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Effects of T Cell Frequency and Graft Size on Transplant Outcome in Mice

Chunshui He,* Soren Schenk,* Qiwei Zhang,* Anna Valujskikh,* Jörg Bayer,* Robert L. Fairchild,∗† and Peter S. Heeger2∗†

The features that determine whether graft-reactive T lymphocytes develop into effector cells capable of mediating organ destruction are not well understood. To investigate potential factors involved in this process, we first confirmed that female recipient mice acutely rejected minor Ag-disparate male skin, but not heart transplants. Despite this difference in outcome, heart and skin transplantation induced antidonor T cell responses of similar magnitude, specificity, and cytokine profile. The heart-graft-primed T cells transiently infiltrated the graft and ultimately induced the development of chronic transplant vasculopathy. Increasing the frequency of donor-reactive T cells by presensitization or by using TCR (CD8⁺ antimale)-transgenic recipients did not mediate acute rejection but accelerated the pace and severity of the vasculopathy. Surprisingly, decreasing the tissue mass of the donor heart by 50% resulted in acute rejection of these smaller grafts without increasing the frequency of antidonor effector T cells in the recipients. In complementary studies, placement of one or two male skin grafts on a single recipient did not affect the frequency or cytokine profile of the induced antimale T cell repertoire. Nonetheless, the recipients of single grafts acutely rejected the transplanted skin while the recipients of two skin grafts did not. These results provide new insight into the pathogenesis of transplant vasculopathy and provide an explanation for the difference in outcome between murine skin and heart transplants by highlighting the novel concept that the efficiency of transplant-reactive T cell immunity is heavily influenced by the tissue burden it encounters at the effector stage. The Journal of Immunology, 2004, 172: 240–247.

Immune-mediated rejection of a transplanted organ involves several discernable steps: activation of donor-reactive lymphocytes within secondary lymphoid organs (1), trafficking of the activated T lymphocytes to the graft (2, 3), re-encounter with the specific ligand at the graft site (4, 5), and elicitation of effector functions that lead to target organ injury (4, 6–9). If left untreated, this induced antigraft T cell immune response generally results in destruction of the transplanted organ. There are, however, selected situations in which transplantation does not result in graft loss (10, 11) and in fact may lead to graft tolerance (12).

The specific factors that define whether antidonor T cell immunity is induced following transplantation and whether this induced response will be pathogenic are not well understood. The number and activation state of graft-derived dendritic cells capable of priming recipient T cells (13, 14), the presence of ischemia-reperfusion injury to the graft (15, 16), and the number of foreign Ags expressed by the graft (11, 17) are among the donor characteristics that may contribute to the immunogenicity of a transplanted organ. Once the recipient T cells are activated, potentially relevant factors that determine whether these lymphocytes will induce graft destruction include the quantity and distribution of Ag expressed on the individual graft cells (4, 5, 17, 18), the subtype (CD4 vs CD8) of T cells activated in response to the transplant (19), the specific chemokine receptor ligand of the T cells to graft site (2, 3), the effector functions utilized by the activated T cells when they re-encounter their ligand at the graft site (cytokines produced, cytotoxicity) (4, 8, 9, 17, 18, 20, 21), and the frequency of effector T cells (11, 17, 22).

Transplantation of a fully allogeneic organ graft primes a high frequency of donor-reactive CD4⁺ and CD8⁺ T cells capable of mediating cytotoxic T lymphocyte (CTL) activity and producing type 1 proinflammatory cytokines (e.g., IFN-γ) (9, 17, 21, 23, 24). Untreated recipients rapidly reject fully allogeneic heart and skin grafts (10, 11, 22). In contrast, minor Ag-disparate transplants prime lower frequencies of effector T cells, often with the same proinflammatory phenotype as T cells induced to a fully allogeneic graft (7, 10, 17, 22, 25, 26). Nonetheless, such minor Ag-disparate grafts are rejected at a slower pace than MHC-disparate grafts, and in some situations minor Ag-disparate transplants are not rejected at all (7, 10, 17).

Although the frequency of induced effector T cells seems to influence the outcome of a transplanted organ, it has been suggested that the size of the target organ might also contribute to the resultant pathology (11, 27, 28). Proinflammatory, cytolytic, and Th1 cytokine-producing effector T lymphocytes have limited life spans and limited capacities to kill target cells. Thus, larger grafts may be more resistant than smaller grafts to the consequences of a defined number of activated donor-reactive effector T cells. Selected studies suggest that this may indeed be true (11, 27, 28), but a detailed assessment of the interrelationship between T cell frequency and graft size has not been performed.

In an effort to better understand these relationships, we performed a series of experiments using minor Ag-disparate skin and
heart transplantation in mice. We found that skin and heart grafts prime similar numbers of donor-reactive effector T cells and that a threshold number of these effector T cells is required to result in graft destruction. Consistent with previous work, larger numbers of effector T cells are required to destroy a heart vs a skin graft derived from the same donor strain. Our data additionally show, however, that given a limited antidonor T cell repertoire, it is the tissue mass of the graft rather than the type of graft (skin vs heart) that determines whether the graft is accepted or rejected. Finally, the data show that even in the absence of graft destruction, low frequency antidonor T cell immunity has adverse effects on the target organ and contributes to chronic graft injury.

Materials and Methods

Animals

Male and female C57BL/6 (H-2b) and A/J (H-2a) mice, age 6–8 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME). Female C57BL/10NA- (Tg)TCR MataHari-(KO) Rag1 (H-2b, MataHari Rag KO) (4), age 6–8 wk, were obtained as a generous gift from P. Matzinger (National Institutes of Health, Bethesda, MD) and O. Lantz (Institut National de la Santé et de la Recherche Médicale, Paris, France). Female MataHari RAG+/− mice were obtained as the F1 generation of a cross between MataHari Rag KO mice and wild-type B6 animals. All animals were maintained and bred in the pathogen-free animal facility at the Cleveland Clinic Foundation.

Peptides

HYDβp (NAGFSNRANSSRS), HYUrpy (NAGFSNSRANSSRS), and βgal96–103 (DAPYTVN) were synthesized by Research Genetics (Huntsville, AL) at >90% purity.

Placement and evaluation of skin and cardiac grafts

Full-thickness trunk skin grafts were placed using standard techniques (17, 23). Bandages were removed on day 7, and the grafts were then visually scored daily for evidence of rejection. Grafts were considered fully rejected when they were >90% necrotic. Vascularized heterotopic cardiac grafts obtained from 8- to 10-wk-old donors were placed in the abdomen as described and palpated daily for evidence of a heartbeat (4, 8, 29). For selected experiments, donor hearts were obtained from 3-wk-old donors. Rejection was defined as a loss of palpable heartbeat. Technical failures were defined as loss of heartbeat within 48 h of graft placement. Technical failure rate was <10% for standard heart grafts, but was ~40% in recipients of small, 3-wk-old donor hearts. Grafts were harvested at the time of rejection or at predetermined time points posttransplantation.

Histology and immunohistochemistry

Formalin-fixed paraffin sections of graft tissues were stained with H&E and for elastin as described elsewhere (4, 8, 29). Vasculopathy was quantified by calculating the neointimal index (NI) as described by Armstrong et al. (30, 31) and by reporting the number of vessels with >30% vasculopathy per graft.

ELISPOT assays

Assays were performed as outlined previously in detail (4, 22, 29, 32). Briefly, ELISPOT plates (Millipore, Bedford, MA) were coated overnight with the capture Ab (obtained from BD Pharmingen, San Diego, CA) in sterile PBS blocked with sterile 1% BSA in PBS, and washed three times with sterile PBS. Spleen cells (0.2–1 × 10^6/well) were plated in HL-1 medium (BioWhittaker, Walkersville, MD) with or without mitomycin C-treated male stimulator cells (400,000/well) and/or soluble Ags (HYDβp, HYUrpy, βgal96–103 at 0.1–10 μM) and then incubated at 37°C in 5% CO_2 for 24 h. After washing with PBS followed by PBS/0.025% Tween (PBST), detection Abs (obtained from BD Pharmingen) were added over night. After washing with PBST, alkaline phosphatase-conjugated anti-biotin Ab (Vector Laboratories, Burlingame, CA) diluted 1/2000 in PBST was added for 90 min at room temperature. The plates were developed as previously described (4, 22, 29, 32). The resulting spots were counted on an ImmunoSpot Series 1 Analyzer (Celular Technologies, Cleveland, OH).

In vivo CTL assays

This assay was adapted from published studies (33). Spleen cells were isolated from naive B6 females and RBCs were removed by osmotic lysis. The B6 spleen cells were suspended in RPMI 1640 plus 10% FBS at 10 × 10^6/ml and pulsed with HYUrpy (experimental) or βgal96–103 (control), 2 μg/ml final concentration for 90 min at 37°C in 5% CO_2. After washing five times in PBS, cells were resuspended at 10 × 10^6/ml in PBS/0.1% BSA. The control peptide-loaded cells were labeled with a high concentration of CFSE (20 μM) (CFSE low cells) and the HYUrpy-loaded target population was labeled with a low concentration of CFSE (3 μM) (CFSE high cells) for 10 min at 37°C in 5% CO_2. The labeled cells were washed three times in PBS. Equal numbers of cells from each population were mixed together and injected i.v. into the tail vein of recipient mice at predetermined time points posttransplantation. Injections were performed such that each mouse received a total of 5 × 10^6 experimental (CFSE low) cells and 5 × 10^6 control (CFSE high) cells in a total of 400 μl of PBS. Sixteen hours later, the animals were sacrificed and the cell suspensions were analyzed by flow cytometry. Each population was detected by its differential CFSE fluorescence intensities. Up to a total of 500,000 gated events were acquired per sample. For each animal, we calculated a ratio of the number of CFSE low cells to the total number of CFSE high plus CFSE high cells. Specific lysis was calculated as (1 – (ratio in naive B6 mice – ratio experimental mice)) × 100.

Results

Consistent with previously published work (10, 11, 17), B6 female recipients acutely rejected minor Ag-disparate male B6 skin grafts, but male B6 heart grafts survived indefinitely in female B6 mice (Fig. 1). Skin-derived Langerhans cells are more frequent in number and may be more immunogenic than heart-derived dendritic cells (34, 35). In addition, because skin grafts are not initially vascularized, induced ischemia-reperfusion injury is likely to be more prolonged than in vascularized heart transplantation, possibly leading to increased activation and immunogenicity of donor dendritic cells migrating to the recipient secondary lymphoid organs. Potential consequences of these differences might be higher frequencies of anti-HY effector T cells and/or induction of more potent (pathogenic) T cell effector functions in recipients of skin vs heart transplants. To assess this possibility, we analyzed the donor-reactive T cell repertoire on day 14 posttransplantation in skin vs heart graft recipients. Fig. 2A shows donor-reactive IFN-γ ELISPOT recall responses for spleen cells obtained from individual skin or heart graft recipients. Spleen cells from naive mice were included as controls. We tested for reactivity to two known immune dominant determinants derived from the male Ag (36, 37), HYDβp (MHC class II, IA b -restricted) and HYUrpy (MHC class I, D b -restricted), and for reactivity to mitomycin C-treated donor male spleen cells. The frequency of donor-reactive CD4 + and CD8 + T cells specific for male Ags was of similar magnitude in heart and skin graft recipients. Although placement of a skin graft induced a significantly higher frequency of donor-reactive IFN-γ-producing T cells (~200–250 per/million donor-reactive cells) than heart graft recipients (~100–150 per/million), this difference was only 2-fold, could be partially attributed to differences in kinetics of activation (see Fig. 3), and was of unclear biologic significance. For comparison, the frequency of antidonor T cells induced in response to fully allogeneic transplanted hearts or skin grafts (both

FIGURE 1. B6 females acutely reject syngeneic male skin but not heart transplants. Graft survival is shown and median survival is statistically different between groups by Kaplan-Meier analysis, p < 0.05.
antihy immunity was detectable in naive B6 females (Fig. 2A). Specific target and control cells detectable in the spleens of the transplanted or naive control recipients, and then the relative number of target cells along with a control target population into the transplanted directly in vivo is determined by transferring specific target cells loaded with \( \beta gal_{96–103} \) or specific target cells loaded with HYUtype are labeled with different concentrations of CFSE so that they can be differentiated by flow cytometry (a 5-fold concentration difference permits distinguishing between the two populations). For our experiments, the HYUtype-loaded targets cells were labeled with CFSE\(_{\text{low}} \) and the control syngeneic cells were labeled with CFSE\(_{\text{high}} \). Equal numbers of the two populations (5 \( \times \) 10\(^6 \) of each) were then mixed and injected i.v. into the tail vein of a naive or a grafted mouse. Animals were sacrificed the following day (<16 h) and spleen cells were studied by flow cytometry for the presence of the CFSE-labeled cells. As shown in Fig. 2B, skin and heart grafts both induced potent antimalle CTL activity compared with naive controls. Thus, on day 14 posttransplant, the frequency, cytokine profile, ability to mediate cytotoxicity, and relative immune dominance of the antimalle T cell immune response did not differ substantially between skin and heart graft recipients. Nevertheless, skin grafts were rapidly rejected but heart grafts were not (Fig. 1).

We next performed a kinetic analysis of T cell immune function and graft histopathology following transplantation of male heart grafts to syngeneic female recipients. As shown in Fig. 3, anti-HY T cell immunity was readily detectable on days 7–14 posttransplant. The immune response to the MHC II-restricted peptide peaked on day 7, several days before the detected response to the dominant HMC class I-restricted peptide (as assessed by IFN-\( \gamma \) ELISPOT (Fig. 3A) and in vivo CTL activity (Fig. 3B). Both antimalle CD4\(^+\) and CD8\(^+\) T cell immunity rapidly diminished by days 21–30 posttransplant.

Kinetic histologic evaluation revealed a diffuse intragraft infiltrate detectable in male hearts placed into female recipients on days 7–21 but few mononuclear cells were detectable in the recipients by day 30 (Fig. 4). Male heart grafts exhibited early vas-

![FIGURE 2. Male heart and skin grafts prime similar antidonor T cell immune responses. A, Frequencies of IFN-\( \gamma \)-producing cells reactive to MHC class I-restricted HYUtype (left), MHC class II-restricted HYDhyp (middle), and male stimulator cells (right) per million splenocytes in heart graft recipients (black circles), skin graft recipients (white circles), or naive mice (gray circles). Each symbol represents the mean value of duplicate or triplicate wells for an individual animal (<20% well-to-well variability). Animals were sacrificed on day 14 posttransplant. Dashes are placed at the mean value for each group. There was no response to control peptides \( \beta gal_{96–103} \) or OVA\(_{23–39} \) (data not shown). The filled square represents the mean value of donor-reactive IFN-\( \gamma \)-producing spleen cells tested at the time of cessation of heartbeat (days 8–10) in three B6 recipients of allogeneic A/J heart grafts. B, Representative flow cytometry plots for day 14 in vivo cytotoxicity assays are shown for a naive mouse (left), a skin-grafted mouse (middle), and a heart-grafted mouse (right). Average percent cytotoxicity for three to four animals per group is shown below each graph.](https://example.com/figure2)

![FIGURE 3. Antimale immunity wanes with time. Antimale T cell immunity was tested by IFN-\( \gamma \) ELISPOT (A) and in vivo cytotoxicity assay (B) on days 7, 14, 21, and 30 posttransplant. The day 14 data are the same data as shown in Fig. 2. Each point represents the mean response for three to four animals per group tested at that time point. Error bars fell within the size of the symbols for the cytotoxicity assay.](https://example.com/figure3)

![FIGURE 4. Chronic immune injury and transplant vasculopathy develop in male hearts transplanted into female recipients. Representative H&E-stained sections from hearts obtained on days 7, 14, 21, 30, 60, and 90 posttransplant. Original magnification, \( \times 10 \). Insets in the two lower panels are elastin stains demonstrating endothelial cell proliferation consistent with transplant vasculopathy. Original magnification, \( \times 10 \).](https://example.com/figure4)
culopathy on day 60 posttransplant and progressive vasculopathy by day 90. Quantitative analysis revealed significantly increased frequency and severity of transplant vasculopathy in male hearts transplanted into females when compared with isogenic female heart grafts placed into female recipients by day 90 posttransplant (Table I). The female grafts exhibited an essentially normal histologic appearance. The progressive vasculopathy in male but not female grafts suggested that the female recipients of male heart grafts were not tolerant to male transplantation Ags.

To formally assess the presence or absence of immune tolerance, male B6 heart transplants were placed into female B6 recipients and 30 days later, the animals were transplanted with male B6 trunk skin. The skin grafts were rejected by day 18 (n = 3, data not shown), clearly showing that the female recipients were not tolerant to the male Ag. Recall assays performed 3 wk after skin graft rejection (55–60 days after heart graft placement) revealed antidonor immunity as assessed by both IFN-γ ELISPOTs (frequency) and in vivo CTL assays (Fig. 5), confirming the absence of tolerance. In striking comparison, there was minimal detectable antimalle immune in B6 female recipients of B6 male hearts alone on days 50–60 (no additional skin graft placed, Fig. 5).

Interestingly, the rejection of male skin did not precipitate acute rejection of the previously placed male hearts; the heart grafts in these animals continued to beat for more than 3 wk after placement (Table I). The female grafts exhibited an essentially normal histologic appearance. The progressive vasculopathy in male but not female grafts suggested that the female recipients of male heart grafts were not tolerant to male transplantation Ags.

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Interestingly, the rejection of male skin did not precipitate acute rejection of the previously placed male hearts; the heart grafts in these animals continued to beat for more than 3 wk after placement of the skin graft. Histology of these heart grafts performed on day 30, however, revealed more extensive vasculopathy compared with male hearts placed into female recipients that did not receive a skin graft (Fig. 5C and Table I). The mononuclear cell infiltrates were more diffuse and the vasculopathy more severe in the animals given male heart grafts followed by a subsequent male skin graft compared with male grafts placed into females without a skin graft. Overall, the data suggest that the heart transplant primed a proinflammatory, antimalle T cell immune response but that this response was insufficient in quantity to mediate acute rejection of the heart grafts. Nonetheless, the sublethal immune response led to graft injury that eventually became manifest as chronic rejection. “Boosting” of antimalle T cell immunity via rejection of a male skin graft was still insufficient to induce destruction of the heart (over the 3-wk time period tested) but led to an accelerated form of chronic graft injury.

An additional possibility that could account for the absence of rapid graft destruction (persistent graft function) in female recipients of male hearts is that amount of male Ag expressed on the parenchymal cells of the graft might be too low to be recognized by primed T cells trafficking through the organ. To assess this possibility, we placed male B6 heart grafts into female MataHari RAG KO recipient mice. MataHari females bred onto a B6 RAG KO background are TCR-transgenic animals in which every T cell in the recipient is CD8+ and specific for the immune dominant determinant HY/Up+ plus Dα+ (4). As shown in Fig. 6, MataHari RAG KO females rapidly rejected the male (but not female) B6 heart grafts. As might be anticipated for a TCR-transgenic RAG KO recipient, the rejection was associated with a high frequency of primed antidonor effector T cells as assessed by IFN-γ production at the time of rejection (>11,000 per million spleen cells, n = 3, data not shown). These results demonstrate that the male grafts do express sufficient Ag to facilitate rejection by primed T cells if large enough numbers of these effector T cells are present in the recipients.

To further assess the role of T cell frequency as a mediator of heart graft rejection, we next transplanted male B6 heart grafts into MataHari RAG−/− recipients. Female MataHari RAG−/− mice only contain ~20% MataHari T cells (data not shown) with the remainder of the CD8+ and CD4+ T cell repertoire deriving endogenously. Despite the increased frequencies of donor-reactive T cells compared with wild-type female B6 recipients (Table II), the MataHari RAG−/− recipients did not acutely reject male B6 heart grafts. The grafts transplanted into these animals did, however, exhibit clear evidence of chronic injury/vasculopathy by an early 30-day time point (Fig. 6 and Table I). ELISPOT recall assays performed on days 7 and 30 posttransplant revealed 10- to 50-fold higher absolute numbers of antidonor effector T cells compared with wild-type B6 recipients (Table II). Overall, the ability of MataHari RAG KO females to reject male heart grafts is consistent with the interpretation that a threshold number of T cells is required to acutely reject a cardiac transplant (11). The data further suggest that subthreshold numbers of donor-reactive T cells mediate chronic injury and that the severity and rapidity of the induced injury is partially dependent on the frequency of donor-reactive T cells in the recipient; below the threshold number that

### Table I. Quantification of vasculopathy in recipients of B6 male heart grafts

<table>
<thead>
<tr>
<th>Donor → Recipient</th>
<th>Day Posttransplant</th>
<th>Average NI (%)</th>
<th>No. of Vessels with NI &gt; 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male B6 → female B6 (n = 5)</td>
<td>30–35</td>
<td>&lt;10</td>
<td>0/26 (0%)</td>
</tr>
<tr>
<td>Male B6 → female MataHari RAG−/− (n = 5)</td>
<td>30–35</td>
<td>30.5</td>
<td>14/35 (40%)</td>
</tr>
<tr>
<td>Male B6 → female B6 (n = 5)</td>
<td>55–63</td>
<td>22.9</td>
<td>8/43 (18.6%)</td>
</tr>
<tr>
<td>Male B6 skin followed by male B6 heart → female B6 (n = 3)</td>
<td>50</td>
<td>42</td>
<td>15/24 (62.5%)</td>
</tr>
<tr>
<td>Male B6 → female B6 (n = 3)</td>
<td>87–94</td>
<td>38.2</td>
<td>14/33 (42%)</td>
</tr>
<tr>
<td>Male B6 → male B6 (control, n = 3)</td>
<td>88–92</td>
<td>13.7</td>
<td>0/10 (0%)</td>
</tr>
</tbody>
</table>

**FIGURE 5.** Female recipients of male hearts are not tolerant to the male Ag. Mean frequencies of splenic antimalle IFN-γ producers (A) and individual in vivo cytotoxicity assay results (B) are shown for female recipients of male heart grafts 55–60 days posttransplant. ■ and ○, Results for recipients of male hearts alone (n = 4). □ and ▽, Results for recipients of male hearts that additionally received male skin grafts on day 30 following the heart transplant. No responses were detected to control peptides (data not shown). C, Representative H&E-stained and elastin-stained (inset) section of a male heart graft placed into a female recipient followed by placement of a male skin graft on day 30. The histology was assessed on day 55 after the heart transplant. 1 wk after the skin graft was visually rejected. Inset is an elastin-stained section from the same graft. Original magnification, ×10.
leads to acute organ destruction, higher frequencies of donor-reactive T cells induce chronic injury at a more rapid rate than lower frequencies.

We next sought to directly determine whether the frequency of donor-reactive T cells required to reject a heart graft was influenced by the size of the donor organ. We therefore challenged wild-type female B6 mice with cardiac allografts from 3-wk-old male donors. The weight of the hearts from these 3-wk-old donors (52.2 ± 11 mg) is 50% the weight of normal adult donors obtained from mice 8–10 wk of age (98 ± 5.5 mg). Interestingly, ~60% of the technically successful small heart transplants were acutely rejected by day 25 (Fig. 7). Immune-mediated injury was confirmed by histologic examination in all grafts that stopped beating (Fig. 7). Recall immune responses on day 14 posttransplant revealed that the frequency of antidonor T cells was no higher in recipients of small vs large heart grafts (10–60 per million HYUtyp-specific IFN-γ producers and 30–300 per million HYDtyp-specific IFN-γ producers, n = 5, data not shown), showing that transplantation of small vs large hearts was no more efficient at priming recipient T cells. Thus, the data are consistent with the interpretation that the ability of a given number of effector T cells to reject a target organ is largely dependent on the size of the organ.

The above data suggest that the difference in outcome of skin vs heart grafts (rejection vs acceptance) is the tissue burden of the graft rather than the ability of a graft to prime recipient T cells. A limited frequency of induced antidonor effector T cells seems to only be able to destroy a graft of a given size (regardless of whether it is a heart or a skin graft) before the response wanes. If this is indeed true, then increasing the tissue burden of a skin graft should prolong graft survival but should not affect the number of primed effector antidonor T cells induced by the transplantation. We assessed this possibility by placing either one or two 0.5-cm male skin grafts onto wild-type recipient female mice. As shown in Fig. 8A, skin graft survival was markedly prolonged in recipients of two vs one skin graft. Moreover, placement of either one or two skin grafts primed similar numbers of antidonor T cells in the recipients. If one skin graft was placed, the antidonor immune response was detectable in the lymph nodes draining the graft and in the spleen. If two grafts were placed, the antidonor response was detectable in both draining lymph nodes and the spleen, but the overall number of antidonor T cells was the same as in the animals with a single graft.

**Discussion**

It has long been noted that skin grafts are more readily rejected than heart transplants by minor Ag-disparate recipients, but the reasons for this difference in outcome has remained unclear (10, 11, 27, 38, 39). Skin grafts are thought to be more “immunogenic” than cardiac allografts because they contain large numbers of Langerhans cells (34, 35), a population of APCs with excellent Ag presentation capability. Skin transplants may also induce a more potent immune response than heart grafts because of the prolonged ischemic injury they suffer while recipient vasculature grows in to feed the graft (days for skin grafts vs hours for heart grafts). The prolonged ischemia may provide inflammatory signals (16), including up-regulation of chemokines (40–44) that could increase the efficiency of T cell priming and facilitate/accelerate trafficking of the primed T cells to the graft.

We did find more antidonor T cells in the skin vs heart graft recipients, consistent with the interpretation that skin is more immunogenic than heart tissue. Still, the highest detectable frequencies were much below the frequency of antidonor T cells induced in response to a fully allogeneic graft (Fig. 2) and could in part be related to differences in activation kinetics following skin vs heart transplantation. More importantly, even a >10- to 50-fold increase in the frequency of donor-reactive effector T cells as found in the MataHari RAG+/- recipients (Fig. 6 and Table II) did not result in acute rejection of male heart grafts. Thus, the detected difference in the frequency of antidonor IFN-γ-producing T cells in skin vs heart graft recipients was of unclear biologic significance and alone could not account for the difference in graft outcome between the two sets of recipients. Also, there was no significant difference in the relative immunodominance of MHC class I-restricted or MHC class II-restricted anti-HY responses between skin

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**Table II. Frequency and absolute number of anti-HYUtyp-specific IFN-γ producers in recipients of male heart grafts**

<table>
<thead>
<tr>
<th>Day posttransplant</th>
<th>B6 Male → B6 Female</th>
<th>Day posttransplant</th>
<th>B6 Male → MataHari B6 RAG+/− Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (per 10^6 spleen cells)</td>
<td>Total per spleen</td>
<td>Frequency (per 10^6 spleen cells)</td>
</tr>
<tr>
<td>7</td>
<td>13 ± 6</td>
<td>970 ± 478</td>
<td>7</td>
</tr>
<tr>
<td>14</td>
<td>119 ± 9</td>
<td>7,110 ± 539</td>
<td>30</td>
</tr>
<tr>
<td>21</td>
<td>18 ± 7</td>
<td>1,050 ± 404</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>46 ± 9</td>
<td>2,751 ± 546</td>
<td></td>
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*The results represent mean values ± SE for four to six animals per group.*
and heart transplant recipients (Fig. 2). Nor was there a difference in the development of type 1 cytokine secretion or the ability to mediate CTL activity in recipients of heart vs skin grafts at 7–14 days posttransplant. Thus, our data strongly suggest that the immunogenicity of the donor graft alone cannot account for the difference in outcome between skin and heart grafts in this model.

The results delineated by this work raise the possibility that one major difference between skin and heart grafts occurs not at the priming stage, but instead at the effector stage. The larger amount of tissue found in the heart vs the skin grafts seems to resist destruction by a limited T cell repertoire. This conclusion is supported by experiments showing that adequate Ag is expressed on heart tissue to permit graft destruction if sufficient numbers of high-affinity antidonor T cells are present in the recipient (Fig. 6). Additionally, female recipients rejected small male heart grafts despite the fact that they did not prime higher numbers of anti-HY T cells than the larger grafts. Complementary experiments further showed that increasing the amount of skin graft tissue resulted in prolonged graft survival despite priming of the same frequency of donor-reactive T cells as single graft recipients (Fig. 8). The results confirm and extend previous studies in heart and skin graft models suggesting that the size of the graft tissue can influence graft survival (11, 27, 28). Importantly, these previous studies did not address whether the noted effect of tissue burden was attributable to T cell priming or an influence at the effector stage issue, an issue clarified by this work.

Our data also confirm, using a polyclonal system, the findings of Jones et al. (11) in which the authors demonstrated that a threshold number of TCR-transgenic T cells are required to reject an allograft and that this threshold number is larger for heart vs skin grafts. The data from the present manuscript provide additional insight by showing that a subthreshold number of donor-reactive T cells, while not capable of acutely rejecting the graft, induce chronic graft injury as manifested by fibrosis and transplant vasculopathy (Figs. 4–7 and Table I). Male-to-female heart grafts exhibited evidence of vasculopathy at 60–90 days posttransplant, while syngeneic female-to-female hearts were essentially normal at this time point. If the frequency of antidonor effector T cells was increased, but remained below the threshold required for rejection, chronic injury occurred at an accelerated rate; vasculopathy was detectable by day 30. This was true for both monoclonal TCR-transgenic T cells and for polyclonal effector T cells boosted by a skin allograft. It is important to note that no anti-HY Abs develop in this model and essentially no vasculopathy was detectable in syngeneic female transplants. The resultant vasculopathy can therefore only be attributed to antidonor (anti-HY) T cell immunity.

These findings have important implications for human transplantation and suggest that understanding and monitoring the frequency of donor-reactive T cells may be relevant to predicting long-term graft function in a clinical setting. In many respects, the concept of antidonor T cell frequency as a risk for long-term outcome is reminiscent of the relationship between elevated blood pressure and chronic organ injury (45, 46). Extremely high blood pressure can result in acute injury (encephalopathy, renal failure, heart failure) but subthreshold elevations in blood pressure produce chronic injury to the same organs. Moreover, the risk of chronic injury is directly related to the level of blood pressure and the time over which the blood pressure is elevated. Analogously, our data, in conjunction with recently published work by our group in human allograft recipients (47, 48), suggest that the frequency of donor-reactive T cells in the recipient over time may directly correlate with the risk of developing chronic injury.

One additional issue that we found particularly intriguing was the observation that although anti-HY T cell immunity was induced by heart grafts, the immune response was not sustained in wild-type recipient mice. Understanding in detail why such potent immune rapidly exhausts is an important issue that could potentially be exploited to prolong allograft survival in other situations. One potential interpretation is that each effector T lymphocyte has a limited life span and a limited ability to kill a certain number of target cells. Once that limit is reached, the T cell dies. The number of donor Ag-expressing cells in a heart graft (but not a skin graft) may be sufficiently large to withstand the initial onslaught, with the result being sublethal injury without graft destruction. New thymic immigrants would only be primed inefficiently, as the number of donor APCs capable of priming naïve T cells is markedly reduced over time. If an additional immunogenic stimulus such as a skin graft is placed, then the new T cells (or memory T cells) expand, reject the skin graft, and produce additional injury to the male heart. Overall, such a model is analogous to the inadequate immune response induced to a chronic viral infection where the initial infection primes a response, but this antiviral immunity becomes “exhausted” over time (49, 50). Vaccination in the correct context can boost the response and cure the infection.

An alternate but not mutually exclusive explanation for the observed decrease in antidonor immunity beyond 3 wk following heart transplantation is that regulatory T cell responses develop as a natural means of controlling the induced response. It has been
hypothesized that one mechanism to control any immune response is the coincident increase in regulatory T cells (T_{reg}) (51–53). Although detailed mechanisms are not understood, it is clear that T_{reg} expand in concert with most proinflammatory immune responses and it is thought that such regulatory cells may be essential down-modulators of the immune system. The natural activation and expansion of T_{reg} following heart transplantation might be able to control a low frequency response to a minor Ag-disparate heart graft, but be inadequate to control the high frequency responses induced to a fully MHC-disparate allograft. Depletion of anti-CD25^+ CD4^+ T cells in vivo can result in minor Ag-disparate heart graft rejection accompanied by an increase in antidonor T cell immunity (54), consistent with this hypothesis.

In summary, the results from these studies clarify an important interrelationship between T cell frequency and size of a target organ. A threshold number of T cells is required to reject a heart graft, and this number is dependent in part on the tissue burden of the target organ. Subthreshold numbers of effector T cells, while insufficient to acutely destroy the graft, induce chronic injury in a frequency- and time-dependent manner. The findings provide an experimental basis for the clinical observation that graft function in human recipients of transplants with larger tissue mass is better than in recipients of allografts with smaller tissue mass (55, 56). Moreover, the studies highlight the need for immune monitoring in human allograft recipients so as to be able to maintain the antiodonor immune response at levels that are as low as possible without inducing significant side effects.

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References

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