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The Allograft Defines the Type of Rejection (Acute versus Chronic) in the Face of an Established Effector Immune Response

Geetha Chalasani,* Qi Li,* Bogumila T. Konieczny,‡ Lonnette Smith-Diggs,* Barbara Wrobel,‡ Zhenhua Dai,* David L. Perkins,§ Fady K. Baddoura,‡ and Fadi G. Lakkis 2

Transplanted organs fail due to either acute or chronic rejection. The prevailing view is that the nature or magnitude of the recipient’s immune response to donor Ags determines the type of rejection. In variance with this view, we show in this study that the status of the graft itself plays a dominant role in defining the type of rejection even in the face of an established alloimmune response. Using adoptive transfer mouse models in which the graft is exposed to a constant number of effector lymphocytes, we found that newly transplanted heart allografts are rejected acutely, while healed-in allografts undergo chronic rejection. Acute rejection of healed-in allografts was largely recapitulated by subjecting the grafts to ischemia-reperfusion injury similar to that present in newly transplanted organs. Ischemia-Reperfusion injury altered the outcome of rejection by enhancing the accumulation of effector T cells within the graft. The accumulation of effector T cells in the graft was dependent on the presence of both ischemia-reperfusion injury (inflammation) and alloantigens. These findings demonstrate that the graft plays a dominant role in shaping the outcome of rejection by controlling the trafficking of effector T cells. The Journal of Immunology, 2004, 172: 7813–7820.

The host’s immune response to donor Ags leads to two types of allograft rejection that differ histologically and clinically. Acute rejection is characterized by an intense cellular and humoral attack on donor tissue that results in rapid graft loss. Chronic rejection in contrast is a more insidious process characterized by obliterator vasculopathy and parenchymal fibrosis that lead to progressive graft failure (1). The risk of acute rejection in humans is highest in the early posttransplantation period, but declines dramatically over the ensuing months. In contrast, the risk of chronic rejection increases gradually and becomes a significant cause of graft loss after the first year of transplantation.

The mechanisms that underlie the divergent histological and clinical characteristics of acute and chronic rejection are not well understood. The prevailing view is that the nature or magnitude of the recipient’s immune response to donor Ags determines the type of rejection that ensues. It is hypothesized that host exposure to intact MHC alloantigens displayed on donor APCs (direct allorecognition) results in acute rejection because of substantial expansion of T cells of multiple specificities, while host exposure to donor alloantigenic protein and presented by host APCs (indirect allorecognition) leads to the activation of a limited T cell repertoire with restricted ability to recognize graft targets, and thus, chronic instead of acute rejection (2–5). This hypothesis is supported by experiments in which blocking the direct allorecognition pathway or, alternatively, limiting the size of the alloreactive T cell clone shifted the rejection process from an acute to a chronic form (6–9). Furthermore, the abundance of donor APCs in newly transplanted allografts correlates with the high risk of acute rejection early after transplantation, while their gradual replacement with host APCs over time ushers in the period of chronic rejection (10, 11).

In addition to the transition from direct to indirect allorecognition during the afferent (sensitization) phase of the immune response, long-term surviving grafts undergo adaptive changes that protect them against the effector arm of the response (12). Graft adaptation was originally described by Woodruff and Woodruff (13), who found that thyroid allografts parked in the anterior eye chamber of guinea pigs are rejected if the recipients receive a simultaneous thyroid allograft under the skin, but become resistant to rejection if s.c. grafting is delayed by several weeks. Subsequent experiments provided evidence that skin allografts also become less vulnerable to rejection with time (14, 15). Despite these findings, the contribution of graft adaptation to the long-term survival of transplanted organs remains a matter of debate (16, 17), and its relative importance in defining the pattern of rejection (acute vs chronic) after the alloimmune response has been initiated is unclear. Using adoptive transfer models in which the graft is exposed to a constant number of effector lymphocytes, we demonstrate in this study that newly transplanted heart allografts are rejected acutely, while healed-in allografts survive long-term, but undergo chronic rejection. We also provide evidence that resolution of ischemia-reperfusion injury is a central mechanism of graft adaptation that protects vascularized organ transplants against acute rejection by limiting the accumulation of effector T cells within the graft.

Materials and Methods
 Murine cardiac transplantation

All heart donors were 6- to 8-wk-old C3H (H-2k) or BALB/c (H-2b) mice, and all recipients were 6- to 8-wk-old C57BL/6 (H-2b) mice. All mice were

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Role of allograft in rejection

Isolation of graft-infiltrating cells

Histological analysis

Gene expression analysis by real-time PCR

Results

The status of the graft determines the type of rejection

References

Abbreviations used in this paper: aly, al amyloidotic; C10, cycle threshold; Mig, monokine induced by IFN-γ; MT, Masson-Trichrome.
Graft adaptation plays a dominant role in de- 
jecting a healed-in allograft to ischemia-reperfusion injury reca-
mechanism of graft adaptation. We therefore asked whether resub-
ment than newly transplanted grafts (31), raising the possibility 
Flmia-reperfusion, constitute a much less proin-
Healed-in grafts, which have recovered from the sequelae of isch-
acute rejection is recapitulated by subjecting a healed-in 
allograft to ischemia-reperfusion injury.

Activated T cells fail to accumulate in healed-in allografts

Allograft rejection is mediated by activated T cells that home to 
the transplanted organ. Therefore, we hypothesized that a healed-in allograft is protected from acute rejection.
because of reduced homing or accumulation of activated T cells in the graft. To test this hypothesis, we transferred activated T cells, harvested from CD8 TCR-transgenic 2C\textsuperscript{vif−/−} (H-2\textsuperscript{b}) mice 4 days after immunization with BALB/c (H-2\textsuperscript{d}) splenocytes, to aly/aly-spleen recipients of BALB/c cardiac allografts either 2 or 50 days following transplantation. Approximately 30–50% of CD8 T cells in 2C\textsuperscript{vif−/−} mice express the transgenic TCR specific to the MHC class I Ag L\textsuperscript{d} present on BALB/c cells and are detected with the clonotypic Ab 1B2. Cardiac grafts were harvested either 6 or 24 h after T cell transfer, and infiltrating T cells were phenotyped and quantitated. As shown in Fig. 4\textit{a}, the total number of infiltrating T cells 6 h post-cell transfer was comparable in newly transplanted (day 2) and healed-in cardiac (day 50) allografts. However, at 24 h, the T cell number had increased by ~10-fold in newly transplanted hearts, but declined by 3- to 4-fold in healed-in hearts. This observation was true for both CD4 and CD8 T cells and for CD8\textsuperscript{−}1B2\textsuperscript{+} (Ag-specific) T cells that infiltrated the grafts (data not shown). The vast majority of infiltrating T cells had an activated (CD44\textsuperscript{high}) phenotype, and the higher number of activated T cells present in newly transplanted hearts could not be attributed to increased proliferation, as CFSE dilution profiles were comparable in day 2 and 50 grafts (histograms not shown). We then asked whether resubjecting healed-in allografts to ischemia-reperfusion injury recapitulates the accumulation of activated T cells observed in newly transplanted hearts. To do so, we retransplanted BALB/c cardiac allografts that had been parked in aly/aly-spleen mice for 50 days into new aly/aly-spleen recipients and transferred activated T cells 2 days later. As shown in Fig. 4\textit{a}, activated T cells accumulated in retransplanted hearts to the same extent that they would have if the hearts had been newly transplanted. These findings indicate that ischemia-reperfusion injury is a critical determinant of activated T cell accumulation in transplanted organs.

Because T cell entry into nonlymphoid tissues can occur in the context of a nonspecific, Ag-independent response to ischemia-

FIGURE 2. Healed-in allografts undergo chronic rejection in immunosuppressed wild-type hosts following the transfer of activated T cells. \textit{a}, Cardiac allografts are acutely rejected in immunosuppressed wild-type hosts if activated T cells are transferred 2 days after transplantation (C, n = 6). In contrast, acute rejection is not observed if activated T cells are transferred 50 days after transplantation (□, n = 5). Control mice that did not receive any exogenous T cells did not reject their grafts (●, n = 5). \textit{b} and \textit{c}, MT (×200) and Verhoess Van Giesen (×400) staining of cardiac allograft tissue removed 50 days after T cell transfer in immunosuppressed mice that received activated T cells 50 days following transplantation. Note diffuse fibrosis (\textit{b}) and vascular intimal thickening (\textit{c}, arrow), which are consistent with chronic rejection. \textit{d} and \textit{e}, MT (×100) and Verhoess Van Giesen (×400) staining of cardiac allograft tissue removed 100 days after transplantation from immunosuppressed mice that did not receive exogenous T cells. Note lack of significant fibrosis (\textit{d}) and normal vessel wall morphology (\textit{e}, arrow).
H&E staining of retransplanted cardiac allograft tissue exposed to exogenous activated T cells and removed at the time of cessation of contractions shows splenectomized aly/aly tractants persists in the transplanted organ even after sufficient time is allowed for the graft to heal. The data are also consistent with our finding that T cells home in equal numbers to day 2 and day 50 allografts early (at 6 h) after adoptive transfer (Fig. 4a).

We then asked whether newly transplanted and healed-in allografts differ in the expression of molecules required for arresting activated T cells that have homed to the graft. To address this question, we performed oligonucleotide microarray analysis on cardiac allografts removed either 2 or 50 days after transplantation and before adoptive T cell transfer. Differential gene expression analysis revealed that mRNA species corresponding to 13 extracellular matrix/cell adhesion proteins were significantly less abundant in day 50 than day 2 allografts (Table I). Many of these proteins, particularly laminin and collagens types I and IV, bind to integrins on activated T cells and cause their arrest within inflamed tissues (33, 34). No extracellular matrix protein mRNA species was up-regulated in day 50 relative to day 2 grafts, and no difference in chemokine mRNA expression was detected between the two time points, except for Mig mRNA, which was 3-fold elevated in day 50 grafts. Likewise, there were no significant differences between day 2 and day 50 allografts in the expression of integrin receptor ligands required for the firm adhesion of T cells to the endothelium. Taken together, our gene expression data suggest that diminished accumulation of T cells in healed-in allografts could be attributed at least in part to reduced arrest of effector T cells within the graft.

**Discussion**

We have provided direct evidence that the status of the graft plays a dominant role in both allograft survival and the type of rejection that ensues. When exposed to identical populations of activated T cells, newly transplanted heart allografts were rejected acutely, while healed-in grafts survived long-term, but developed histologic manifestations of chronic rejection. This finding was confirmed in two independent adoptive transfer models in which the status of the graft was varied while keeping the alloimmune response constant. In the first model, vascularized cardiac allografts were parked in mice that lack secondary lymphoid organs (aly/aly-spleen) for either 2 days (newly transplanted) or >50 days (healed-in) before adoptively transferring wild-type, allosensitized T cells. In the second model, allografts were parked in wild-type mice in which primary immunity was inhibited by agents that block T cell costimulation. Allograft rejection in both models is mediated exclusively by the adoptively transferred, Ag-experienced T cells, and not by endogenous lymphocytes (20, 24, 29, 30). The principal advantage of these models is that they allow one to investigate how graft adaptation, defined as resistance of the graft to the effector arm of the immune response, shapes the outcome of rejection independent of alterations in afferent immunity that occur after transplantation. The finding that healed-in allografts underwent chronic instead of acute rejection in both aly/aly-spleen and wild-type hosts makes it unlikely that our results are biased by the immunologic abnormalities present in aly mice (26, 27). Moreover, the wild-type model simulates the usual clinical situation whereby transplant recipients are immunosuppressed to
Prevent acute rejection, yet develop chronic rejection later on. Therefore, our data provide direct evidence that adaptive changes that occur in the graft itself play a dominant role in defining the type of rejection that occurs.

The adaptive mechanisms that account for graft resistance to immune attack are not completely understood. Proposed mechanisms include gradual replacement of graft endothelium by host endothelial cells, reduced expression of MHC Ags in the graft, and resistance of graft cells to apoptosis (12, 35). In this study, we addressed the general hypothesis that ischemia-reperfusion injury that occurs at the time of transplantation favors acute rejection and, conversely, the resolution of ischemia-reperfusion injury over time sways rejection toward a chronic form. To test this hypothesis, we resubjected healed-in allografts to ischemia-reperfusion injury by retransplanting them into new hosts before transferring allosensitized T cells. We found that retransplantation recapitulates acute rejection, albeit partially, indicating that resolution of ischemia-reperfusion injury is an important, but not the only mechanism of graft adaptation. Other changes that occur in long-term surviving grafts, such as the replacement of donor APCs with host APCs and up-regulation of antiapoptotic genes, may protect transplanted tissues against immunologic attack.

Ischemia-Reperfusion injury is a complex inflammatory process that encompasses up-regulation of adhesion molecules, induction of inflammatory mediators, and activation of the complement system (36). Because these events participate in leukocyte migration into peripheral tissues, we asked in this study whether ischemia-reperfusion injury influences the type of rejection by modulating activated T cell entry into the transplanted organ. We found that the status of the allograft (newly transplanted vs healed-in) is a critical determinant of T cell homing to the graft. Interestingly, the number of T cells that entered the allograft at an early time point (6 h after lymphocyte transfer) appeared to be independent of the graft status, while T cell accumulation observed 18 h later occurred only if the graft had been subjected to ischemia-reperfusion injury. This finding suggests that the accumulation, rather than initial homing, of activated T cells is dependent on the presence of inflammation within the target tissue. Moreover, T cell accumulation was also dependent on the presence of foreign Ags, as activated T cells failed to accumulate in newly transplanted syngeneic grafts. These findings are relevant not only to transplantation, but also to the migration of Ag-experienced T cells to sites of infection and autoimmunity. The adoptive transfer models described in this work, therefore, are well suited for analyzing in more depth the

Table I. Extracellular matrix protein mRNA species down-regulated in healed-in allografts

<table>
<thead>
<tr>
<th>Gene Accession No.</th>
<th>Description</th>
<th>Fold Reduction (day 50 vs day 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L02918</td>
<td>Procollagen type V, α2</td>
<td>4.8</td>
</tr>
<tr>
<td>XS6304</td>
<td>Tenasin C</td>
<td>4</td>
</tr>
<tr>
<td>AF011450</td>
<td>Procollagen type XV</td>
<td>3.6</td>
</tr>
<tr>
<td>AF064794</td>
<td>Collagen type V1, α3</td>
<td>3.4</td>
</tr>
<tr>
<td>U03419</td>
<td>Procollagen type 1, α1</td>
<td>3.4</td>
</tr>
<tr>
<td>X58251</td>
<td>Procollagen type I, α2</td>
<td>3.1</td>
</tr>
<tr>
<td>M15832</td>
<td>Procollagen type IV, α1</td>
<td>2.9</td>
</tr>
<tr>
<td>U69176</td>
<td>Laminin α4</td>
<td>2.7</td>
</tr>
<tr>
<td>X04647</td>
<td>Procollagen type IV, α2</td>
<td>2.6</td>
</tr>
<tr>
<td>Z18272</td>
<td>Procollagen type V1, α2</td>
<td>2.4</td>
</tr>
<tr>
<td>U12147</td>
<td>Laminin α2</td>
<td>2.3</td>
</tr>
<tr>
<td>AB009993</td>
<td>Collagen type V, α1</td>
<td>2.1</td>
</tr>
<tr>
<td>X05212</td>
<td>Laminin B1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Three samples were analyzed per group. Values shown are average fold reduction determined by differential gene expression analysis of microarray data.
factors that govern the accumulation of effector and memory T cells in nonlymphoid tissues.

Diminished T cell accumulation in healed-in allografts could be due to either decreased arrest and survival of T cells or a reduction in T cell infiltration after the initial homing stage. Chemokine and adhesion molecule gene expression analysis reported in this study does not definitively differentiate between these possibilities, but suggests that diminished T cell accumulation in healed-in allografts is caused by reduced arrest of activated T cells due to decreased expression of extracellular cell matrix proteins involved in cell adhesion. Additional analysis is needed to exclude the possibility that following adoptive T cell transfer, infiltrating cells increase chemokine expression and lead to further T cell accumulation in newly transplanted, but not healed-in allografts. Moreover, it is possible that the final outcome of rejection (acute vs chronic) is not simply determined by the number of activated T cells that accumulate in the graft, but also by the locale of T cell accumulation (37). For example, it is conceivable that vascular T cell accumulation leads to chronic rejection, while parenchymal T cell localization leads to acute rejection. This hypothesis remains to be tested.

Bingaman et al. (31) observed that healed-in skin or vascularized cardiac allografts parked for 50 days in Reg−/− lymphocyte-deficient recipients undergo acute rejection upon the transfer of exogenous T cells. In contrast, we found in this study that healed-in vascularized allografts undergo chronic rejection. The discrepancy between our results and theirs could be attributed to a fundamental difference in the models used. In the model used by Bingaman et al., the homeostatic proliferation of T cells transferred to lymphocyte-deficient Reg−/− mice could make these cells more aggressive, leading to acute instead of chronic rejection (38). In contrast, T cells transferred to aly/aly-spleen mice do not undergo significant homeostatic proliferation, as these mice are T cell replete (39).

Our study differs from previous investigations into the role of the graft in the rejection process in that we used experimental models in which the afferent and effenter limbs of the immune response are separated. Earlier studies by Lechler and Batchelor and by Rosengard and colleagues (10, 11) clearly demonstrated the importance of the afferent arm of the immune response, specifically that of passenger APCs, in determining the survival of a transplanted organ and the type of rejection that ensues. In this study, we focused on how the graft modulates the effenter limb of the immune response and found, contrary to prevailing view, that the graft itself plays a dominant role in defining the type of rejection even after a full-blown alloimmune response has been initiated. Our finding may explain why many patients who stop their immunosuppression several years after transplantation develop chronic instead of acute rejection, and may provide insights into harnessing graft adaptation to achieve long-term allograft acceptance.

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