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Differential Susceptibility of Heart, Skin, and Islet Allografts to T Cell-Mediated Rejection[1]

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Although it is widely accepted that there is a hierarchy in the susceptibility of different allografts to rejection, the mechanisms responsible are unknown. We show that the increased susceptibility of H-2K^{b+} skin and islet allografts to rejection is not based on their ability to activate more H-2K^{b}-specific T cells in vivo; heart allografts stimulate the activation and proliferation of many more H-2K^{b}-specific T cells than either skin or islet allografts. Rejection of all three types of graft generate memory cells by 25 days posttransplant. These data provide evidence that neither tissue-specific Ags nor, surprisingly, the number of APCs carried in the graft dictate their susceptibility to T cell-mediated rejection and suggest that the graft microenvironment and size may play a more important role in determining the susceptibility of an allograft to rejection and resistance to tolerance induction. ***The Journal of Immunology,* 2001, 166: 2824–2830.**

In transplantation biology a well-established hierarchy exists among organ and tissue allografts in their susceptibility to rejection and conversely their resistance to tolerance induction. Skin, small bowel, and lung seem to be the most susceptible to rejection; whereas pancreatic islets, vascularized pancreas, heart, kidney, and liver are progressively more easily accepted (1–5). Liver allografts perhaps should be classified separately in that they are often accepted without the need for immune modulation in the pig, rat, and mouse, and liver grafts are able to induce acceptance of and in some cases tolerance to other coexistent donor-type allografts (6). A careful elucidation of the immune response generated by different types of allograft in this hierarchy may provide an understanding of the factors responsible for the differences in the susceptibility to rejection of allografts may allow more effective and selective strategies for immunosuppression to be developed.

A number of explanations have been proposed to account for the differences in susceptibility to rejection of the various types of allografts. These explanations include differences in the mode of revascularization, the lymphatic drainage, the presence of tissue-specific Ags, differences in the immunogenicity of the grafts, and graft size.

It is well established that nonvascularized grafts are subject to ischemic degeneration that can lead to inflammation and necrosis, even in syngeneic grafts (7). This nonspecific damage may well render nonvascularized grafts more susceptible to subsequent immune destruction. However, it has been demonstrated that MHC class I-mismatched nonvascularized heart allografts are rejected much more slowly than skin allografts in the same strain combination and are even on occasion accepted indefinitely (2). Furthermore, surgically vascularized skin allografts were found to be as vulnerable to rejection as conventional, naturally revascularized skin allografts (Ref, 8; A. R. Bushell and M. Liddington, unpublished observation). Thus, on balance, differences in the mode of vascularization are unlikely to fully explain the differential survival of heart, skin, and islet allografts. Other inherent differences between these grafts must therefore be responsible for the differences in their susceptibility to rejection.

A second potential explanation is the presence of tissue-specific Ags. Evidence for the existence of tissue-specific alloantigens arose from observations in hemopoietic chimeras. In this situation, although allogeneic hemopoietic cells survived indefinitely, such chimeras would not accept skin grafts from the same donor, suggesting that the skin-expressed Ags not found on the hemopoietic cells (5, 9). Indeed, Skn and Epa-1, two skin-specific Ags, have been implicated as targets for skin graft rejection in some models (5, 10, 11).

A third possible factor responsible for the sensitivity of skin [1]grafts to rejection is the special immunological function of the skin, which may be subverted after transplantation to promote a vigorous rejection response. Skin contains numerous Langerhans cells (LC)[5] which are professional APCs with the capacity to migrate from the graft and efficiently stimulate recipient T cells (12, 13). Thus, the potential for a skin graft to stimulate large numbers of alloreactive T cells may explain why these grafts are so susceptible to rejection.

The susceptibility of allografts to rejection may also in part be dictated by the actual size of the graft. Obviously, the smaller the graft the smaller is the number of cells that must be destroyed for the graft to be rejected. Evidence that graft size has a bearing on graft rejection has recently been presented by Sun et al. (14), who demonstrated that whereas a single allograft was acutely rejected, grafting a recipient with multiple grafts resulted in the prolonged

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[5] Abbreviations used in this paper: LC, Langerhans cells; tg-TCR, transgenic TCR; AT, adoptive transfer; FasL, Fas ligand.

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survival of the transplanted organs. This observation is of particular relevance to small cell allografts such as islets.

Previously, it has not been possible to compare directly the immune response generated by different types of allografts due to an inability to identify alloantigen-specific T cells in vivo and the potentially confounding effects of T cells responding to tissue-specific Ags. To overcome these issues, we have developed an experimental strategy in which the rejection response is mediated by defined numbers of $CD8^+$ T cells that are specific for the allogeneic MHC class I molecule $H\text{-}2K^b$. In this system, tissue-specific Ags do not play a role in rejection because rejection is dependent solely on the activation of the $H\text{-}2K^b$-specific T cells. Using four-color flow cytometry, we monitored the proliferation, activation, and homing of the alloreactive cells after exposure to heart, skin, and islet allografts. Heart, skin, and islet allografts were chosen as challenge grafts because these graft types are distributed evenly across the transplantation hierarchy.

Materials and Methods

Animals

CBA/Ca (H-2K) and C57BL/10 ($H2^b$) mice were purchased from Harlan Olac, (Bicester, U.K.). BM3 (H-2K, BM3.3 TCR transgenic) mice have been described previously (15). All mice were housed in the part-barrier facilities of the Biomedical Services Unit, John Radcliffe Hospital (Oxford, U.K.). All donor and recipient experimental mice were sex and age matched between 8 and 12 wk of age at the time of first experimental procedure.

mAbs and hybridomas

YTA3.1 and YTS169 hybridomas were a gift from Professor H. Waldmann (Sir William Dunn School of Pathology, Oxford University, Oxford, U.K.) (16). Ti98 hybridoma has been described previously (17). All hybridomas and cell lines were grown in either RPMI 1640 or α-MEM (Life Technologies, Paisley, U.K.), supplemented with 2 mM glutamine, antibiotics, and 10% (v/v) FCS (Life Technologies). Anti-CD4 (YTA3.1) and anti-CD8 (YTS169) mAbs were purified and dialyzed into PBS before being used in vivo. Anti-CD8-APC (53-6.7), anti-clonotypic TCR-biotin (Ti98), anti-CD25-PE (PC61), anti-CD44-PE (IM7), anti-CD45RB-PE (16A), anti-CD62L-PE (MEL-14), and anti-CD69-PE (H1.2F3) mAbs were used in vitro for FACS analysis. For intracellular cytokine staining anti-IL-2-PE (JES6-5H4), anti-IFN-γ-PE (XMG1.2), anti-IL-4-PE (11B11), and anti-IL-10-PE (JES5-16E3) were used. Isotype-matched mAbs R3-34-PE (rat IgG1), and R35-38-PE (rat IgG2b) were used as controls. All mAbs were obtained from PharMingen, Becton Dickinson (Oxford, U.K.), unless stated otherwise.

Surgical procedures

Thymectomy. CBA/Ca mice were thymectomized under direct visualization, as previously described (18).

Heterotopic heart transplantation. Abdominal vascularized heterotopic heart transplants were performed essentially as documented by Corry et al. (19). The function of the transplanted hearts was followed by abdominal palpation, electrocardiogram, and laparotomy.

Skin transplantation. Individual full thickness tail skin grafts were prepared to fit the graft bed on the left lateral thorax of anesthetized recipients. The grafts were inspected regularly until they were completely destroyed, at which time the grafts were considered to have been rejected.

Islet transplantation. Islets were isolated from the donor pancreas using standard collagenase digestion followed by centrifugation through a discontinuous Ficoll gradient. Approximately, 600 freshly isolated islets were then mixed with a drop of the recipient's blood before transplantation under the left kidney capsule.

Induction of diabetes and monitoring of blood glucose levels

Male CBA recipient mice were rendered diabetic by a single i.v. dose of streptozotocin (250 mg/kg; Sigma, St. Louis, MO), and blood glucose levels were monitored regularly (Glucose Analyser II; Beckman, Bucks, U.K.). Only those animals with blood glucose concentrations of >20 mmol/L were used as recipients. After islet transplantation, blood glucose levels were monitored every other day for the first week and then twice weekly thereafter. Those animals with three consecutive blood glucose readings of >12.5 mmol/L were considered to have rejected their islet grafts.

Cell purification and CFSE labeling

A single-cell leukocyte suspension was made from spleens and mesenteric lymph nodes harvested from BM3 TCR-transgenic mice. $CD8^+$ T cells were purified by positive selection using anti-CD8 MACS beads (Miltenyi Biotec, Bergische Gladbach, Germany). Typically, $CD8^+$ T cells were isolated to $>95\%$ purity; $>95\%$ of the $CD8^+$ T cells expressed the $H\text{-}2K^b$-specific transgenic TCR (tg-TCR). The isolated cells were incubated for 10 min at 37°C with between 5 and 10 μM CFSE; Molecular Probes, Leiden, The Netherlands), washed twice, and resuspended in PBS ready for i.v. injection.

Standard experimental protocol

CBA/Ca mice were thymectomized (day −25) and rested for 2 wk before being treated with depleting anti-CD4 (YTA3.1; 100 μg/dose) and anti-CD8 (YTS169; 100 μg/dose) mAbs on day −12 and day −11. To allow time for the depletion of the majority of T cells, the mice were rested for a further 10 days at which time they were termed "empty" mice because they were severely depleted of peripheral T cells (95% T cell depletion). The mice were then reconstituted with an i.v. injection of the CFSE-labeled purified $CD8^+$tg-TCR^+ (BM3) T cells (day −1). The day after adoptive transfer (AT), i.e., day 0, mice received an $H\text{-}2K^{b+}$ (C57BL/10) heart, skin, or islet allograft.

Flow cytometric analysis

Leukocytes (1.5×10^6) were stained with anti-CD8-APC and Ti98-biotin mAbs. After washing, a streptavidin-conjugated fluorochrome was added (streptavidin-CyChrome (PharMingen)). The samples and fluorochrome were then incubated at 4°C before being washed twice. Finally, the samples stained with PE-labeled mAbs for detection of activation markers. All samples were then fixed with 250 μl PBS with 2% (v/v) paraformaldehyde, before being acquired on a FACSort (Becton Dickinson), and analyzed using the Cellquest software package (Becton Dickinson).

Intracellular cytokine staining

Spleen cells (1×10^6/ml) were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) for 4 h at 37°C, with brefeldin A (10 μg/ml) added for the last 2 h. Surface staining using anti-CD8-APC and Ti98-biotin mAbs was performed in PBS-FCS-azide + brefeldin A. After two washes, streptavidin-CyChrome was added for 15 min. The cells were then washed and resuspended in PBS-brefeldin A-2% (v/v) formaldehyde. The following day, cells were washed and preincubated for 10 min in permeabilization buffer (PBS-1% FCS-0.5% saponin (Sigma)), and incubated with anti-IL2 (2.5 μg/ml), anti-IFN-γ (5.0 μg/ml), anti-IL4 (5.0 μg/ml), anti-IL10 (5.0 μg/ml), or an isotype control for 30 min. All of the cytokine Abs were PE conjugated. After two washes with permeabilization buffer, the cells were washed in PBS-1% FCS without saponin to allow membrane closure. Samples were analyzed on a FACSort flow cytometer (BD, Oxford, U.K.). Results were analyzed using CellQuest software (BD).

Immunohistochemistry

Thin frozen sections (7 μm) were cut, air-dried overnight, and fixed in acetone (BDH, Cardiff, U.K.). After inhibition of endogenous peroxidase activity and blockade of endogenous biotin, sections were incubated with the primary anti-CD8 mAb (YTS169). After incubation with a HRP-conjugated anti-primary species Ab, staining was developed by addition of diaminobenzidine substrate (Vector Laboratories, Burlingame, CA). Finally, sections were counterstained with Gill's hematoxylin (BDH) and prepared for permanent mounting.

Statistical analysis

Statistical analysis was performed using Student's *t* test.

Results

Determination of the number of $H\text{-}2K^{b+}$-specific T cells required to reject $H\text{-}2K^{b+}$ heart, skin, and islet allografts in vivo

In an initial set of experiments, we sought to quantify the number of alloreactive T cells required to mediate rejection of heart, skin, and islet allografts, where allograft rejection was dependent on the response of a defined number of alloantigen-specific T cells recognizing a single allogeneic MHC class I molecule ($H\text{-}2K^b$). T

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cell-depleted recipient mice were generated by thymectomy and T cell depletion. These animals were severely immunocompromised and were unable to reject fully mismatched H-$2K^{b+}$ (C57BL/10 (H-2^b)) heart (median survival time (MST), >50 days; $n = 8$), skin (MST >50 days; $n = 8$) or islet (MST >50 days; $n = 10$) allografts (Table I).

We have previously demonstrated that AT of 6×10^6 H-$2K^b$-specific T cells into such immunocompromised mice resulted in the rejection of H-$2K^{b+}$ heart allografts with an MST of 18 days (18). However, transfer of 4×10^6 H-$2K^b$-specific T cells failed to reconstitute rejection ($n = 8$; Table I).

In contrast to heart allograft rejection, as few as 1×10^3 H-$2K^b$-specific $CD8^+$ T cells were able to reject H-$2K^{b+}$ skin allografts (MST = 35 days; $n = 3$; Table I). In similar experiments, 1×10^3 H-$2K^b$-specific $CD8^+$ T cells were found to reject H-$2K^{b+}$ islet allografts (MST 12 days; $n = 3$) (Table I). Thus, many more alloreactive T cells (~6000-fold more in this model) were required to mediate the rejection of a heart allograft than were necessary to reject skin or islet allografts.

Alloantigen-specific T cell activation in vivo in response to heart, skin, and islet allografts

It was possible that the vast discrepancy in the number of alloreactive T cells required to reject skin and islets, compared with heart allografts, was a reflection of the ability of the different graft types to activate alloreactive T cells. To characterize the kinetics of T cell division in vivo after transplantation, H-$2K^b$-specific T cells were labeled with the fluorescein-based dye CFSE before transfer. CFSE has been shown to segregate evenly between daughter cells on cell division, resulting in sequential halving of the fluorescent intensity which can be analyzed by flow cytometry (20, 21). Using four-color flow cytometry, we gated on the $CD8^+$ tg-TCR^+ cells, which allowed us to analyze the response of H-$2K^b$-specific T cells independently of other cells within the recipient (detailed in Ref. 18).

We analyzed the proliferative response of alloreactive T cells within the spleen 1, 3, and 7 days after transplantation of either H-$2K^{b+}$ heart, skin, or islet allografts into immunocompromised recipients which had been reconstituted with 6×10^6 $CD8^+$ H-$2K^b$-specific T cells. No proliferation occurred within 24 h after transplantation of any of the grafts. By 3 days after transplantation, H-$2K^b$-specific T cells had begun to divide in response to H-$2K^{b+}$ heart allografts, but not in response to H-$2K^{b+}$ skin or islet grafts (Table II). When proliferation was examined 7 days after transplantation, almost 50% of the H-$2K^b$-specific T cell population within the spleen had divided in response to the heart allografts, whereas in animals that had received either skin or islet allografts no proliferation above that seen in nongrafted mice was observed (Table II). The proliferation of the H-$2K^b$-specific T cells to the three different grafts was also shown to correlate with the up-regulation of CD69 and CD44 (markers of activation). Activated H-$2K^b$-specific T cells were found in the spleen only after transplantation of a heart allograft (data not shown).

These data were at first sight surprising given that the islet and skin allografts were rejected by far fewer H-$2K^b$-specific T cells. One possible explanation as to why proliferating, activated T cells were not found after transplantation of either skin or islet grafts was that the activated cells had rapidly infiltrated the grafts. Consequently, the timing of graft infiltration by the $CD8^+$ H-$2K^b$-specific T cells was determined by immunohistochemistry; graft-infiltrating cells were identified by their expression of CD8. Three days after transplantation, $CD8^+$ cells were absent from the skin and islet allografts. In contrast, small numbers of $CD8^+$ cells had infiltrated the heart allografts (Fig. 1). By 7 days posttransplant, $CD8^+$ T cells were readily detected in all three graft types. These data indicated that the H-$2K^b$-specific T cells had infiltrated the skin and islet grafts with slower kinetics than the heart grafts.

Analysis of allo-specific T cell numbers in the spleen also suggested that the failure to detect proliferating cells was not due to rapid migration of these cells from the spleen as analysis of the absolute numbers of H-$2K^b$-specific T cells showed that although there was a significant loss of K^b-specific T cells from the spleen of mice receiving an islet allograft 1 day after transplant no preferential loss of cells from the spleen in mice that had received a skin or islet graft compared with mice that received a heart allograft was observed (Fig. 2).

Preferential homing but not activation of allospecific T cells to the draining lymph node after skin transplantation

In the transplantation setting, T cell activation is thought to occur within the lymphoid tissues draining the graft site (12, 13). Therefore, the spleen may not be the optimal location in which to observe the activation of alloreactive T cells after skin and islet transplantation. To address this possibility, we compared the pattern of T cell proliferation and homing within the left axillary lymph nodes 1, 3, and 7 days after either skin or heart transplantation. The percentage of dividing H-$2K^b$-specific T cells within the draining axillary lymph nodes after skin transplantation was not increased compared with either mice that had not received a transplant or over the percentage of dividing cells within the spleen of skin grafted mice (Fig. 3*A*). In fact, heart allografts induced more alloantigen-specific T cell proliferation in the left axillary lymph nodes, than the skin grafts. However, the absolute number of H-$2K^b$-specific T cells present in the left axillary nodes was dramatically increased in mice that had received a skin graft (Fig. 3*B*; $p < 0.05$).

In the case of islet grafts transplanted under the renal capsule, it is likely that lymphatic drainage is initially directed toward the local renal lymph node (22). In the CBA recipients used in this study, the renal lymph nodes were too small to allow analysis of T cell activation occurring at this site after renal subcapsular islet transplantation.

Table I. *Rejection of different H-$2K^{b+}$ allografts by defined numbers of H2H^b-specific T cells*[a]

No. of tg-TCR^+ $CD8^+$	Individual Graft Survival Time		
	Heart	Skin	Islet
No cells	8 × >50	8 × >50	10 × >50
6×10^6	3 × 15, 18, 21, 2 × >50	4 × 20, 3 × 23	1, 3 × 2
4×10^6	8 × >50	22, 2 × 26	2 × 5
2×10^6		2 × 26, 30	5, 11
1×10^6		16, 28, 31	2 × 12
1×10^5		22, 2 × 27	2 × 4, 26
1×10^4		37, >50	2 × 12, >50
1×10^3		32, 35, >50	4, 12, >50
500		3 × >50	3 × >50
100			3 × >50

[a] Athymic, T-depleted CBA (H-2^k) recipients were reconstituted with graded numbers of purified H-$2K^b$-specific $CD8^+$ T cells the day before transplantation of a H-$2K^{b+}$ heart, skin, or islet allograft. Grafts were deemed to be rejected according to the criteria defined in *Materials and Methods.*

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Table II. *CD8⁺tg-TCR⁺ T cells proliferate in the spleen after transplantation of H2K^{b+} heart but not H2K^{b+} skin or islet allografts*[a]

Day	AT Only	B10 Heart	B10 Skin	B10 Islet
+1	1.5 ± 0.1	1.7 ± 0.2 NS	1.4 ± 0.4 NS	1.5 ± 0.8 NS
+3	5.8 ± 0.5	16.3 ± 2.9 $p < 0.05$	2.6 ± 0.2 NS	5.2 ± 0.3 NS
+7	15.6 ± 4.4	44.8 ± 2.7 $p < 0.05$	14.6 ± 4.2 NS	19 ± 1.8 NS

[a] Purified CFSE-labeled CD8$^+$ tg-TCR$^+$ T cells (6×10^6) were adoptively transferred into immunodeficient mice. These mice then received either a heart skin or a islet allograft or were not transplanted the day after transfer. Spleen leukocytes were isolated from such mice 1, 3, and 7 days after transplantation and stained for expression of CD8 and the tg-TCR. This allowed analysis of the proliferation of the CD8$^+$tg-TCR$^+$ T cells as assessed by loss of CFSE fluorescence. Numbers indicate the percentage of CD8$^+$tg-TCR$^+$ cells that had divided at least once ± SD. Two to seven mice were used per group.

Tc1-like memory cell generation after rejection of heart, skin, and islet allografts

Finally, H-2K^b-specific T cells were analyzed in the spleen 25 days posttransplantation (i.e., after all three graft types had been rejected). Analysis of the CFSE profile of H-2K^b-specific T cells showed that in mice that had received a heart allograft, ~50% of cells had divided at least six times in response to the graft (Fig. 4*A*). An identical assessment of mice that had received a skin or islet graft revealed that these mice also contained a significant proportion of H-2K^b-specific T cells that had divided at least six times, although the percentage of these cells was much lower than in heart allograft recipients. Mice that were left untransplanted did not contain cells that had divided more than six times (Fig. 4*A*). Therefore, although too few cells responded to the skin and islet grafts to be detected at early time points, by 25 days posttransplant a clear population of donor alloantigen-specific T cells that had divided in response to the H-2K^{b+} skin and islet allografts could be identified. No difference in terms of the absolute number of CD8$^+$ K^b-specific T cells was noted between groups.

Next, we determined whether these heavily divided cells exhibited the characteristics of memory cells. We analyzed these cells for the production of cytokines after a short in vitro stimulation with PMA and ionomycin. We found that H-2K^b-specific T cells that had responded to the grafts by dividing at least six times were capable of producing both IL-2 and to a greater extent IFN-γ on restimulation (Fig. 4*B*). In contrast, the H-2K^b-specific T cells that had failed to divide in response to the grafts were unable to produce IL-2, and only a small percentage (between 4 and 7%) were able to produce IFN-γ. No IL-4 or IL-10 was produced by the H-2K^b-specific T cells in any group irrespective of division (Fig. 4*B*). Cells analyzed from mice that did not receive a transplant failed to produce any cytokine on restimulation, consistent with their naive phenotype (data not shown). Not only did the CFSE-negative cells rapidly produce cytokine on restimulation but also phenotypic analysis of these cells revealed a memory phenotype in that they expressed high levels of CD44 and low levels of CD45RB (data not shown).

Discussion

We have developed an experimental model in which the immune response generated against different allografts can be compared and contrasted directly. This allowed us to examine the behavior of alloantigen-specific T cells in response to heart, skin, and islet allografts, which exhibit marked differences in their susceptibility to rejection and resistance to the induction of tolerance. By reconstituting immunocompromised recipients with defined numbers of H-2K^b-specific alloreactive T cells, this model gave us the unique opportunity to compare the immune response generated against

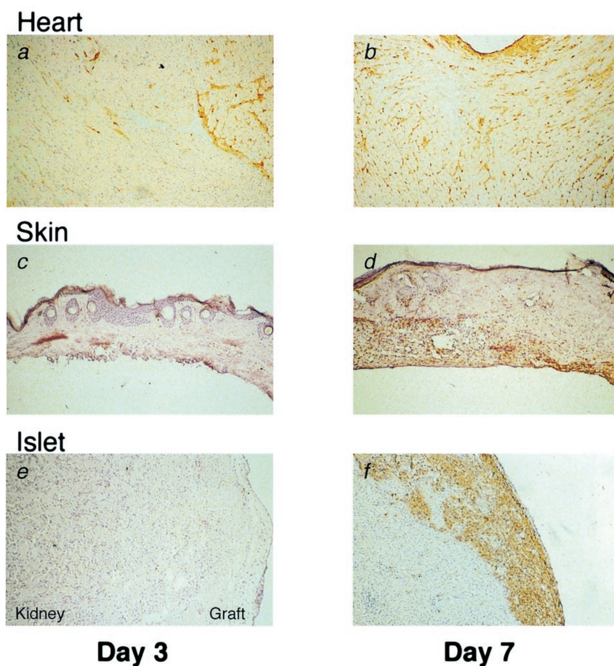

FIGURE 1. H-2K^b-specific T cells infiltrate heart, skin, and islet allografts by 7 days posttransplantation. Reconstituted mice (6×10^6 CD8$^+$tg-TCR$^+$ T cells) were either transplanted with a heart, skin, islet H-2K^{b+} allograft or not transplanted. Heart (*a* and *b*), skin (*c* and *d*), and islet (*e* and *f*) allografts were harvested on 3 and 7 days posttransplantation and stained with an anti-CD8 mAb. Results are indicative of at least three mice studied per group.

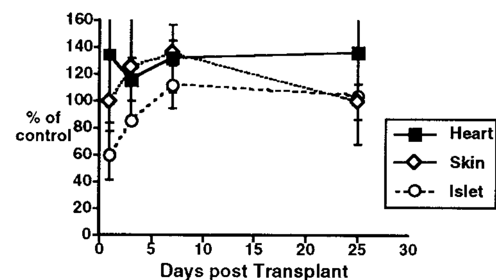

FIGURE 2. Absolute number of CD8$^+$tg-TCR$^+$ T cells in the spleen during rejection of heart, skin, and islet allografts. Reconstituted mice (6×10^6 CD8$^+$tg-TCR$^+$ T cells) were either transplanted with a heart, skin, islet H-2K^{b+} allograft or not transplanted. Spleen leukocytes were isolated from such mice 1, 3, 7, and 25 days after transplantation. The total leukocyte number of each spleen was determined by cell counting using trypan blue exclusion. The leukocytes were then stained for expression of CD8 and the tg-TCR and the percentage of these cells in a given spleen was determined. The percentage and total leukocyte count were then used to calculate the absolute number of CD8$^+$tg-TCR$^+$ cells per spleen. The graph shows the absolute number of CD8$^+$tg-TCR$^+$ cells per spleen in transplanted mice as a percentage of the number of CD8$^+$tg-TCR$^+$ cells per spleen in control nontransplanted mice. Results are expressed as mean percentage ± SD. Three mice were used per group. Only the islet transplant group on day +1 was significantly different from the AT only group ($p < 0.05$).

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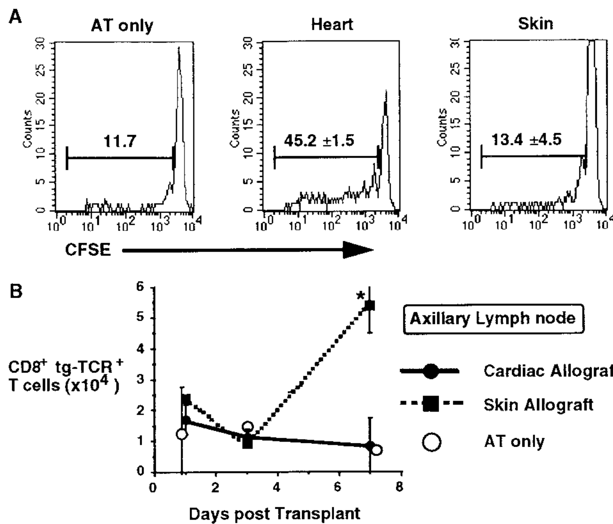

FIGURE 3. $CD8^+$tg-TCR^+ T cells home to but do not become activated in the axillary lymph nodes after skin transplantation. Reconstituted mice (6×10^6 $CD8^+$tg-TCR^+ T cells) were either transplanted with a heart, skin, islet H-$2K^{b+}$ allograft or not transplanted. The left axillary lymph nodes (draining the skin graft) were removed, and single-cell suspensions were prepared. *A,* Analysis of the CFSE profiles of $CD8^+$tg-TCR^+ T cells taken from the left axillary nodes 7 days after transplant of either heart or skin graft. Cells from mice that had not received a transplant are shown as a control. Histograms are representative of three mice studied. *B,* Absolute numbers of $CD8^+$tg-TCR^+ T cells in the left axillary nodes 1, 3, and 7 days after transplantation. Three mice were used per group. *, $p < 0.05$.

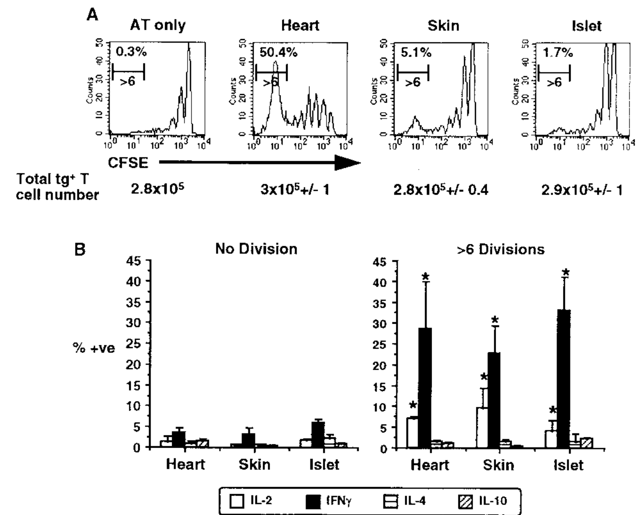

FIGURE 4. Detection of memory/effector $CD8^+$tg-TCR^+ T cells after heart, skin, or islet transplantation. Reconstituted mice (6×10^6 $CD8^+$tg-TCR^+ T cells) were either transplanted with a heart, skin, islet H-$2K^{b+}$ allograft or not transplanted. Spleens were harvested 25 days after transplantation. *A,* The CFSE profiles of $CD8^+$tg-TCR^+ T cells was determined. Three mice in each group were analyzed; all showed similar results. *B,* Splenocytes were stimulated in vitro with PMA and ionomycin for 4 h. Using three-color flow cytometry, a gate was placed around the $CD8^+$tg-TCR^+ T cells and either cells within the far right CFSE peak (*A*) or cells within the far left CFSE peak as gated in *A*. This allowed the evaluation of intracellular cytokine production by the H-$2K^b$-specific T cells that had not divided or had divided at least six times in response to the allografts. Results are expressed as the mean of three mice ± SD. *, $p < 0.05$ compared with cells from the same animal that had not divided. No significant difference was found in the percentage of cells that stained cytokine positive (+ve) between mice that had received a heart, skin, or islet graft.

different types of allografts in recipients with an identical precursor frequency of alloreactive T cells.

Initially, we defined the number of H-$2K^b$-specific T cells that were required to reject the different H-$2K^{b+}$ allografts. In concordance with many others studies and the current dogma, it was found that indeed skin and islet grafts were far more susceptible to rejection than heart allografts (requiring some 6000-fold less cells; Table I). Importantly, because rejection was mediated by T cells recognizing a single allogeneic donor MHC class I molecule, a response to tissue-specific Ags could not explain the differential susceptibility of these grafts to rejection. The same was true with anti-MHC class II responses given that transfer of 6×10^6 naive $CD4^+$ T cells into immunocompromised recipients resulted in acute skin allograft rejection but failed to reconstitute rejection of allogeneic heart allografts (C. I. Kingsley and A. R. Bushell, unpublished observations).

The observation that skin and islet grafts remain susceptible to graft rejection in the absence of tissue-specific Ags has important clinical and experimental ramifications when attempting the induction of transplantation tolerance to these different grafts. For example, many experimental models of tolerance induction involve some form of Ag pretreatment before transplantation (23, 24). Although most of these models are highly successful at inducing heart allograft acceptance, many fail to prevent skin or islet graft rejection (3, 25). This finding has often been attributed to the presence of tissue-specific Ags on the graft that were not on the original donor cell inoculum. However, the data presented here suggest an alternative explanation in that if a proportion of donor-reactive T cells escapes the initial tolerization process while there may be an insufficient number of these cells to adversely affect a resistant graft such as a heart graft. there may be high enough numbers to reject a susceptible graft such as a skin or islet graft.

This hypothesis could also explain why donor reactive T cells can frequently be detected by in vitro analysis in animals that have accepted a resistant allograft (although at much reduced numbers compared with naive controls), and why animals tolerant of a primary graft that is relatively resistant to rejection often reject a skin graft from a genetically identical donor (25–27).

Tolerance induction strategies are unlikely to regulate the activity of all potentially alloreactive T cells. Although solid vascularized organs, such as the heart allografts used in this study, were able to resist destruction mediated by large numbers of activated alloreactive T cells (4×10^6); nonvascularized grafts, such as islets and skin, were rapidly rejected by a very limited number of alloreactive T cells (1×10^3). In a similar manner, the lack of success of clinical islet transplantation may, in part, be due to the inability of pharmacological immunosuppression to regulate the activity of the complete alloreactive T cell repertoire.

In addition to the presence of tissue-specific Ags, the susceptibility of skin grafts to rejection has often been attributed to the large numbers of LC found within the skin. After transplantation, these LC become activated, increase their expression of MHC class II and costimulatory molecules, and migrate via the lymphatics to the T cell areas of the draining lymph nodes where they can provide a powerful stimulus for graft rejection. However, when we directly examined the ability of a skin graft to activate donor-reactive T cells, it was clear that the skin graft was relatively inefficient at stimulating T cell activation and proliferation even in the lymph nodes draining the graft bed, which is exactly the site where the potent immunostimulatory LC have been shown to migrate (Fig. 3) (12, 13). Indeed, the H-$2K^b$-specific T cells failed to proliferate or become activated (data not shown) in any lymphoid

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tissue at any time-point studied within the first 7 days after transplantation (Table II). However, by 7 days post transplant the H-$2K^b$-specific T cells were present in increased numbers in the draining node and within the graft itself.

It is likely that either migrating LC had activated a small number of peripheral T cells (which our system was not sensitive enough to detect) which had then homed to the graft or that a proportion of the naive T cells had migrated to the graft and did not proliferate in the peripheral lymphoid organs. We favor the former explanation as entry into the graft was not immediate (Fig. 1); and few $CFSE^+$ cells were present in any of the grafts 7 days after transplantation, suggesting that the infiltrating T cells had divided outside the graft (data not shown).

Thus, these data suggest that the susceptibility of skin allografts to rejection is not based on a profound T cell response generated by the migratory, immunostimulatory LC. However, even with the activation of a relatively small number of donor-specific T cells, the skin graft itself may amplify and exacerbate the inflammatory response. Keratinocytes possess the ability to secrete an array of inflammatory cytokines and will act as APC after up-regulation of MHC class II molecules (28). In addition, expression of T cell adhesion molecules, such as E-selectin (29), and the high concentration of extracellular matrix glycoproteins, including fibronectin and laminin (30), allow the skin graft to attract and engage activated T cells efficiently.

Islet allografts, like skin allografts, were rejected by as few as 1×10^3 donor-specific T cells (Table I). Islets contain only limited numbers of intragraft APC, and islet endocrine cells are unlikely to express significant levels of MHC class II during an inflammatory response (31, 32). As a result, islets may be less efficient at activating alloreactive T cells than other graft types. This fact was borne out by the finding that relatively few heavily divided memory cells were detected 25 days after transplantation. Inflammation, and the production of IFN-γ, would up-regulate MHC class I expression by the islets rendering them more susceptible to killing by activated, directly alloreactive cytotoxic $CD8^+$ T cells (33). In addition, IL-1 is a potent modulator of insulin secretion and at high concentrations is cytotoxic to β-cells (34, 35). Thus, as expected, migrating APC and the subsequent activation of peripheral H-$2K^b$-specific T cells did not correlate with the susceptibility to rejection of islet grafts. Therefore, the small graft size, sensitivity to cytokines, and requirement for revascularization are likely to be the main contributors to the sensitivity of islet allografts to rejection.

Equally interesting is the relative resistance of heart allografts to rejection despite the marked activation of the alloreactive T cell population (Tables I and II). This resistance to rejection, compared with islet and skin grafts, may be simply a reflection of the larger size of the heart. Sun et al. (36) demonstrated that increasing the mass of transplanted organs prolonged graft survival. Single heart or kidney transplants in the rat strains investigated were acutely rejected, whereas simultaneous transplantation of two hearts and two kidneys into a single recipient resulted in prolonged survival of all the grafted organs.

Alternatively, vascularized grafts may also be afforded some protection from alloreactive T cells due to the expression of Fas ligand (FasL) by vascular endothelial cells (37). In this situation, when an activated T cell expressing Fas comes into contact with the vascular endothelium expressing FasL, the activated T cell may be induced to undergo apoptosis. Although the in vivo relevance of FasL expression by graft endothelial cells is unknown, it may be an important factor in down-regulating T cell-mediated rejection of vascularized grafts.

It has been well documented that there is greater $CD8^+$ T cell dependency on $CD4^+$ T cells during cardiac allograft rejection than with skin and islet allografts. We would argue that because heart allografts are more resistant to rejection than skin or islet grafts, either the response would need to be more aggressive per cell or a greater number of cells would need to respond (as evidenced in Table I) to cause rejection. $CD4^+$ T cells therefore may be required to a greater extent in heart allograft rejection to provide growth factors such as IL-2 to either enable $CD8^+$ T cells with low affinity for alloantigen to respond optimally or to increase the ability of $CD8^+$ T cells to induce effector responses. However, when we have performed an analysis of the response of the transgenic $CD8^+$ T cells to a cardiac allograft in the presence or absence of $CD4^+$ T cells, no difference was noted in the response; we therefore favor the former explanation.

In conclusion, our results suggest that islet and skin allografts lie at the "difficult" end of the transplantation hierarchy due to the ability of very small numbers of alloreactive T cells to orchestrate their destruction in vivo, rather than the presence of tissue-specific Ags or the intrinsic ability of these nonvascularized grafts to stimulate T cells. The need for rigorous control of the alloreactive T cell population to ensure engraftment of skin and islet allografts has potential implications for the design of tolerance induction strategies and the need to tailor immunosuppressive drug regimens for these vulnerable allografts.

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References

1. Nash, J. R., M. Peters, and P. R. F. Bell. 1977. Comparative survival of pancreatic islets, heart, kidney, and skin allografts in rats, with and without enhancement. *Transplantation 24:70.*
2. Davis, W. C., I. F. C. McKenzie, and R. W. Melvold. 1980. A comparison of skin and heart graft rejection patterns in *H-2* mutant mice. *Transplantation 29:189.*
3. Mason, A., P. L. Mottram, A. Mirisklavos, G. J. Clurne, and T. E. Mandel. 1988. A comparison of cyclosporine, donor-specific transfusion, and antilymphocyte serum suppression of skin, heart, and fetal pancreatic islet allograft rejection in mice. *Transplantation 45:1155.*
4. Zheng, Z., L. Zhu, D. Quan, B. Garcia, N. Ozcay, J. Duff, C. Stiller, A. Lazarovits, D. Grany, and R. Zhong. 1996. Pattern of liver, kidney, heart, and intestine allograft rejection in different mouse strain combinations. *Transplantation 62:1267.*
5. Steinmuller, D. 1998. The enigma of skin allogaft rejection. *Transplant. Rev. 12:42.*
6. Calne, R. Y., R. A. Sells, J. R. Pena, D. R. Davis, P. R. Millard, B. M. Herbertson, R. M. Binns, and D. A. L. Davies. 1969. Induction of immunological tolerance by porcine liver allografts. *Nature 223:472.*
7. Medawar, P. B. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits. *J. Anat. 78:176.*
8. Cho, S. I., F. S. Marcus, and S. I. Kountz. 1972. A new model for study of allograft rejection in the rat: use of skin with an intact vascular pedicle. I. Effect of vascularity on allograft survival. *Transplantation 13:486.*
9. Boyse, E. A., and L. J. Old. 1968. Loss of skin allograft tolerance by chimeras. *Transplantation 6:619.*
10. Steinmuller, D., and J. S. Lofgreen. 1977. The "Sk" (skin-specific) rejection phenomenon as evidence of the natural role of acquired tolerance. *Transplant. Proc. 9:1385.*
11. Tyler, J. D., and D. Steinmuller. 1982. Establishment of cytolytic T lymphocyte clones to epidermal alloantigen *Epa-1. Transplantation 34:140.*
12. Larsen, C. P., R. M. Steinman, M. Witmr-Pack, D. F. Hankins, P. J. Morris, and J. M. Austyn. 1990. Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med. 172:1483.*
13. Richters, C. D., E. V. Gelderop, J. S. D. Pont, M. J. Hoekstra, R. W. Kreis, and E. W. A. Kamperdijk. 1999. Migration of dendritic cells to the draining lymph node after allogeneic or congenic rat skin transplantation. *Transplantation 67: 828.*
14. Sun, J., A. G. R. Sheil, C. Wang, L. Wang, K. Rokahr, A. Sharland, S. Jung, L. Li, G. W. McCaughan, and G. A. Bishop. 1996. Tolerance to rat liver allografts. IV. Acceptance depends on the quantity of donor tissue and on donor leukocytes. *Transplantation 62:1725.*
15. Spoonas, A. M., P. D. Tomlinson, J. Antoniou, N. Auphan, C. Langlet, B. Malissen, A.-M. Schmitt-Verhulst, and A. L. Mellor. 1994. Induction of tolerance to self MHC class I molecules expressed under the control of milk protein or β-globulin gene promoters. *Int. Immunol. 6:277.*
16. Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann. 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature 312:548.*

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17. Hua, C., C. Boyer, A. Guimezanes, F. Albert, and A.-M. Schmitt-Verhulst. 1986. Analysis of T cell activation requirements with the use of alloantigens or anti-clonotypic monoclonal antibody. *J. Immunol. 136:1927.*
18. Jones, N. D., A. van Maurik, M. Hara, B. J. Gilot, P. J. Morris, K. J. Wood. 1999. T cell activation, proliferation and memory following cardiac transplantation in vivo. *Ann. Surg. 229:570.*
19. Corry, R. J., H. J. Winn, and P. S. Russell. 1973. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation 16:343.*
20. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods 171:131.*
21. Wells, A. D., H. Gudmundsdottir, and L. A. Turka. 1997. Following the fate of individual T cells throughout activation and clonal expansion: signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J. Clin. Invest. 100:3173.*
22. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. A. P. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med. 184:923.*
23. Bushell, A. R., P. J. Morris, and K. J. Wood. 1995. Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on $CD4^+$ T cell regulation during the induction phase of the response. *Eur. J. Immunol. 25:2643.*
24. Sayegh, M. H., X. Zheng, C. Magee, W. W. Hancock, and L. A. Turka. 1997. Donor antigen is necessary for the prevention of chronic allograft rejection in CTLA4Ig-treated murine cardiac allograft recipients. *Transplantation 64:1646.*
25. Nakafusa, Y., J. A. Goss, T. Mohanakumar, and M. W. Flye. 1993. Induction of donor-specific tolerance to cardiac but not skin or renal allografts by intrathymic injection of splenocyte alloantigen. *Transplantation 55:877.*
26. Ilano, A. L., M. V. McConnell, K. E. Gurley, A. Spinelli, N. W. Pearce, and B. M. Hall. 1989. Cellular basis of allograft rejection in vivo. V. Examination of the mechanisms responsible for the differing efficacy of monoclonal antibody to $CD4^+$ T cell subsets in low- and high-responder rat strains. *J. Immunol. 143: 2828.*
27. Pearson, T. C., C. R. Darby, A. R. Bushell, L. J. West, P. J. Morris, and K. J. Wood. 1993. The assessment of transplantation tolerance induced by anti-CD4 monoclonal antibody in the murine model. *Transplantation 55:361.*
28. Barclay, A. N., and D. W. Mason. 1982. Induction of Ia antigen in rat epidermal cells and gut epithelium by immunological stimuli. *J. Exp. Med. 156:1665.*
29. Berg, E. L., T. Yoshino, L. S. Rott, M. K. Robinson, R. A. Warnock, T. K. Kishimoto, L. J. Picker, and E. C. Butcher. 1991. The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. *J. Exp. Med. 174:1461.*
30. Kupiec-Weglinski, J. W., A. J. Coito, and L. V. D. Water. 1997. Extracellular matrix proteins and their interactions with the cellular repertoire of transplant recipients. *Transplant. Proc. 29:2601.*
31. Parr, E. L., K. J. Lafferty, K. M. Bowen, and I. F. C. McKenzie. 1980. H-2 complex and Ia antigens on cells dissociated from mouse thyroid glands and islets of Langerhans. *Transplantation 30:142.*
32. Markmann, J. F., J. D. Jacobson, H. Kiumura, M. Choti, W. F. Hickey, I. J. Fox, W. K. Silvers, C. F. Barker, and A. Naji. 1989. Modulation of the major histocompatibility complex antigen and the immunogenicity of islet allografts. *Transplantation 48:478.*
33. Hao, L., Y. Wang, R. G. Gill, F. G. L. Rosa, D. W. Talmage, and K. J. Lafferty. 1990. Role of lymphokine in islet allograft rejection. *Transplantation 49:609.*
34. Bendtzen, K., T. Mandrup-Poulsen, J. Nerup, J. H. Nielsen, C. A. Dinarello, and M. Svenson. 1986. Cytotoxicity of human *p*I 7 interleukin-1 for pancreatic islets of Langerhans. *Science 232:1545.*
35. Ling, Z., P. A. I. Veld, and D. G. Pipeleers. 1993. Interactions of interleukin-1 with islet β-cells: distinction between indirect, aspecific cytotoxicity and direct, specific functional suppression. *Diabetes 42:56.*
36. Sun, J., G. W. McCaughan, N. D. Gallagher, A. G. R. Sheil, and G. A. Bishop. 1995. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation 60:233.*
37. Sata, M., and K. Walsh. 1998. TNFα regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. *Nat. Med. 4:415.*

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