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Pre-transplant immune regulation predicts allograft tolerance

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Abstract

CD4⁺ Tregs specific for non-inherited maternal antigens (NIMA^d) are detectable in some but not all B6 × BDF₁ backcross, H-2^b homozygous offspring, and their presence is strongly correlated with extent of maternal (BDF₁) microchimerism. We hypothesized that the level of pre-transplant donor antigen-specific Tregs could predict allograft tolerance. To test this idea, mice were screened for bystander suppression in a DTH assay, followed 1 wk later by DBA/2 heterotopic heart transplantation. NIMA^d-exposed, H-2^b offspring that failed to suppress DTH uniformly rejected heart allografts (12/12) by d15. In contrast, 5/6 NIMA^d-exposed DTH “regulators” accepted their allografts >100d. The defect in ‘non-regulator’ offspring could be corrected by transfer of CD4⁺CD25⁺, but not CD4⁺CD25^{neg} or CD8⁺T cells from transplant acceptor mice.

Conclusion—Donor-specific T reg screening of F1 backcross offspring correctly predicted which recipients would accept a heart allograft. If translated to the clinic, similar pre-transplant Treg screening could greatly enhance the effectiveness of tolerance as a clinical strategy in transplantation between family members.

Keywords

transplantation tolerance; regulatory T cells; NIMA

Introduction

Kidney graft survival is optimal when the donor and recipient are HLA identical, with graft half-lives of ≥20 years being common between HLA-identical siblings. Since HLA-identical siblings are relatively uncommon in small families, HLA haploidentical parents or siblings are more commonly considered as potential living related donors. In these cases, formation of anti HLA antibodies and HLA allospecific T cells heighten the risk of immune-mediated graft rejection. Unfortunately, immunosuppressive drugs administered to prolong graft survival increase the risk of systemic infections, are nephrotoxic, and may encourage tumor growth (1). Therefore, transplant immunology seeks alternative ways to maintain a functional allograft without the co-morbidities of lifelong immunosuppression. Taking advantage of natural tolerance induced by exposure to non-inherited maternal antigens

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(NIMA) is one of the more promising, but still relatively unexplored, approaches for this purpose.

The clinical benefit of NIMA tolerance was first noted by Owen et. al. (2) who showed that Rh⁻ women with an Rh⁺ mother failed to produce antibody (Ab) against the Rh antigen when they gave birth to Rh⁺ children. Claas et al. (3) later showed that exposure to an HLA alloantigen in fetal life can induce humoral tolerance to the same alloantigen later in life. The impact of tolerance to NIMA in HLA haploidentical living related kidney transplantation was first noted in siblings, where a 25% increase in graft survival at 10 yrs was noted in the pre-cyclosporin era (4). Interestingly, recent efforts to find alternatives to calcineurin-based immunosuppression (IS), including complete IS withdrawal (5) have achieved variable degrees of success in HLA haploidentical recipients. Understanding the “natural”, but variable, tolerance induced by maternal and fetal antigen exposures has the potential to enhance selection of optimal donor-recipient combinations for tolerance, in advance of transplant surgery.

How exposure to NIMA induces lifelong tolerance to the NIMA is not clear. Offspring get exposed to NIMA through placenta both in fetal life and orally during nursing in neonatal life (6). This exposure can lead to exchange of progenitor cells across the maternal-fetal interface (7-9), which often results in maternal microchimerism (MMc) in different organs of the offspring (10). Maternal alloantigens promote T cell proliferation in the human fetal lymph node, where the proliferating CD4⁺ T cells are induced toward a Foxp3⁺/Treg fate by a TGFβ-dependent mechanism, protecting the maternal cells from elimination by suppressing anti-NIMA alloreactivity during pregnancy (11, 12). In an F₁ backcross breeding (BDF1 female × B6 male), persistence of H-2^{d+} MMc in multiple organs and cell lineages of adult H-2^{b/b} mice was strongly correlated with NIMA^d-specific Tregs (10) indicating a causative link between the two. Variability in tolerance to NIMA^d-expressing DBA/2 (H2^{d/d}) heart grafts in offspring of BDF1 female × B6 male breedings has been attributed to differences in the ability of individual offspring to mobilize NIMA^d-specific, IL10- and TGFβ-producing, CD4⁺CD25⁺ Tregs into graft-draining lymph nodes, as well as to the graft itself (13).

Whether or not pre-transplant evidence of donor-specific Tregs could be used to predict successful post-transplant allograft outcome is currently unknown. For this reason, we tested the hypothesis that pre-transplant NIMA-specific Treg status predicts the post-transplant outcomes of NIMA-expressing allografts.

Materials and methods

Source of mice, breeding, and typing

C57 BL/6 (B6, H-2^{b/b}), DBA/2 (H-2^{d/d}), B6D2F₁ (BDF₁, a cross of B6 and DBA/2; H-2^{b/d}), and B6C3F₁ (a cross of B6 and C3H/He; H2^{b/k}) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The care and breeding of animals were performed under institutional guidelines.

When BDF₁ female mice were crossed with B6 males, half of the offspring inherited H2^{b/b} MHC, thus having been exposed to maternal H2^d in fetal and neonatal life (NIMA^d-exposed). When BDF₁ males were bred with B6 females, the H2^{b/b} offspring (NIPAd controls) have similar background genes but were not exposed to maternal H2^d since their mother did not have H2^d. It is possible that, since multiparous B6 females will have acquired some H-2^{d+} cells from their H-2^{bxd} offspring, this may contribute a low level of Mc transmitted to subsequent litters. However, in the absence of breast-feeding by a H-2^{d+}

mother this small amount of Mc has no immunologic consequence. Thus the NIPA^d offspring served as excellent controls.

Hemisplenectomy

A left mid-flank incision is made in the skin followed by a similar small incision in the body wall. The spleen is gently retracted out of the peritoneal cavity. The spleen is ligated in half using a 6-0 silk suture. The splenic artery and splenic vein of one side of the spleen are ligated, cut, and cauterized. Half of the spleen is transected and the remaining half of the spleen is cauterized. The muscle/fascial layer is closed with 4-0 vicryl and the skin is closed with a running 6-0 vicryl intradermal sutures.

Heterotopic heart transplantation

Heterotopic vascularized heart transplantation was conducted using an intraabdominal microsurgical technique as described before (14).

Adoptive transfer DTH assay

Splenocyte were collected from TT/DT vaccinated NIMA^d-exposed and NIPA^d-control mice. Ten million splenocytes were injected into footpads of naïve B6 recipients with coinjection inoculums: PBS, BDF₁ or B6C3F₁ Ag, or 0.25 lf of TT/DT. To measure bystander suppression, splenocytes were co-injected with BDF₁ Ag and 0.25 lf of TT/DT. Changes in footpad swelling were measured after 24 hours post-injection using a dial-thickness gauge to measure DTH reaction.

Cell sorting

Splenocytes were incubated with magnetic bead conjugated antibodies against CD4 and CD8 (Miltenyi Biotech, Auburn, CA) for 15 minutes. CD4⁺ and CD8⁺ cells were sorted using the autoMACS sorter (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocols. The purity of sorted cells was >97%.

Multiplex Quantitative RT-PCR

RNA was extracted from donor hearts using RNeasy Protect Mini Kit (Qiagen, Valencia, CA). Genomic DNA was removed using DNAase during RNA extraction. RNA was quantified and one microgram of RNA was used for reverse transcriptase reaction to produce cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystem, Foster City, CA). cDNA was used to amplify target and control (beta actin) transcripts in a single tube with multiplex quantitative PCR using a Bio-Rad iCycler (BioRad, Hercules, CA). Primers and probes specific for IFN- γ , TGF- β , IL10, and Foxp3 transcripts were purchased from Applied Biosystems. Primers and probe specific for beta actin was designed by University of Wisconsin Biotechnology Center. For data analysis, comparative threshold (Ct) values of beta actin were used to normalize variation in loading cDNA. Fold differences in the expression of the transcripts over syngeneic control were reported.

Statistics

Data was analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). To analyze graft survivals in different groups, log rank test was used. One-way ANOVA followed by Bonferroni's multiple comparison test was used to analyze multiplex quantitative RT-PCR data. For rest of the data, the non-parametric Mann-Whitney test was used.

Results

Pre-transplant screening for NIMA^d-specific Tregs

As depicted in Figure 1A, we performed hemisplenectomy to obtain splenocytes from NIMA^d-exposed (see materials and methods section) and non-exposed offspring that had been immunized with tetanus toxoid (TT) two weeks previously. Adoptive trans-vivo DTH assays were performed for each mouse to measure suppression of the recall TT response in presence of maternal (BDF₁) antigen. DTH suppression in the presence of BDF₁ (maternal) antigen is a measure of NIMA^d-specific Tregs. Figure 1B depicts typical DTH transfer assay results. Splenocytes from mouse NIMA#1 responded well to TT, but suppressed its swelling response by 40% in presence of the maternal antigen (regulator). NIMA#2 did not exhibit bystander suppression (non-regulator) and in this respect was similar to the NIPA control. Overall, we found that 11/23 (48%) of hemi-splenectomized NIMA^d-exposed offspring inhibited recall TT-induced DTH swelling to a variable extent (Fig. 1C). In contrast, none of the non-exposed “NIPA” controls (n=9) exhibited DTH suppression (p=0.03). None of the NIMA^d-exposed offspring inhibited TT-induced DTH swelling in presence of a third party antigen (B6C3F₁, H2^{k/k}) indicating that DTH suppression was alloantigen-specific.

Heterotopic heart transplantation was performed one week after hemi-splenectomy (Fig 1A). We observed that NIPA^d control offspring (n=9) uniformly rejected DBA/2 heart allografts (Fig. 1D), whereas 5/18 NIMA^d-exposed offspring accepted DBA/2 heart allografts long term. When broken down by pre-transplant regulation status, all non-regulator mice (n=12) rejected DBA/2 heart allografts by d. 15. One mouse in this group was found to be pre-sensitized to maternal antigens, i.e. $\geq 20 \times 10^{-4}$ inches swelling response to BDF₁ antigen alone. This one rejected its allograft on d.6 (*, Fig. 1D); all others rejected on d.9 or later. Remarkably, 5/6 NIMA regulator mice accepted a DBA/2 heart allograft >100d (p=0.001 vs. NIMA^d non-regulators or NIPA^d controls; Fig 1D).

The tolerance to DBA/2 heart allografts in the NIMA-exposed mice was robust, with substantial preservation of cardiomyocyte and vascular integrity at d.100 as previously reported (14), without evidence of chronic allograft vasculopathy, and featured not only less CD8⁺ and more CD4⁺ cell infiltration, but also elevated levels of Foxp3⁺ cells --mostly concentrated in lymphoid aggregates in the allograft – as compared to the rejected allografts of non-regulator and control offspring (data not shown).

Elevated levels of regulatory cytokines in the donor hearts of regulator offspring post-transplantation

We previously found that NIMA-specific DTH suppression was IL-10 and TGF- β -dependent (15). To investigate whether these cytokines are involved in the observed tolerance, we harvested DBA/2 donor heart allografts from recipients on day 8 post transplant when all the hearts (with the exception of the lone NIMA^d-sensitized mouse) were still beating. Figure 2 shows the levels of cytokine transcripts in the different groups as fold differences compared to heart isografts (B6 into B6). We found that the regulator offspring had significantly higher levels of IL-10 and TGF- β mRNA transcripts in their heart transplants compared to both non-regulator and control offspring (both p<0.05; Fig. 2A & 2B). In contrast, NIMA^d non-regulator and NIPA^d control offspring both had significantly higher levels of IFN- γ mRNA transcripts in their donor heart grafts on day 8 than the pre-transplant regulator mice (p<0.05) (Fig. 2C).

CD4⁺CD25⁺ Treg cells from allograft acceptor mice transfer NIMA-specific tolerance to “non-regulator” offspring

The intra-graft cytokine gene expression pattern on d. 8 suggested that the tolerance predicted by pre-transplant screening was being mediated by Tregs. To determine if we could correct the tolerance defect in “non-regulator” mice by Treg transfer, regulator offspring tolerant to DBA/2 hearts >100 d. were sacrificed and splenocytes were sorted from the remaining half of the spleens. Subsets of T cells (5×10^5) were injected i.v. into NIMA^d-exposed non-regulator offspring. Each mouse received a DBA/2 heart allograft 1d. later (Fig. 3A). As shown, 4/4 “non-regulator” offspring that received CD4⁺CD25⁺ T cells accepted their allografts long term, whereas those treated with CD4⁺CD25^{neg} or CD8⁺ T cells rejected their grafts by day 11 post transplant (Fig. 3B). The tolerance obtained after Treg transfer required antigen specificity, since an equal dose of Tregs isolated from a naïve B6 mouse did not induce tolerance to DBA/2 allografts in non-regulator offspring (n=2; Fig. 3B). Furthermore, Tregs transferred from DBA/2 heart allograft acceptors did not induce tolerance to a third party heart allograft (C3H, H2^{k/k}; n=3; Fig. 3B).

Discussion

Inbred homozygous mouse strains derived from multiple generations of brother-sister matings are still the gold standard in basic immunology research. They are prized by transplant biologists precisely for the relative uniformity of their response to allografts, allowing comparisons of treatment protocols. In contrast, investigation of natural tolerance to alloantigens requires a partially outbred breeding model in which maternal heterozygosity is present. Recent data using a mouse F2 breeding system involving 3 different H-2 haplotypes on a uniform non-MHC “background” suggests that T effector cell hyporesponsiveness to non-inherited maternal MHC antigens might be useful for predicting freedom from GVHD after H-2 mismatched bone marrow transplantation (16). T effector cell frequency to panels of allogeneic stimulator cells has also shown promise in prediction of acute rejection of kidney transplants (17). While these approaches may identify alloantigen “low vs. high responder” individuals, they do not directly measure specific tolerance susceptibility. Here we show using F₁ backcross mice, a direct correlation between pre-transplant, maternal BDF1 antigen-specific Tregs detected by bystander suppression assay, and subsequent tolerance to a fully allogeneic DBA/2 heart allograft. The Tregs detected prior to transplant were of the “indirect” allorecognition variety, since only BDF1 cell lysates were used to trigger “bystander suppression” of host recall DTH response to TT. However it is quite possible that a subset of these Tregs have dual function, both on indirect and on the direct allorecognition pathway recognizing naturally presented peptide/MHC.

The reasons for the non-uniformity of NIMA-specific Treg development and maternal microchimerism are still unknown. It is worth noting that unlike the situation in humans, the mouse immune system is not fully developed at birth, and natural CD4⁺CD25⁺ Tregs do not emigrate from the mouse thymus until 2d. after birth, a fact that facilitated their initial identification (18). Not surprisingly therefore, nursing exposure is a critical variable in NIMA-specific Treg development in mice (10, 13); however, we did not attempt to measure the actual amount of breast milk received by each F₁ backcross offspring. Whatever the reason, the non-uniformity of NIMA-specific tolerance makes it possible to test whether one can reliably predict allograft tolerance in a recipient population with heterogeneous prior exposure to alloantigens due to maternal-fetal cell exchange. The finding here of a predictive correlation between pre-transplant NIMA-specific regulation and subsequent tolerance to an organ allograft suggests that it may indeed be possible to predict, using a similar approach, which HLA haploidentical family members are prone to develop specific transplantation tolerance to a living related donor and which recipient-donor pairs are poor tolerance candidates. The pronounced Treg-orientation of human T cells developing from

hematopoietic stem cells present during fetal life (13), makes a compelling case for novel assays to assess the profound impact of maternal non-inherited antigens on recipients' and donors' T and B cell repertoire prior to organ transplantation.

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Non-standard abbreviations used

ILNs	inguinal lymph nodes
Mc	microchimerism
MMc	maternal microchimerism
NIMA	non-inherited maternal antigen
NIMA^d	homozygous H-2 ^b offspring of a BDF1 mother
NIPA	non-inherited paternal antigen
NIPA^d	homozygous H-2 ^b offspring of a B6 mother and a BDF1 father
T_{regs}	T regulatory cells
T_E	T effector cells
APC	antigen presenting cells
MHC	major histocompatibility complex
LN	lymph nodes

Figure 1. Prediction of NIMA-specific tolerance based on pre-transplant maternal-fetal immune status

A) Experimental design to predict NIMA-specific tolerance based on pre-transplant maternal fetal immune status. NIMA^d-exposed and NIPA^d control offspring were immunized with tetanus toxoid (TT). After two weeks, hemisplenectomy was performed to obtain splenocytes, which were used in a bystander suppression assay to determine NIMA-specific Treg/T effector status of the offspring. Fully allogeneic DBA/2 (H2^{d/d}) hearts were transplanted into the offspring after two weeks. Heart beating was monitored daily by abdominal palpation. B) Example of a DTH assay showing NIMA^d-exposed regulator, non-regulator, and NIPA. Hemisplenectomy was performed to obtain splenocytes from the offspring immunized with tetanus toxoid (TT) two week prior to the surgery. The splenocytes were used in a bystander suppression assay to measure suppression of recall TT-induced DTH swelling, which correlates with NIMA-specific Treg activity. NIMA#1 suppressed recall TT-induced swelling in presence of the maternal antigens (regulator). However, NIMA#2 did not suppress the DTH swelling (non-regulator). C) Summary of percentage of DTH suppression in NIMA^d-exposed and NIPA^d control offspring in presence of maternal (BDF₁) and a third party (B6C3F₁) antigens. D) Graft survival curves in different groups of mice transplanted with either syngeneic (B6) or allogeneic (DBA/2) hearts. NIMA^d-exposed regulator offspring had significantly better graft survival than the NIMA^d-exposed non-regulator and NIPA^d control offspring. The sensitized NIMA^d-exposed offspring is marked as *.

Figure 2. Higher levels of regulatory cytokines and lower level of inflammatory cytokine in NIMA^d-exposed regulators

mRNA was extracted from the donor DBA/2 hearts from the NIMA^d-exposed and NIPA^d control offspring, and B6 syngeneic control mice at day 8 post transplants when all the donor hearts were functional. cDNA was prepared and a qPCR was performed to detect IL-10, TGF- β , and IFN- γ transcripts. The levels of cytokine transcripts are expressed as fold differences compared to the syngeneic (B6 into B6) control.

Figure 3. NIMA-specific tolerance is transferable and CD4⁺ CD25⁺ cell-dependent

Spleens were collected from NIMA^d-exposed regulator offspring tolerant to DBA/2 hearts. CD4⁺ CD25⁺, CD4⁺ CD25⁻, and CD8⁺ splenocytes were sorted with a FACS sorter. Half a million of the sorted cells was injected i.v into NIMA^d-exposed non-regulators one day before DBA/2 heart transplants.