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Vigorous Allograft Rejection in the Absence of Danger

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Tolerance to self is a necessary attribute of the immune system. It is thought that most autoreactive T cells are deleted in the thymus during the process of negative selection. However, peripheral tolerance mechanisms also exist to prevent development of autoimmune diseases against peripheral self-Ags. It has been proposed that T cells develop tolerance to peripheral self-Ags in the absence of inflammation or “danger” signals. We have used immunodeficient Rag 1−/− mice to study the response of T cells to neo-self peripheral Ags in the form of well-healed skin and vascularized cardiac allografts. In this paper we report that skin and cardiac allografts without evidence of inflammation are vigorously rejected by transferred T cells or when recipients are reconstituted with T cells at a physiologic rate by nude bone graft transplantation. These results provide new insights into the role of inflammation or “danger” in the initiation of T cell-dependent immune responses. These findings also have profound implications in organ transplantation and suggest that in the absence of central deletional tolerance, peripheral tolerance mechanisms are not sufficient to inhibit alloimmune responses even in the absence of inflammation or danger. The Journal of Immunology, 2000, 164: 3065–3071.

The adaptive immune system has a remarkable capacity to respond to and control a myriad of pathogens and at the same time only rarely causes destructive immune responses directed against autologous tissues. Several conceptual models have been proposed to explain how the immune system so effectively prevents the development of pathologic autoimmune responses. The classic model of self-tolerance holds that developing T cells undergo an education process resulting in a repertoire of T cells that distinguish between self- and non-self-Ags (1–3). It is thought that most autoreactive T cells are deleted in the thymus during the process of negative selection (4–6). However, some self-molecules appear to be expressed exclusively in peripheral (nonthymic) tissues such as inducible proteins, hormones, or proteins with a developmentally restricted pattern of expression. It has been proposed that self-reactive T cells that escape from the thymus are deleted or inactivated by additional, less understood mechanisms in the periphery (7–10).

More recently, an alternative model has been proposed to explain the ability of T cells to control pathogens while failing to injure host tissues (11, 12). This model suggests that the immune system does not primarily distinguish self from non-self but rather recognizes the context in which Ags are presented. In the setting of inflammation or tissue injury, “danger” signals induce the expression of critical costimulatory molecules on APC, thus permitting T cells to fully respond to Ags presented in this context. Conversely, it is proposed that under homeostatic conditions, in the absence of inflammation, Ag presentation occurs without costimulation, leading to specific T cell unresponsiveness and ultimately to tolerance.

Previous studies addressing mechanisms of peripheral tolerance have explored the immunogenicity or tolerogenicity of neo-self-Ags expressed in the periphery using transgenic murine models. These studies have yielded inconsistent results. Some models have demonstrated development of T cell tolerance to the transgenic Ag (13–15), whereas other models have resulted in T cell reactivity against immunogenic forms of the same Ag (16–21). In this report, we have used immunodeficient Rag 1−/− mice to study the response of T cells to neo-self peripheral Ags in the form of well-healed skin or vascularized cardiac allografts. This approach ensures no thymic expression of the alloantigens and enables us to study whether quiescent allografts can tolerate circulating T cells. Surprisingly, we show that well-healed allografts with no evidence of inflammation are promptly rejected by adoptive transfer of T cells and by newly emerging T cells in recipients reconstituted by bone graft transplantation. Furthermore, skin grafts with isolated multiple major or multiple minor histocompatibility differences are also rejected by newly emerging T cells. These experiments demonstrate for the first time in a nontransgenic model that newly emerging T cells are neither ignorant to nor tolerized by alloantigens expressed on well-healed transplanted organs.

Materials and Methods

Mice

Adult male 6- to 8-wk-old C57BL/6, BALB/c, C3H/HeJ, B6CBy F1, B6.CH-2b, C.B10H-2b, C57BL/6 nude, and B6CBy F1 nude mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 Rag 1−/− mice were obtained from The Jackson Laboratory and bred as homozygotes under sterile conditions at Emory University (Atlanta, GA).

Skin grafting

Full-thickness ear skin grafts (~1 cm2) were transplanted onto the thorax of recipient mice and secured with a Band-Aid (Johnson & Johnson, Arlington, TX) for 7 days. Mice were housed individually under sterile conditions with sterile food and water for the duration of all experiments. Rejection was defined as the complete loss of viable epidermal graft tissue.
Bone grafting

Femurs were harvested from donor mice and cleaned of connective tissue under sterile conditions. Femurs were cut into 8–10 small (1- to 2-mm) fragments, and fragments of one femur were transplanted under the kidney capsule of recipient mice.

Heart transplantation

Vascularized heterotopic heart transplantation was performed using microsurgical techniques essentially as described (22). Graft survival was followed by daily palpation. Rejection was defined by the loss of palpable cardiac contractions, which was confirmed with direct visualization at laparotomy.

Cell preparation for adoptive transfer

Spleenic and mesenteric lymph node cells were harvested from B6 mice. After red blood cell lysis with Tris-buffered ammonium chloride (Sigma, St. Louis, MO), T cell-enriched populations were prepared as nylon wool-nonadherent cells. T cell subsets were prepared from nylon wool-nonadherent cells by incubation with rat anti-mouse CD11b (M1/70), anti-mouse CD45R (B220), and either anti-mouse CD4 (GK1.5) or anti-mouse CD8 (TIB105) (all from American Type Culture Collection, Manassas, VA) for 20 min on ice. Cells were then washed and incubated with goat anti-rat IgG biotin (Pierce, Rockford, IL) and avidin-fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Sections were then incubated with an avidin/biotin block kit (Vector Laboratories). T cell-enriched populations were prepared as nylon wool-nonadherent cells and stained with hematoxylin and eosin according to standard procedures.

Histology and immunocytochemistry

Fresh tissues were fixed in molecular biology fixative (Streck Laboratories, Omaha, NE) for 2 h and then in 70% alcohol until ready for use. When ready, tissues were processed and embedded in paraffin (Fisher Scientific, Pittsburgh, PA). Five-micron thick sections were cut on a microtome (Leica, Nussloch, Germany) and stained with hematoxylin and eosin according to standard procedures. For immunohistochemical staining, sections were deparaffinized and then rehydrated. Sections were then incubated with an avidin/biotin block kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions before quenching with 3% H2O2 for 5 min. Sections were then stained with biotinylated anti-Ly-6-G or IgG2b (PharMingen) before incubation with ABC complex (Vector Laboratories). Peroxidase activity was visualized using diaminobenzidine substrate (Pierce, Rockford, IL).

RT-PCR

At specified intervals after transplantation, the skin allografts were removed. Assessment of transcript expression was performed using RT-PCR on a Perkin-Elmer 9600 Thermocycler (Norwalk, CT) as described (23). The annealing temperature for IL-1β was 57°C; for macrophage-inflammatory protein-2 (MIP-2), 39°C; for B7.1, forward 5′-AGGTTGGTACATACATCGGTT-3′, and reverse 5′-ATGAGGATTCCCTATCCGGG-3′, 20 cycles; for IL-1β, forward 5′-GCCAATTGTCGAAGAAT-3′ and reverse 5′-CTCGGAGCCTGATATAAG-3′, 22 cycles; for MIP-2, forward 5′-ATGTTGCGTCCGTTACAT-3′ and reverse 5′-CCGACTGCATCTATTTGTCCCC-3′, 20 cycles; for B7.1, forward 5′-AGGTTGGTACATACATCGGTT-3′, and reverse 5′-ATGAGGATTCCCTATCCGGG-3′, 17 cycles. PCR products were analyzed on ethidium bromide-stained, 1.5% Separide (Life Technologies, Gaithersburg, MD) gels.

Results

Well-healed allogeneic skin grafts on Rag 1−/− mice have no evidence of inflammation or “danger”

To study the role of acute tissue injury (“danger”) in the initiation of allograft rejection and the induction of T cell tolerance to transplanted tissues, we developed an experimental model using T cell-deficient C57BL/6 (H-2b, B6) Rag 1−/− mice, which are unable to reject allografts. By reconstituting these mice with T cells at various times after transplantation of a BALB/c (H-2k) skin graft, we were able to study responses to acutely inflamed, freshly transplanted allografts or to uninflamed, well-healed allografts (quiescent allografts). First, we compared the freshly transplanted allografts with allografts that had been allowed to heal for 50 days on unconditioned B6 Rag 1−/− recipients. Freshly transplanted skin grafts appeared thickened and edematous, with granulation tissue around the edges of the graft. Histologically, these grafts showed prominent neovascularization and diffuse infiltration with Ly6-G+ polymorphonuclear leukocytes (Fig. 1A). In contrast, 50 days after transplantation, epithelialization of the surrounding graft bed was complete and the BALB/c skin grafts appeared grossly well-healed. Histologically, the quiescent allografts were indistinguishable from normal skin with complete resolution of the granulocytic infiltrate (Fig. 1A). To determine whether the freshly transplanted and quiescent allografts differed in expression of mediators of inflammation or costimulation, we analyzed transcript expression by RT-PCR. Analysis of freshly transplanted skin harvested from Rag 1−/− recipients demonstrated markedly increased expression of transcripts for IL-1β, MIP-2, and B7.1, which is indicative of a local innate immune response consistent with acute inflammation associated with tissue injury. By 50 days, the expression of these transcripts had returned to basal levels observed in normal skin, confirming the resolution of inflammation or “danger” within the quiescent allografts (Fig. 1B).

Adoptively transferred T cells mediate prompt rejection of well-healed skin allografts

Next, we used this model to test the hypothesis that alloantigens presented in an inflammatory or dangerous environment would elicit immunity and rejection, whereas the same alloantigens presented in a quiescent environment would fail to generate a rejection response. For this, we adoptively transferred 1 × 107 B6 T cells into B6 Rag 1−/− mice that had freshly transplanted skin allografts or well-healed allografts that had been transplanted 50 days before reconstitution (Fig. 2A). As expected, Rag 1−/− mice that were not reconstituted with T cells accepted allogeneic skin grafts indefinitely. Surprisingly, Rag 1−/− mice that were reconstituted with B6 T cells promptly rejected both well-healed and acutely transplanted skin allografts with identical kinetics. Because there is evidence that the costimulatory requirements of CD4 and CD8 T cells differ (24, 25) and that high-affinity CD8 T cells may be activated in the absence of any costimulation (26, 27), we performed similar experiments using purified CD4 and CD8 T cells for the reconstitution. As for the mixed T cell population, we observed that either CD4 or CD8 T cells could mediate prompt rejection of both acute and well-healed BALB/c skin allografts (Figs. 2, b and c). These results suggest that acute tissue injury is not required in order for transplanted allogeneic tissues to elicit prompt rejection responses. However, it is possible that other danger signals provided by the innate immune system play an important role in this process. Although Rag 1−/− mice do not develop T or B cells, they have a
normal population of NK cells that may be activated by the absence of self-MHC class I molecules on the transplanted allograft (28, 29). Therefore, it is possible that NK cells in the Rag1/2 recipients may have provided danger signals that were responsible for the activation of the adoptively transferred T cells and rejection of the well-healed allografts. To explore this possibility, we performed similar experiments in which semiallogeneic B6-BALB/c F1 (H-2 bxd) skin grafts were transplanted onto B6 Rag1/2 recipients. In this experiment the transplanted skin graft would express the same alloantigens as a fully allogeneic BALB/c skin graft. However, because the semiallogeneic grafts also express recipient (H-2b) MHC class I genes, NK cell recognition of “missing self” on the transplanted grafts will not occur, and therefore, NK cells will not be activated against the graft. As with the previous experiments, adoptive transfer of T cells resulted in prompt rejection of both the acutely transplanted and well-healed allografts (Fig. 2d). Thus, adoptively transferred T cells do not require inflammation or NK cell activation via recognition of “missing self” to initiate or mediate skin allograft rejection.

Developing T cells populate the periphery and mediate rejection of well-healed skin allografts

These initial experiments indicated that acute tissue injury was not required for the initiation of T cell responses to transplanted tissues. Furthermore, these results suggested that encounter with Ags in the absence of costimulation failed to induce tolerance. It is possible that some of the transferred cells became activated during ex vivo preparation before adoptive transfer. Additionally, there is evidence that peripheral tolerance mechanisms can be overcome by a large dose of potentially autoreactive cells (36, 37). To address these possibilities, we studied whether alloreactive T cells...
FIGURE 2. Freshly transplanted and quiescent skin allografts are rejected by adoptively transferred T cells with similar kinetics. B6 Rag1−/− recipients of freshly transplanted (day 0 with respect to adoptive transfer, ○) or quiescent (day 50, □) BALB/c skin allografts were reconstituted with $1 \times 10^7$ syngeneic (B6) unseparated T cells (a), CD4+ T cells (b), or CD8+ T cells (c). Freshly transplanted and quiescent allografts were promptly rejected in all groups with similar kinetics. d, B6 Rag1−/− recipients of freshly transplanted and quiescent B6 C skin allografts were reconstituted with $1 \times 10^7$ syngeneic (B6) unseparated T cells with similar prompt rejection in both groups. e, Adoptive transfer of $5 \times 10^7$ B6 thymocytes resulted in prompt rejection of well-healed and freshly transplanted BALB/c skin allografts. a, Recipients with quiescent allografts reconstituted with saline (○) did not reject allografts. Similar results were obtained in experiments in which T cell reconstitution was delayed for >100 days.

Emerging from the thymus at a physiologic rate would be tolerized or activated by encounter with alloantigens in the form of a well-healed skin allograft. To investigate this possibility, we developed a model to physiologically reconstitute Rag1−/− mice by transplanting bone marrow grafts from T cell-deficient B6 nude mice under the kidney capsule of the Rag1−/− recipient. In this system, progenitors from the nude bone graft traffic to the Rag1−/− thymus mature into CD4+ and CD8+ T cells and populate the periphery between 4 and 8 wk after transplantation. Therefore, B6 Rag1−/− recipients of BALB/c skin grafts were reconstituted by transplantation of B6 nude bone marrow grafts. Flow cytometry of peripheral blood confirmed that recipient mice contained no detectable T cells 4 wk after transplantation (data not shown). Furthermore, skin allografts harvested from Rag1−/− recipient mice 4 wk after transplantation appeared well-healed and demonstrated no evidence of inflammation or “danger” by histology or RT-PCR (Fig. 1). Nonetheless, as T cells emerged over the ensuing several weeks, recipients of B6 nude bone grafts uniformly rejected their BALB skin allografts (Fig. 3a). To confirm that rejection was specific for the allogeneic skin grafts, we performed similar experiments in which B6×BALB/c F1 (B6C) nude bone grafts were transplanted into B6 Rag1−/− recipients simultaneously with BALB/c and C3H/HeJ (H-23) skin grafts. After 6–12 wk, recipients of B6C nude bone grafts rejected all well-healed C3H/HeJ skin grafts, whereas BALB/c grafts remained well-healed indefinitely (data not shown). These data indicate that naive alloreactive T cells emerging from the thymus at a physiologic rate are not tolerized by encounter with alloantigens in the form of a quiescent skin allograft. Rather, even uninfamed allogeneic tissues elicit a vigorous rejection response. Thus, whereas T cell recognition of Ag in the thymus confers robust tolerance, encounter with the same Ag in the periphery elicits immunity. Therefore, in the setting of an allogeneic transplant, peripheral tolerance mechanisms appear to be unable to fully compensate for failure to delete Ag-specific T cells in the thymus.

Previous studies have demonstrated peripheral tolerance using transgenic mice that express single allogeneic MHC class I or class II genes on peripheral tissues (13–15). Because our studies examined allogeneic skin grafts with multiple major and minor histocompatibility differences, it is possible in the setting of these multiple antigenic differences that peripheral tolerance mechanisms alone might not be sufficient to tolerate a strong polyclonal T cell response with a wide range of affinities. Additionally, it is possible that unidentified superantigens expressed by the BALB/c genome could induce an immune response in the absence of costimulation. To address these possibilities, we performed similar experiments with simultaneous transplantation of B6 nude bone graft and

FIGURE 3. Emerging T cells reject well-healed skin allografts. B6 Rag1−/− mice were transplanted with a B6 nude bone graft under the kidney capsule (□) and skin grafts from BALB/c (a), C57BL/H-2b (b), and B6.C5H-2d (c) donors. On day 28 after transplantation, skin grafts were well-healed (Fig. 1) and recipients had no detectable T cells by flow cytometry (data not shown). In nude bone graft recipients, skin grafts in each group were rejected with similar kinetics. Rag1−/− recipients that did not receive B6 nude bone grafts (○) did not reject allografts. Similar results were obtained in experiments in which transplantation of the nude bone graft was delayed for 90 days.

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isolated multiple major (B6.C.H-2^d) or minor (C.B10.H-2^b) histocompatibility disparate skin grafts from congenic mice onto B6 Rag^1^-/- mice. After 6 wk, all skin grafts were smooth and had healthy hair growth. However, grafts slowly began to show evidence of rejection with small ulcerations, and all skin grafts from both groups with multiple major and multiple minor histocompatibility differences were completely rejected (Fig. 3, b and c). Thus, emerging T cells populate the periphery and reject well-healed skin grafts with isolated multiple major or multiple minor histocompatibility differences.

**T cells mediate rejection of vascularized cardiac allografts in the absence of danger**

Our data demonstrate that well-healed skin allografts can be rejected by circulating T cells and that they do not induce tolerance of newly emerging T cells. Recent evidence in a transgenic model suggests that adult mice fail to develop tolerance to allogeneic MHC class I Ag in the skin due to low levels of intradermal T cell traffic (38). Therefore, it is possible that circulating naive T cells are never exposed to the well-healed skin allograft and thus are not rendered tolerant to it. Furthermore, because the skin allograft is exposed to the external environment, minor trauma could elicit a cutaneous inflammatory response and rejection of the allograft. Additionally, there is evidence that vascularized allografts can induce tolerance to themselves under some circumstances (39) and therefore may influence the responses of peripheral alloreactive T cells more effectively than nonvascularized grafts. Thus, it was possible that well-healed vascularized allografts with higher levels of T cell traffic through the donor-derived endothelium could induce peripheral tolerance in this model. To address this possibility, we performed similar experiments to determine whether adoptively transferred T cells or newly emerging T cells could reject or be tolerized by the presence of a well-healed heterotopic vascularized cardiac allograft (Fig. 4). Surprisingly, adoptive transfer of T cells into Rag^1^-/- mice on the day of cardiac transplantation or 50 days after transplantation resulted in prompt rejection and extensive lymphocytic infiltration and myocardial damage of the vascularized allografts. Furthermore, simultaneous cardiac allograft and nude bone graft transplantation resulted in rejection of cardiac allografts between 8 and 12 wk, which was associated with a diffuse lymphocytic infiltrate and destruction of myocytes. Thus, well-healed vascularized cardiac allografts are also promptly rejected by T cells and are unable to independently promote peripheral tolerance induction of naive thymic emigrants.

**Discussion**

The results presented here provide new insights into the role of inflammation or “danger” in the initiation of T cell-dependent immune responses. In our model, T cell-deficient mice reject well-healed skin and vascularized cardiac allografts upon adoptive transfer of T cells or when they develop T cells from a transplanted bone marrow graft. There are several possible explanations for the immunogenicity of the well-healed allografts. It is well known that
the immune response to transplanted tissues is particularly vigorous. In contrast to the low frequency of responding T cells to most conventional Ags, as many as 1–15% of T cells respond to a fully MHC disparate allogeneic stimulus (40–42). Thus, it is possible that such a high frequency of responding T cells could trigger a critical local level of cytokine and/or costimulatory molecule induction that is sufficient to initiate an immune response in the absence of inflammation or danger signals. Indeed, Kunts et al. (36) have demonstrated that transfer of OVA-specific CD8+ T cells into transgenic mice that express OVA in the β cells of the pancreas and in the kidney become activated in the draining lymph nodes of OVA-expressing tissues and may cause diabetes if given in sufficient numbers. This observation combined with our current results suggest that in the setting of the well-healed allograft, sufficient Ag presentation and T cell activation may take place in the draining lymph nodes even in the absence of danger and may lead to prompt allograft rejection.

In addition to the high frequency of T cells responding to transplanted tissues, there also may be a wide range of TCR affinities for the many MHC-peptide complexes presented during an allogeneic response (43). Because the need for costimulation is diminished in the presence of high-affinity receptors (26, 27), it is possible that responding T cell clones with high affinity account for the danger-independent rejection response. It is thought that such high-affinity TCRs for self-molecules would have been deleted in the recipient thymus during selection (3, 4). Thus, peripheral tolerance mechanisms may not be sufficient to prevent an immune response by high-affinity alloreactive cells. Additionally, it has been hypothesized that the high density of MHC-peptide complexes presented by allogeneic cells may contribute to the strength of an allogeneic response (44). It is possible that high ligand densities increase TCR triggering (45), diminishing the need for costimulation to initiate an immune response. Thus, the immune response to well-healed transplanted allografts may differ in several respects from responses to conventional Ags or transgenes expressed on peripheral tissues.

These studies do not suggest that danger may not play a critical role in some immune responses; rather, it is possible that in the setting of responses against Ags with lower affinity and diminished T cell precursor frequencies, the presence of danger and/or costimulation may be critical for the initiation of an immune response. Future studies of the T cell response to well-healed allografts with single minor histocompatibility complex differences (H-Y, for example) or utilizing allografts from transgenic donors may help to clarify these issues.

Although our results suggest that danger-induced costimulation is not required for allograft rejection, we have previously demonstrated that skin and cardiac allograft rejection are significantly inhibited by costimulation blockade (46). Resolution of this apparent paradox may lie in the differences between the two models. In the current studies, we have investigated whether the absence of danger-induced costimulation at the inception of the immune response would promote tolerance or rejection. Interestingly, we have found that although costimulation is absent in quiescent allografts, B7-1 and B7-2 transcripts were strongly induced after adoptive transfer of T cells (data not shown). Thus, danger-induced costimulation is not necessary to initiate an allogeneic response; however, T cell-induced costimulation may be necessary to sustain the response. In addition, the absence of costimulation in our model is apparently brief. It is possible that sustained TCR signaling in the presence of costimulation blockade therapy could induce T cell anergy or development of a regulatory population of T cells.

Finally, these results have profound implications in organ transplantation. These findings suggest that in the absence of central deletional tolerance, peripheral tolerance mechanisms are not sufficient to inhibit alloimmune responses even in the absence of inflammation or danger. These data are consistent with reports of patients with well-functioning allografts many years after transplantation who promptly reject their allografts upon cessation of immunosuppressive drugs (47–49). Because new T cells continue to develop into late adult life (50), future tolerance-induction protocols cannot rely on the well-healed allograft to induce peripheral tolerance of emerging T cells. Rather, strategies to effect thymic selection, such as bone marrow transplantation to induce hematopoietic chimerism or strategies to induce active donor-specific regulatory T cells, may be required to maintain long-term allograft acceptance in the absence of chronic immunosuppression.

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lead to the amplification in vivo of cytotoxic T cell responses. J. Exp. Med. 186:47.


