response genes of both recipient and donor that together determine the type of immune response invoked by the allograft. Recent data suggests a key role for recipient IFN-γ in the establishment of T_R cell dominance and tolerance to a heart allograft potentially explaining why offspring from the D1 model tolerate well (25).

We used flow cytometry as an index of maternal microchimerism. This same technique was used by Zhang and Miller (15) who found in the same D1 model used in our study, that NIMA-exposed mice showed enhanced survival of maternal skin grafts which directly correlated with the number of maternal⁺ T cells in their lymph nodes. In their study, they detected similar levels of H-2^d cells in the graft draining lymph nodes as we found in the PBMC of pre-transplant NIMA-exposed offspring. We were surprised at the high levels of maternal antigen positive cells detected in the PBMC of pre-transplant NIMA-exposed mice. Because flow cytometry measures only antigen positive cells and not maternal DNA positive cells, the “true” levels of maternal microchimerism are likely to be much lower. Indeed, our preliminary studies using qPCR indicate that maternal microchimerism is generally below the level of detection by flow cytometry, on the order of 1–5 cells per 10⁵ in the D1 model (Dutta and Molitor-Dart, manuscript in preparation). Therefore, what we are seeing in the PBMC is likely to be cells that have acquired H-2^d antigen from maternal cells that present in the tissues of the NIMA-exposed mice. The process of transfer and acquisition of MHC molecules between allogeneic

cells is termed trogocytosis; several studies have shown that T cells, B cells, macrophages, and dendritic cells (DCs) are all capable of this process (26–31).

Vernochet et al (32) found inter-strain differences in a NIMA effect when they found that maternal cells influence development of fetal and neonatal allospecific B cells, and that affinity played a large role in the outcome of this maternal influence. The results from their study indicate that receptor affinity for the NIMA plays a major role in the timing and quality of the response elicited by the interaction. Therefore, depending on the pool of responding T and B cells selected according to the unique set of background genes in the offspring, immunogenicity of the NIMA major and minor antigens will vary resulting in different allograft survival outcomes. This could explain why NIMA-exposed offspring from the B1 model produce significantly more NIMA-specific antibodies post transplant.

Exposure to NIMA appears to prime T effector cells in certain strains, as seen in the increased IFN- γ production of NIMA-exposed splenocytes obtained from mice in the K model after maternal-type semi-allogeneic cell stimulation. This indicates that NIMA H-2^k exposure resulted in sensitization of T cells in this model. It is possible that this T effector sensitization may occur in all strains, but is counter balanced in the D models by the simultaneous induction of adaptive T_R cells (9). Depending on the pool of responding T and B cells selected according to the unique set of MHC and background genes in the offspring, immunogenicity of the NIMA major and minor antigens will vary resulting in different allograft survival outcomes.

Our finding that male NIMA-exposed mice fared much better than female NIMA-exposed mice is of interest to clinical transplant tolerance protocols. It is well known in human transplantation that female recipients and grafts have poorer graft survival than do male recipients and male-donated grafts (33–35). This is also true for murine heart transplantation (36) and is believed due to differences in renal cortex nephron mass and influence of the hormone estradiol on immune responsiveness (37). In addition, parous females may become sensitized by exposure to fetal-derived inherited paternal antigens. This may limit beneficial NIMA effects in female recipients.

In conclusion, the data establishes that genetic background is critically important in the establishment of a NIMA effect on transplantation tolerance. This may help to account for differences in results obtained from centers in different parts of the worlds, and emphasizes the importance of large single center studies(6) rather than mixing data from multiple centers with different genetic makeup(38). Donor selection based on NIMA could still be of benefit for transplantation outcome and tolerance trials in patients for whom no HLA-identical donor is available. Given that levels of maternal microchimerism and extent of NIMA-induced T regulatory cell development differs markedly between strains and within a single strain, pre-transplant screening becomes essential for identifying patients who will benefit from NIMA, a naturally induced form of tolerance.

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Figure 1. Heterotopic heart allograft survival of all strain combinations tested

A) D1 model in which H-2^d non-exposed controls and NIMA H-2^d exposed offspring are transplanted with a DBA/2 donor heart. n=61 for NIMA-exposed and n=12 for non-exposed controls. B) D2 model in which H-2^k non-exposed controls and NIMA H-2^d exposed offspring are transplanted with a DBA/2 donor heart. n=12 for NIMA-exposed and n=6 for non-exposed controls. C) D3 model in which H-2^b non-exposed controls and NIMA H-2^d exposed offspring are transplanted with a BALB/c donor heart. n=8 for NIMA-exposed and n=5 for non-exposed controls. D) B1 model in which H-2^k non-exposed controls and NIMA H-2^b exposed offspring are transplanted with a B6 donor heart. n=11 for NIMA-exposed and n=6 for non-exposed controls. E) B2 model in which H-2^d non-exposed controls and NIMA H-2^b exposed offspring

are transplanted with a B6 donor heart. n=8 for NIMA-exposed and n=7 for non-exposed controls. F) K model in which H-2^b non-exposed controls and NIMA H-2^k exposed offspring are transplanted with a C3H donor heart. n=9 for NIMA-exposed and n=6 for non-exposed controls. P values in graft represent comparison between NIMA-exposed and non-exposed controls.

A

B

Figure 2. Heterotopic heart allograft survival of D1 model separated by gender

A) Female (n=22 for NIMA-exposed and n=6 for non-exposed controls) and B) male (n=39 for NIMA-exposed and n=6 for non-exposed controls). P values in graft represent comparison between NIMA-exposed and non-exposed controls.

A

B

Figure 3. Post transplant serum analysis for allo-antibody against the non-inherited H-2 shows that NIMA-exposed mice from the D1 model produce significantly less complement fixing antibody compared to NIMA-exposed from the B1 model, who appear sensitized

Serum samples were obtained from mice post transplant at various time points and analyzed for the production of complement fixing anti-NIMA IgG₂ antibodies and anti-NIMA IgG₁ in mice from the A) D1 model and B) B1 model. n=5 for each group of mice from D1 model, and n=3 for NIMA-exposed and n=5 for non-exposed controls from the B1 model. *p= <.05 for NIMA-exposed versus non-exposed controls.

Figure 4. Pre-transplant analysis for alloreactive IFN- γ producing T cells indicates that NIMA-exposed mice from the K model are sensitized to maternal antigens compared to NIMA-exposed mice from the D1 and B1 models

Splenocytes were harvested from NIMA-exposed and non-exposed control mice and stimulated with irradiated B6D2F₁ (D1 model) or B6C3F₁ (K and B1 models) splenocytes for ELISpot analysis of IFN- γ production. Background spots resulting from stimulation with irradiated homozygous splenocytes (B6 for D1 and K models, C3H for B1 model) was subtracted from the number of spots obtained with semi-allo stimulation. Results shown as mean $\pm$ SD spot/million cells over background. n=5–9 mice for all groups tested.

Figure 5. Flow cytometric analysis comparing three stains shows that NIMA-exposed mice from the D1 model have significantly more NIMA⁺ cells compared their non-exposed controls, and to NIMA-exposed mice from the K and B1 models

PBMC obtained from mice ages 4–8 weeks of age was collected and analyzed by flow cytometry for non-inherited antigen⁺ cells. A) Representative flow cytometric analysis for non-inherited antigen⁺ cells (H-2K^d for the D1 model, H-2K^k for the K model, and H-2K^bD^b for the B1 model) in PBMC from NIMA-exposed and non-exposed mice from the D1, K, and B1 models. B) NIMA-exposed mice from the D1 model had significantly more H-2K^d cells compared to non-exposed controls. No differences were found between NIMA-exposed and non-exposed controls in the other two models tested. Results shown as mean $\pm$ SD. n= 6–13 mice for all groups tested.

Table 1

NIMA Mouse Backcross Models

Model Name	Female Parent	Male Parent	MHC Background Offspring of Interest	Donor Heart Challenge
D1	(B6 × DBA/2)F ₁ (H-2 ^{b/d})	C57BL/6 (H-2 ^b)	H-2 ^b (d exposed)	DBA/2 (H-2 ^d)
D2	(C3H × DBA/2)F ₁ (H-2 ^{k/d})	C3H (H-2 ^k)	H-2 ^k (d exposed)	DBA/2 (H-2 ^d)
D3	(B6 × BALB/c)F ₁ (H-2 ^{b/d})	C57BL/6 (H-2 ^b)	H-2 ^b (d exposed)	BALB/c (H-2 ^d)
B1	(B6 × C3H)F ₁ (H-2 ^{b/k})	C3H (H-2 ^k)	H-2 ^k (b exposed)	C57BL/6 (H-2 ^b)
B2	(B6 × DBA/2)F ₁ (H-2 ^{b/d})	DBA/2 (H-2 ^d)	H-2 ^d (b exposed)	C57BL/6 (H-2 ^b)
K	(B6 × C3H)F ₁ (H-2 ^{b/k})	C57BL/6 (H-2 ^b)	H-2 ^b (k exposed)	C3H (H-2 ^k)