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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 alloantigens processed 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 chambers 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-2d) mice, and all recipients were 6- to 8-wk-old C57BL/6 (H-2b) mice. All mice were
purchased from The Jackson Laboratory (Bar Harbor, ME), except for alymphoplastic (aly/aly) and al/+ mice, which were purchased from Clea Japan (Tokyo, Japan). Heterotopic transplantation of primarily vascularized cardiac allografts was performed, as described (18). In this model, the pulmonary artery and ascending aorta of the heart graft are anastomosed to the recipient’s inferior vena cava and abdominal aorta, respectively. Replantation of heart grafts was performed by anastomosis of donor (first recipient) aortic and inferior vena cava to the second recipient’s aorta and inferior vena cava, respectively. The retransplantation procedure took ~80 min to complete with a total cold ischemia time of 20 min and warm ischemia time of 35 min. Mice were monitored daily. Rejection was defined as the cessation of palpable heart beat, at which time the graft was harvested for histological analysis. Wild-type allograft recipients received the following immunosuppression to prevent primary T cell activation: 0.25 mg of MR1 (anti-CD40 ligand mAb) and 0.25 mg of CTLA4-Ig (both generated in the laboratory of C. Larsen, Emory University, Atlanta, GA) i.p. on days 0, 2, 4, and 6 relative to heart transplantation.

T cell preparation before adoptive transfer

T cells were enriched from mouse lymph nodes and spleen by nonadherence to nylon wool (Polysciences, Warrington, PA) (19). Cell suspensions were subjected to hypotonic lysis of RBCs before passage on nylon wool. Final purity of the T cell population ranged between 75 and 85%. A total of \(4 \times 10^6\) enriched cells was transferred i.v. to each heart graft recipient at the indicated time points. To obtain activated lymphocytes, mice were immunized with \(2 \times 10^7\) allogeneic spleen cells i.p. and s.c. 4 days before harvesting the lymph nodes and spleen.

Histological analysis

Fixed, paraffin-embedded cardiac tissue was stained with H&E, Masson-Trichrome (MT), Verhoesen Van Giesen elastin stain, or anti-mouse CD3 (BD PharMingen, San Diego, CA), followed by peroxidase-conjugated secondary Ab. All analyses were performed by the pathologist (F.K.B.), who was blinded to the experimental protocol.

Isolation of graft-infiltrating cells

Heart allografts were perfused in situ with heparinized 0.9% saline, resected allografts of donor (newly transplanted vs healed-in) in the absence of a secondary lymphoid tissues, this mutation is associated with immunologic abnormalities that could potentially influence allograft rejection mediated by exogenous effector T cells (26, 27). With immunologic abnormalities that could potentially influence allograft rejection mediated by exogenous effector T cells (26, 27), except allografts inde-

Results

The status of the graft determines the type of rejection

To investigate the extent to which the allograft influences the outcome of rejection, we used a heart transplantation model in which the status of the graft can be manipulated independent of the alloimmune response. In this model, we transplanted vascularized C3H (H-2k) hearts to splenectomized alymphoplastic mice (aly/aly-spleen; H-2b), which lack secondary lymphoid tissues and accept allografts indefinitely (23, 24). Exogenous activated T cells, obtained from B6 (H-2b) mice 4 days after immunization with C3H splenocytes, were then transferred to aly/aly-spleen recipients either 2 or 70 days after transplantation to precipitate allograft rejection (20, 24). Allografts were harvested on the day of clinical rejection (cessation of heart contractions) or 100 days after T cell transfer if the heart continued to contract. By varying the day on which activated T cells were transferred, this model allowed us to alter the status of the graft (newly transplanted vs healed-in), while keeping the afferent immune response (exogenous activated T cells) constant.

As shown in Fig. 1a, the transfer of activated T cells to aly/aly-spleen mice 2 days after transplantation invariably resulted in clinical rejection (all allografts stopped contracting within 23 days after cell transfer). In contrast, the transfer of an identical population of activated T cells 70 days after transplantation failed to precipitate clinical rejection in any of the aly/aly-spleen recipients (all allografts were still contracting 100 days after cell transfer). Rejection was not observed in control mice that received naive T cells either 2 or 70 days after transplantation (Fig. 1a). Histologic examination of cardiac allografts confirmed that the transfer of activated T cells 2 days posttransplantation resulted in high-grade acute cellular rejection. In contrast, delaying the transfer of activated T cells until day 70 posttransplantation led to a distinct form of allograft pathology characterized by the presence of diffuse interstitial fibrosis, obliterator vasculopathy, and perivascular T cell infiltrates (Fig. 1, b–e). Histomorphometric analysis revealed that the obliterator vasculopathy was due to intimal thickening (Fig. 1f), a hallmark of the vascular lesion associated with chronic rejection (1). These data therefore indicate that, in the face of a constant effector immune response, a newly transplanted allograft undergoes acute rejection, while a healed-in allograft develops chronic rejection.

The results shown so far were generated in aly mice that harbor a mutation in the NF-kB-inducing kinase (25). In addition to the absence of secondary lymphoid tissues, this mutation is associated with immunologic abnormalities that could potentially influence allograft rejection mediated by exogenous effector T cells (26, 27). To rule out this possibility, we transferred activated T cells to wild-type recipients either 2 or 50 days after heart transplantation. The recipients were treated at the time of transplantation with CTLA4-Ig and MR1 to block the CD28/B7 and CD40/CD40 ligand costimulatory pathways, respectively. Because these pathways are required for the activation of naive, but not Ag-experienced T cells (28–30), this model allowed us to vary the status of the allograft (newly transplanted vs healed-in) in the absence of a

3 Abbreviations used in this paper: aly, al lymphoplastic; C\(_T\), cycle threshold; Mig, monokine induced by IFN-\(\gamma\); MT, Masson-Trichrome.
significant host primary immune response. In addition, this model simulates the normal clinical setting in which graft adaptation and allograft rejection occur in immunosuppressed patients. As shown in Fig. 2a, mice that received activated T cells on day 2 rejected their allografts promptly, while those that received activated T cells on day 50 did not reject throughout the observation period (total of 100 days posttransplantation). As in the allograft model, transferring activated T cells on day 2 led to acute rejection, while day 50 transfer caused chronic allograft rejection (Fig. 2, b and c). Control mice that were treated with CTLA4-Ig and MR1, but did not receive activated T cells, did not reject their allografts and had minimal vasculopathy on day 100 posttransplantation (Fig. 2, d and e). These results confirm, in a clinically relevant model, that graft adaptation plays a dominant role in defining the type of rejection.

Acute rejection is recapitulated by subjecting a healed-in allograft to ischemia-reperfusion injury

Healed-in grafts, which have recovered from the sequelae of ischemia-reperfusion, constitute a much less proinflammatory environment than newly transplanted grafts (31), raising the possibility that resolution of ischemia-reperfusion injury is an important mechanism of graft adaptation. We therefore asked whether resubjecting a healed-in allograft to ischemia-reperfusion injury recapitulates the acute rejection process. To answer this question, we retransplanted cardiac allografts that had been parked for 50 days in aly/aly-spleen hosts to a second set of aly/aly-spleen mice and transferred activated T cells 2 days after the retransplantation procedure. As shown in Fig. 3a, all retransplanted hearts underwent clinical rejection (all allografts stopped contracting by day 62 after T cell transfer; median survival time, 20 days, p < 0.05). Histologic examination revealed that retransplanted hearts harvested from recipients that received activated T cells failed due to acute cellular rejection (Fig. 3b), but that these grafts also developed elements of chronic rejection (vascular intimal thickening) (Fig. 3c). Healed-in hearts retransplanted into aly/aly-spleen mice that did not subsequently receive activated T cells continued to contract for the full duration of the experiment (Fig. 3a). Therefore, resolution of ischemia-reperfusion injury is an important mechanism of graft adaptation that protects against acute rejection.

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...
FIGURE 2. Healed-in allografts undergo chronic rejection in immunosuppressed wild-type hosts following the transfer of activated T cells. a, Cardiac allografts are acutely rejected in immunosuppressed wild-type hosts if activated T cells are transferred 2 days after transplantation (□, 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). b and c, MT (×200) and Verhooss 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 (b) and vascular intimal thickening (c, arrow), which are consistent with chronic rejection. d and e, MT (×100) and Verhooss 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 (d) and normal vessel wall morphology (e, arrow).

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+/- (H-2b) mice 4 days after immunization with BALB/c (H-2d) 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+/- mice express the transgenic TCR specific to the MHC class I Ag Ld 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. 4a, 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+1B2+ (Ag-specific) T cells that infiltrated the grafts (data not shown). The vast majority of infiltrating T cells had an activated (CD44high) 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. 4a, 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-reperfusion injury, we quantitated the accumulation of activated exogenous T cells in syngeneic cardiac grafts and of endogenous (aly) T cells in both syngeneic and allogeneic grafts. We found that activated T cells home to newly transplanted syngeneic grafts at 6 h, but do not accumulate further at 24 h (Fig. 4b). A similar population of activated T cells did not infiltrate healed-in syngeneic grafts (c500 cells/graft at either 6 or 24 h) (Fig. 4b). These data suggest that the accumulation of activated T cells in nonlymphoid tissues is dependent on the presence of both inflammation and foreign Ags. The accumulation of naive, endogenous (aly) T cells in newly transplanted heart allografts was transient, independent of foreign Ag (equal numbers of T cells were isolated from allogeneic and syngeneic grafts), and smaller in magnitude than the accumulation of activated exogenous T cells observed in allogeneic grafts (Fig. 4c).

Chemokine and adhesion molecule gene expression in newly transplanted and healed-in allografts

To begin to address the mechanisms responsible for the difference in effector T cell accumulation between newly transplanted and healed-in allografts, we compared the expression of key molecules involved in effector T cell homing (chemotraction) and retention (arrest) in nonlymphoid tissues between day 2 and day 50 cardiac allografts placed in aly/aly-spleen recipients. Grafts were harvested before the transfer of activated T cells, and mRNA expression was analyzed by real-time quantitative PCR and by hybridization to murine oligonucleotide microarrays. As shown in Fig. 5, real-time PCR analysis demonstrated that the mRNA levels of four chemokines involved in activated T cell chemotraction to transplanted organs (IFN-inducible protein-10, monokine induced by IFN-γ (Mig), IFN-inducible T-cell α chemoattractant, and lymphotactin) (3, 32) were significantly elevated in both healed-in and newly transplanted allografts compared with native donor hearts. Although IFN-inducible protein-10 and IFN-inducible T-cell α
Ischemia-Reperfusion injury of healed-in allografts recapitulates acute rejection. a. Cardiac allografts that had been parked for 50 days in splenectomized aly/aly hosts, retransplanted to a second set of splenectomized aly/aly mice, and exposed to exogenous activated T cells 2 days after the retransplantation procedure underwent acute rejection (*, n = 4). In contrast retransplanted cardiac allografts that were not exposed to exogenous activated T cells did not undergo acute rejection (**, n = 3). The fate of primary cardiac allografts in splenectomized aly/aly hosts following the transfer of activated T cells either 2 days (○, n = 6) or 70 days (□, n = 6) after transplantation is shown for comparison (same groups are also depicted in Fig. 1a). b and c. H&E staining of retransplanted cardiac allograft tissue exposed to exogenous activated T cells and removed at the time of cessation of contractions shows combined elements of both acute and chronic rejection. The straight arrow (b) points to the diffuse mononuclear infiltrate characteristic of acute cellular rejection, while the dashed arrows (b and c) point to a vessel with thickened intima suggestive of chronic allograft vasculopathy.

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

chemoattractant mRNA levels were lower in day 50 than day 2 allografts, those of Mig and lymphotactin were higher in day 50 grafts. These data indicate that the expression of T cell chemoattractants 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.
prevented 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 resected 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>
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<th>Gene Accession No.</th>
<th>Description</th>
<th>Fold Reduction (day 50 vs day 2)*</th>
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<td>Procollagen type V, α2</td>
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</tr>
<tr>
<td>XS6304</td>
<td>Tenascin C</td>
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<td>AF064749</td>
<td>Collagen type V1, α3</td>
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</tr>
<tr>
<td>U03419</td>
<td>Procollagen type I, α1</td>
<td>3.4</td>
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<td>X58251</td>
<td>Procollagen type I, α2</td>
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<tr>
<td>M15832</td>
<td>Procollagen type IV, α1</td>
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<tr>
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* Three samples were analyzed per group. Values shown are average fold reduction determined by differential gene expression analysis of microarray data.


