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We investigated the influence of allograft primary vascularization on alloimmunity, rejection, and tolerance in mice. First, we showed that fully allogeneic primarily vascularized and conventional skin transplants were rejected at the same pace. Remarkably, however, short-term treatment of mice with anti-CD40L Abs achieved long-term survival of vascularized skin and cardiac transplants but not conventional skin grafts. Nonvascularized skin transplants triggered vigorous direct and indirect proinflammatory type 1 T cell responses (IL-2 and IFN-γ), whereas primarily vascularized skin allografts failed to trigger a significant indirect alloresponse. A similar lack of indirect alloreactivity was also observed after placement of different vascularized organ transplants, including hearts and kidneys, whereas hearts placed under the skin (nonvascularized) triggered potent indirect alloresponses. Altogether, these results suggest that primary vascularization of allografts is associated with a lack of indirect T cell alloreactivity. Finally, we show that long-term survival of vascularized skin allografts induced by anti-CD40L Abs was associated with a combined lack of indirect alloresponse and a shift of the direct alloresponse toward a type 2 cytokine (IL-4, IL-10) secretion pattern but no activation/expansion of Foxp3+ regulatory T cells. Therefore, primary vascularization of allografts governs their immunogenicity and tolerogenicity.


Transplantation of allogeneic organs and tissues induces a potent inflammatory immune response that invariably results in early acute allograft rejection. The antidonor immune response is initiated by recipient T cells activated in the recipient’s secondary lymphoid organs via two distinct pathways: the direct pathway, in which T cells recognize intact donor MHC molecules on transplanted cells (1), and the indirect pathway, which involves the recognition of donor peptides processed and presented by host APCs (2). Fully allogeneic skin grafts trigger potent proinflammatory T cell responses via both pathways (3). Either direct or indirect alloresponse is sufficient to mediate acute rejection of skin allografts (4). In contrast, the relative contribution of these pathways to acute rejection of vascularized solid organ transplants, including hearts and kidneys, is less clear. Direct alloreactivity is thought to be the driving force behind early acute rejection of these transplants, whereas the indirect pathway is involved in chronic rejection (5), a late process characterized by peripheral inflammation, fibrosis, and arteriosclerosis involving intimal thickening and luminal occlusion of graft vessels (6). This conclusion was drawn based on the assumption that the direct alloresponse is short-lived due to the rapid elimination of donor passenger leukocytes, whereas the indirect alloresponse is perpetuated via continuous presentation of alloantigens by host APCs. In addition, indirect alloimmunity drives alloantibody production that is essential to the chronic rejection process (7). Finally, de novo induction of indirect alloresponses via allopeptide immunization was shown to trigger chronic rejection of allografts in various animal models (5, 8). Therefore, although indirect alloreactivity is presumably an essential element of the chronic rejection process, its contribution to acute rejection of primarily vascularized solid organ allografts remains to be demonstrated.

Advances in surgical techniques and the development of immunosuppressive agents have rendered possible large-scale transplantation of some allogeneic organs in patients, with minimal risks for early acute rejection. However, continuous widespread immunosuppression treatments are associated with susceptibility to infection and neoplasia in transplanted patients. Additionally, these drugs are nephrotoxic and ineffective in preventing chronic rejection. Altogether, this underscores the need for the development of more efficient and selective immune-based strategies in transplantation. Some protocols involving T cell costimulation blockade and/or donor hematopoietic chimerism achieved immunological tolerance (indefinite graft survival without immunosuppression and chronic rejection) to some vascularized solid organ transplants in rodents and primates (9–12). However, tolerance to skin allografts has proven to be more arduous. The high immunogenicity of skin allografts is traditionally attributed to the presentation of highly immunogenic skin-specific Ags (13) by a large population of resident dendritic cells (14–16). However, until now, this has not been demonstrated.

In the current study, we show that initial vascularization of skin allografts renders these transplants susceptible to tolerance via protocols effective with vascularized solid organ transplants. The mechanisms by which vascularization governs the immunogenicity and susceptibility to tolerogenesis of allografts are investigated.
Materials and Methods

Mice and transplantation

Mice were bred and maintained at Massachusetts General Hospital animal facilities under specific pathogen–free conditions. All animal care and handling were performed according to institutional guidelines. Nonvascularized “conventional” full-thickness skin allografts were placed using standard techniques (17). Skin was harvested from euthanized donor mice, the sci. fat was removed, and the skin was cut into 2 cm pieces and placed in sterile PBS until used for transplantation (<30 min). Recipient mice were anesthetized and shaved around the chest and groin. The skin allograft was placed in a slightly larger graft bed prepared over the groin or chest of the recipient and secured using Vaseline gauze and a bandage. For vascularized skin grafts, a 2 × 3-cm full-thickness flap was outlined in the groin and raised. The epigastric vessels were dissected, the distal superficial and deep femoral vessels were ligated, and the femoral artery and vein were separated. The femoral artery and vein were then divided. For the recipient, a same-size defect was created in the groin area. The femoral artery and vein, right below the inguinal ligament, were separated and prepared for anastomosis. End-to-end anastomosis was performed for arteries, and end-to-side anastomosis was performed for the veins (Supplemental Fig. 2). After the patency of the vessels was confirmed, an end-to-side anastomosis was performed for the recipient’s own skin, a process that was frequently completed by day 10 posttransplantation. Therefore, primary vascularization is considered that this difference relies on graft vascularization at the time of transplantation. To test this, we compared the survival of conventional skin allografts with that of vascularized skin and heart allografts in recipients treated with anti-CD40L mAbs (MR1, 0.5 mg injected i.p. at days 0, 2, and 4) using the B6 to C3H donor–recipient combination. This model was chosen because short-term treatment of recipients with anti-CD40L mAbs (MR1) was shown to markedly prolong the survival of heart, but not conventional skin, allografts (18). Delayed rejection of cardiac allografts in this model is essentially due to inhibition of alloreactive CD4+ T cells (20). Our experiments showed that, as anticipated, anti-CD40L treatment had no substantial impact on the survival of conventional skin allografts (MST: 10 d), whereas it delayed the rejection of cardiac allografts (MST: 55 d) (Fig. 1). In contrast, remarkably, MR1 treatment achieved long-term survival of vascularized skin transplants (MST: 82 d) (Fig. 1). Actually, these skin allografts survived significantly longer than did their cardiac counterparts (p = 0.0012). In addition, it is noteworthy that late rejection of vascularized skin allografts did not follow a common course; they shrunk gradually starting at day 60–70 posttransplantation and never exhibited apparent signs of inflammation or necrosis. As they reduced in size, the allografts became replaced by the recipient’s own skin, a process that was frequently completed by day 100 posttransplantation. Therefore, primary vascularization is sufficient to accomplish long-term survival of skin allografts via anti-CD40L mAb costimulation blockade.

Statistical analyses

Statistical analyses were performed using STATView software (Abacus Concepts, Berkeley, CA). The p values were calculated using the log-rank test and paired t test; a p value < 0.05 was considered statistically significant.

Results

Anti-CD40L Ab treatment achieves long-term survival of primary vascularized skin allografts

First, we compared the rejection of skin allografts that were either vascularized at the time of their placement or not. Fully allogeneic skin grafts from C57BL/6J (B6; H-2b) mice were transplanted on C3H/HeJ (C3H; H-2k) or BALB/cJ (BALB/c; H-2d) recipients. All of the nonvascularized grafts were rejected in an acute fashion (mean survival time [MST]: BALB/c: 9 ± 2 d, C3H: 10 ± 1 d). Vascularized skin grafts were also acutely rejected, although in a slightly delayed manner (MST: BALB/c 11 ± 2 d, C3H: 12 ± 2 d). Similar data were obtained when B6 mice were used as recipients of BALB/c grafts (see survival curves in Supplemental Fig. 1).

It is well known that prolonged survival or tolerance of skin allografts is difficult to accomplish. In contrast, this is regularly achieved in heart-transplanted mice via multiple treatments, including costimulation blockade (9, 18). In this study, we hypothesized that this difference relies on graft vascularization at the time of transplantation. To test this, we compared the survival of conventional skin allografts with that of vascularized skin and heart allografts in recipients treated with anti-CD40L mAbs (MR1, 0.5 mg injected i.p. at days 0, 2, and 4) using the B6 to C3H donor–recipient combination. This model was chosen because short-term treatment of recipients with anti-CD40L mAbs (MR1) was shown to markedly prolong the survival of heart, but not conventional skin, allografts (18). Delayed rejection of cardiac allografts in this model is essentially due to inhibition of alloreactive CD4+ T cells (20). Our experiments showed that, as anticipated, anti-CD40L treatment had no substantial impact on the survival of conventional skin allografts (MST: 10 d), whereas it delayed the rejection of cardiac allografts (MST: 55 d) (Fig. 1).

Anti-CD40L Ab treatment

Recipient mice were injected i.p. with 0.5 mg anti-CD40L mAbs (MR1) at the time of transplantation and on days 4 and 6 posttransplantation, as previously reported (18).

Regulatory T cell assays

Cell enrichment. CD4+ cells were enriched using either CD4 (LJT4) MACS MicroBeads or the CD4+ T Cell Isolation Kit II, mouse (Miltenyi Biotec, Auburn, CA). CD4+CD25+ and CD4+CD25− T cells were enriched using the CD4+CD25− Regulatory T Cell Isolation Kit (Miltenyi Biotec). APCs were mouse splenocytes depleted of T cells using CD90.2 MicroBeads, mouse (Miltenyi Biotec). Flow cytometry data were acquired either on a FACSCalibur using CellQuest software or on an LSRII using FACSDiva software (all from BD Biosciences, San Diego, CA). Data analysis was performed using FlowJo software (TreeStar, Ashland, OR).

Live cells were gated using propidium iodide, and singlet cells were gated for analysis as FSC-W− and SSC-A−. The suppression assays were performed as previously described (19). Briefly, 2 × 105 cells/well MACS-enriched CD4+CD25− T cells were plated in triplicate in 96-well, round-bottom plates along with 1 × 106 irradiated (3000 cGy) CD90-depleted splenocytes/well. MACS-enriched CD4+CD25+ T cells were added at an effecter/regulatory T cell (Treg) ratio of 2:1, 4:1, 8:1, or 16:1. Medium consisted of RPMI 1640 supplemented with 15% Hybrid-MAX (Sigma-Aldrich), 1 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 25 μM 2-ME, with a total volume of 200 μl in each well. Cultures were incubated for 4 d at 37 °C in 5–7% CO2. [3H]thymidine (2 μCi/well) was added for the last 18 h of culture. The percentage of inhibition was calculated as 100 − (avg. cpm sample cultures with CD4+CD25+ T cells/avg. cpm sample cultures without CD4+CD25+ T cells × 100).

Statistical analyses

Statistical analyses were performed using STATView software (Abacus Concepts, Berkeley, CA). The p values were calculated using the log-rank test and paired t test; a p value < 0.05 was considered statistically significant.

Primary vascularized allografts induce direct, but not indirect, T cell–mediated alloreponses

These observations suggested that graft vascularization could influence the nature and/or magnitude of the T cell alloreresponse. Typical nonvascularized skin allografts are known to induce potent direct and indirect proinflammatory alloreponses by recipient T cells (3). In this study, we investigated the effect of primary skin...
The graft vascularization on the recipient’s antidonor T cell response. T cells were isolated from the lymph nodes and spleens of C3H mice transplanted with a conventional or primarily vascularized B6 skin allograft (postoperative days 9–11). The frequencies of T cells secreting proinflammatory IL-2 and IFN-γ cytokines following a 24–48 h culture in the presence of intact donor APCs (direct pathway) or recipient APCs + donor sonicates (indirect pathway) were measured by ELISPOT, as previously described (3). As anticipated, recipients of standard skin allografts mounted potent direct and indirect T cell alloresponses characterized by the expansion of activated T cells secreting proinflammatory cytokines, IL-2 and IFN-γ (Fig. 2A, 2B). In contrast, recipients of vascularized skin grafts mounted a direct, but not indirect, alloresponse (Fig. 2A, 2B). No significant production of type 2 cytokines (IL-4 and IL-10) was recorded with either type of skin graft (Supplemental Fig. 3A, 3B). Third-party allogeneic APCs from an irrelevant donor induced a low direct response corresponding to a primary MLR response (<100 IFN-γ spots/million T cells) (Supplemental Fig. 3C). Therefore, vascularization of skin grafts is associated with the lack of a proinflammatory indirect alloresponse by recipient T cells.

To further investigate the influence of graft vascularization on the nature of T cell alloactivity, we measured direct and indirect responses of T cells in C3H recipients of either vascularized or nonvascularized (placed under the skin) B6 cardiac allografts. Both types of transplants induced a vigorous direct alloresponse (Fig. 2C). In contrast, only recipients of nonvascularized cardiac transplants mounted an indirect alloresponse (Fig. 2D). Together with our observations in the skin graft model, these results suggested that the ability to trigger an indirect alloresponse is not an intrinsic property of the transplant, rather it depends upon its vascularization at the time of placement. In further support of this view was the observation that vascularized kidney allografts also failed to induce an indirect alloresponse, whereas it was triggered by corneal allografts, which are nonvascularized grafts (Fig. 3). Therefore, primary vascularization controls the immunogenicity of allografts in that it governs their ability to initiate a proinflammatory indirect T cell alloresponse.

Anti-CD40L mAb treatment suppresses direct, but not indirect, allosponses by T cells

It is firmly established that anti-CD40L mAb (MR1) treatment inhibits T cell direct alloreactivity and prevents the acute rejection of heart allografts but not conventional skin allografts (21). At the same time, we showed that MR1 delays the rejection of skin allografts when they are vascularized at the time of transplantation (Fig. 1). Because only conventional skin allografts induce an indirect alloresponse, this suggested that MR1 might not prolong the survival of nonvascularized skin grafts because it fails to suppress the indirect alloresponse. To address this possibility, direct and indirect proinflammatory alloresponses (IFN-γ) were measured in mice transplanted with a conventional skin allograft and treated with anti-CD40L mAbs. We observed that the direct alloresponse was thwarted by day 5 posttransplantation (Fig. 4A), whereas anti-CD40L mAb treatment reduced, but did not abolish, the indirect alloresponse by IFN-γ-secreting T cells (Fig. 4B). Similar results were obtained following stimulation of T cells with donor-derived peptides, thus excluding that persistence of the indirect alloresponse was due to a nonspecific effect of the sonicates (Supplemental Fig. 4). It is plausible that this “MR1-resistant” residual proinflammatory indirect alloresponse causes the rejection of conventional skin allografts in MR1-treated mice.

**Tolerance to vascularized skin allografts is donor specific**

Next, we investigated the mechanisms by which MR1 treatment achieved long-term survival of vascularized skin transplants. It was possible that MR1 mediated its effect simply via suppressing the direct alloresponse. At the same time, costimulation blockade (MR1) combined with alloantigen presentation (allograft) could have triggered some regulatory mechanisms inducing transplant tolerance. To address this question, MR1-treated BALB/c mice, which had accepted a B6 vascularized skin allograft for 50 d, were transplanted with a donor-matched (B6) or third-party (C3H) heart allotransplant. B6 cardiac transplants survived indefinitely and showed no signs of chronic rejection, whereas third-party C3H hearts were rejected acutely (Fig. 5). This result shows that anti-CD40L mAb treatment had induced donor-specific tolerance in recipients of vascularized skin allografts. The observation that secondary heart transplants (Fig. 5) survived much longer than did their primary counterparts (Fig. 1) (p < 0.005) presumably reflects the tolerogenic effects exerted by vascularized skin grafts placed in MR1-treated recipients.

**Long-term survival of vascularized skin allografts is associated with directly activated T cells producing type 2 cytokines**

Some evidence has been provided suggesting that short-term blockade of signals given to T cells via CD28/B7 and CD40L/
CD40 suppresses Th1 responses while sparing Th2 immunity (22, 23). This incited us to compare the frequencies of activated T cells producing either type 1 (IFN-γ) or type 2 (IL-4, IL-10) cytokines in mice treated with MR1 anti-CD40L Abs or a control Ab displaying the same isotype 10 d after placement of a vascularized skin allograft. In MR1-treated mice, the frequencies of type 1 cytokine-secreting cells were markedly reduced compared with untreated recipients (Fig. 6A). Concurrently, these mice mounted a potent direct alloresponse mediated by T cells producing type 2 cytokines (IL-4, IL-5, and IL-10) (Fig. 6B). No cytokine secretion was observed with unstimulated T cells or T cells exposed to syngeneic stimulators. Therefore, anti-CD40L mAb treatment of mice transplanted with a typical vascularized B6 heart (V-heart) or a nonvascularized B6 heart (NV-heart). The results are expressed as spots/million T cells for each cytokine ± SD. Five to eight mice were tested in each group.

**Tolerance of skin allografts is not associated with activation or expansion of Foxp3+ Tregs**

Tolerance of vascularized solid organ transplants has been associated with the emergence of CD4+CD25+Foxp3+ suppressive Tregs in murine, primate, and human transplantation models (24–26). Thus, we next evaluated the impact of graft vascularization on Tregs. Treg numbers and suppressive functions were evaluated in naïve C3H mice and recipients of vascularized or nonvascularized skin grafts treated with MR1. The overall frequencies of CD4+CD25+Foxp3+ Tregs and their percentages among CD4+ T cells, measured in the spleen at days 7, 21, and 50 posttransplantation, were similar in both transplanted groups (12.5 ± 2% and 12.7 ± 1% for the nonvascularized and vascularized transplants, respectively). We observed a modest, but insignificant, increase in the percentages of Tregs from the lymph nodes of mice grafted with a conventional skin graft compared with naïve mice and recipients of a vascularized graft (10.8 ± 1, 8.8 ± 1, and 9.3 ± 2%, respectively; p = 0.07). Finally, we compared the suppressive ability of Tregs collected from either spleen or lymph nodes of each group of mice at day 21 (Fig. 7A, 7B) and day 50 (Fig. 7C) after skin grafting. As shown in Fig. 7, lymph node Tregs (filled symbols) were more potent suppressors than were their splenic counterparts (open symbols), but there was no significant difference in the suppressive ability of Tregs isolated from recipients of vascularized or nonvascularized skin grafts. The alloresponses of C3H effector CD4+ T cells to donor BALB/c (Fig. 7) and third-party B6 APCs (Fig. 7) were inhibited in a similar fashion by C3H Tregs, suggesting a lack of donor Ag specificity for Treg suppression. Finally, treatment of recipients with PC61, an anti-CD25 mAb (PC61) known to deplete CD25highFoxp3+ Tregs (27), given
at the time of transplantation did not affect the survival of vascularized skin allografts in MR1-treated mice (data not shown). Collectively, these results support the view that long-term survival of primarily vascularized skin allografts induced by MR1 Abs was not associated with an increase in the frequency and function of peripheral Foxp3⁺ Tregs.

**Discussion**

Primary vascularization of fully allogeneic skin grafts did not improve their survival. This finding confirms previous results obtained in rodents transplanted with skin flaps, composite tissue allografts, and skin grafts parked under kidney capsules (28, 29). Indeed, our study shows that these allografts induce potent direct proinflammatory alloresponses that are sufficient to provoke their early acute rejection.

In contrast to conventional skin allografts that activate T cells via both direct and indirect pathways, primarily vascularized skin transplants did not trigger a substantial indirect alloresponse. Similar results were obtained with vascularized heart and kidney transplants, whereas nonvascularized cardiac allografts triggered a potent indirect alloresponse. Altogether, these observations indicate that primary graft vascularization is generally associated with poor indirect alloreactivity. This suggests that the direct alloresponse is the only driving force behind acute rejection of vascularized transplants. In support of this view, studies from Auchincloss’ group (30) and Gill’s group (31) showed that donor,
five mice tested individually in each group. By Brennan et al. (34) showed induction of vigorous indirect
alloresponses in BALB/c mice transplanted with a B6 heart. However, evidence of the contribution of this response to the acute rejection of these allografts was not provided. Although indirect alloreactivity may not be involved in acute rejection of solid organ transplants, it is possible that the sustained presentation of alloantigens by recipient APCs may lead to perpetuation of some oligoclonal alloresponse associated with chronic graft rejection (8, 30, 35). Finally, it is important to keep in mind that the indirect allorecognition pathway could contribute to acute rejection of vascularized organ transplants in “sensitized” recipients displaying indirectly activated and expanded memory T cells at the time of graft placement (36). We surmise that exposure to allo-MHC molecules following pregnancy, blood transfusion, or a previous transplantation is among the elements accounting for the differentiation of long-lived memory T cells recognizing alloantigens in an indirect fashion in humans.

It is still unclear why the lack of primary vascularization results in potent indirect allosensitization of T cells after transplantation of conventional skin allografts. Because these transplants become vascularized only 4–5 d after their placement, it is conceivable that initial blood deprivation results in cell death, tissue damage, and increased local inflammation. These circumstances are expected to enhance shedding of donor proteins and subsequent presentation of processed allopeptides by recipient APCs to T cells residing in draining lymph nodes. In addition, in the absence of vascularization, donor passenger leukocytes are likely to leave the graft exclusively through the lymphatics and concentrate in the recipient’s draining lymph nodes where the indirect alloresponse is likely to take place (15, 37). Indeed, this process is critical to the rejection process, as evidenced by the seminal “pedicle” experiments of Barker and Billingham (38) and a more recent study from Lakiss’ group (39) that used alv/aly mice to show that alteration of cell trafficking via lymphatics after skin transplantation leads to prolonged allograft survival. Alternatively, primary vascularization of allografts is presumably associated with a rapid emigration of donor passenger leukocytes via blood vessels, rather than lymphatics, and spreading of these cells throughout the body, a process that may not favor indirect priming of proinflammatory alloreactive T cells (40).

Treatment of mice with anti-CD40L mAbs prolonged the survival of cardiac allografts, but not conventional skin allografts, in the B6-to-C3H donor/recipient combination, a result consistent with previous reports from Larsen’s group (18). Remarkably, however, the same treatment significantly extended the survival of vascularized skin allotransplants. This demonstrates that vascularization rendered these skin grafts susceptible to tolerogenesis rather than recipient, MHC class II expression, which triggers the CD4+ T cell direct alloresponse, is required for the rejection of heart allografts. At first glance, this conclusion might appear to disagree with previous reports involving indirect alloresponses in the acute rejection of kidney and cardiac allografts. However, most of these studies were performed either with recipients presensitized with donor MHC peptides emulsified in adjuvant or adoptively transferred with a large number of indirectly activated T cells (32, 33). Although important, these results demonstrated that T cells activated in an indirect fashion can acutely reject vascularized grafts, but they did not allow a conclusion on the relevance of this pathway in unmanipulated hosts. Finally, it is noteworthy that, in contradiction with our results, a recent study by Brennan et al. (34) showed induction of vigorous indirect
alloresponses in BALB/c mice transplanted with a B6 heart. However, evidence of the contribution of this response to the acute rejection of these allografts was not provided. Although indirect alloreactivity may not be involved in acute rejection of solid organ transplants, it is possible that the sustained presentation of alloantigens by recipient APCs may lead to perpetuation of some oligoclonal alloresponse associated with chronic graft rejection (8, 30, 35). Finally, it is important to keep in mind that the indirect allorecognition pathway could contribute to acute rejection of vascularized organ transplants in “sensitized” recipients displaying indirectly activated and expanded memory T cells at the time of graft placement (36). We surmise that exposure to allo-MHC molecules following pregnancy, blood transfusion, or a previous transplantation is among the elements accounting for the differentiation of long-lived memory T cells recognizing alloantigens in an indirect fashion in humans.

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![FIGURE 5.](image-url) Long-term survival of vascularized skin allografts in MR1-treated mice is associated with donor-specific tolerance. MR1-treated C3H mice, which had accepted a vascularized B6 skin allograft for 50 d, were transplanted with a heart from the same B6 donor (black solid line) or a third-party BALB/c heart (black dotted line). Control nontreated C3H mice were transplanted with a B6 heart (gray dotted line). The results are shown as the percentage of graft survival over time after transplantation. Four to eight mice were tested in each group. Graft survival was analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank test.

![FIGURE 6.](image-url) MR1 treatment promotes the activation of T cells secreting type 2 cytokines. C3H mice were transplanted with a B6 conventional skin allograft and injected with medium (A) or MR1 anti-CD40L mAbs (B), as described earlier. Ten days later, spleen T cells were collected and restimulated in vitro with PBS (white bars), syngeneic APCs (gray bars), or irradiated donor APCs (black bars) (direct allorecognition). The frequencies of activated T cells secreting type 1 (IL-2 and IFN-γ) or type 2 (IL-4, IL-5, and IL-10) cytokines were measured by ELISPOT. The results are expressed as numbers of cytokine-forming spots/million T cells ± SD and are representative of three to five mice tested individually in each group.
via costimulation blockade. This shows that, in contrast to traditional beliefs, skin allografts are not intrinsically resistant to tolerance induction compared with solid-organ transplants.

Our study provides new insights into the mechanisms by which conventional skin allografts are rejected acutely, despite costimulation blockade using anti-CD40L mAbs. It is firmly established that MR1 administration to C3H mice blocks the direct activation of alloreactive CD4+ T cells and subsequent differentiation of CD8 cytotoxic T cells (41). Therefore, in the absence of an indirect T cell–mediated alloresponse, inhibition of CD4+ T cell–directed alloreactivity via MR1 treatment was sufficient to prevent acute rejection of cardiac allografts. Our results show that the same reasoning applies to the rejection of primarily vascularized skin allografts. In contrast, because MR1 Abs failed to thwart the indirect activation of T cells, it is likely that the indirect alloresponse was responsible for the acute rejection of conventional skin allografts in MR1-treated mice. Work is underway in an effort to determine why allospecific T cells activated through the indirect pathway are resistant to anti-CD40L mAb treatment.

The mechanisms by which short-term MR1 treatment ensures long-term survival of vascularized skin allografts are not entirely clear. On the one hand, it is possible that anti-CD40L mAbs prevent acute rejection primarily by suppressing the direct alloresponse. Because these allografts trigger a suboptimal indirect alloresponse, inhibition of direct alloreactivity is apparently sufficient to prevent the acute rejection process. On the other hand, MR1 treatment promotes some donor-specific tolerance effect, because mice transplanted with a vascularized skin allograft subsequently accept heart transplants from the same donor but not a third-party one. Transplant tolerance was achieved previously via MR1 treatment combined with donor-specific transfusion (42–44). In this model, tolerance was attributed to Tregs activated in an indirect fashion (45). At the same time, Auchincloss’ group (46) reported that MR1-induced long-term survival of cardiac allografts cannot be achieved in K14 mice, which lack the ability to mount a CD4+ T cell indirect alloresponse. Collectively, these studies suggested that transplant tolerance relies on self-MHC class II–restricted activation (indirect pathway) of Tregs (19). Furthermore, a recent study by Horibe et al. (47) showed that indefinite survival of vascularized skin allografts achieved via injection of rapamycin-conditioned alloantigen-pulsed dendritic cells relied on Treg expansion. However, we did not obtain any evidence indicating the contribution of Foxp3+ Tregs to tolerance of vascularized skin allografts in our model. In turn, we showed that MR1 treatment is associated with immune deviation toward anti-inflammatory type 2 immunity, as evidenced by the high frequencies of activated T cells producing IL-4, IL-5, and IL-10 cytokines found in MR1-treated recipients of a vascularized allograft. Likewise, several studies reported the activation of Th2, Th3, and/or Foxp3+ Tr1 cells secreting IL-10 or TGF-β following systemic Ag administration via blood or oral routes (48–51). However, it is noteworthy that previous studies by Bishop’s group (52, 53) showed that, in the absence of a Th1 response, activation of alloreactive Th2 cells was associated with an aggressive form of cardiac transplant rejection. This suggests that donor-specific Foxp3+ Tr1 cells, which secrete IL-10 and are known to prevent allograft rejection (54, 55), rather than “classical” Foxp3+ Tregs or Th2 cells, contribute to the long-term survival of vascularized skin allografts in MR1-treated mice.

Successful transplantation of allogeneic skin patches is essential to the treatment of patients with major burn injuries and recipients of composite tissue allografts. However, allogeneic skin grafts are invariably rejected in an acute fashion. Although current immunosuppressive treatments are effective in preventing the early rejection of organ transplants, such as kidneys, they have little or no effect in skin transplantation. Additionally, many attempts to engineer artificial skin or to grow autologous skin tissue in vitro had poor results. As a result, clinical skin transplantation is largely confined to autotransplantation of relatively small skin pieces. Seminal “pedicle” studies performed in the 1960s by Barker and Billingham (38) demonstrated the key role for efferent lymphatics, rather than blood vessels, in the early allosensitization to and rejection of skin allografts. However, it has been difficult to adapt this principle to achieve immune tolerance in skin transplantation. Indeed, some studies demonstrated that tolerance to conventional skin allografts can be reliably achieved in some animal models upon accomplishment of high-level and stable donor hematopoietic mixed chimerism (11, 56). However, this procedure involves whole-body irradiation of the recipient, profound depletion of peripheral lymphocytes, donor bone marrow transplantation, and treatment with immunosuppressive drugs. Our study shows that primarily vascularized skin allografts are susceptible to tolerance induction via short-term costimulation blockade, a protocol that has only been effective with kidney and heart transplants. This finding may have important implications in clinical skin transplantation. Further dissection of the tolerogenic effects associated with transplant vascularization and systemic alloantigen delivery through blood vessels will help to unveil the basic mechanisms underlying transplantation tolerance.


Supplemental Figure 1. Conventional and primarily vascularized skin allografts are rejected at the same pace.

The rejection of conventional non-primarily vascularized (dotted lines) and primarily vascularized (solid lines) fully allogeneic skin allografts was monitored in three donor-recipient mouse combinations (A: B6 to C3H, B: B6 to BALB/c and C: BALB/c to B6). The results are shown as percent graft survival over time after transplantation. Five to twelve mice were tested in each group.

Supplemental Figure 2. Primary vascularization of skin allografts.

A 2x3 cm full thickness flap was outlined in the groin and raised. The epigastric vessels were dissected, the distal superficial and deep femoral vessels were ligated and the femoral artery and vein were separated. The femoral artery and vein were then divided. For the recipient, same size defect was created in groin area. The femoral artery and vein, right below the inguinal ligament, were separated and prepared for anastomosis. End-to-end anastomosis was performed for arteries and end to side for the veins.

Supplemental Figure 3. Type 2 cytokine ELISPOT assays and direct T cell responses to control antigens.

Panel A and B: The frequencies of T cells activated either via direct (panel A) or indirect allore cognition (panel B) and secreting pro-inflammatory cytokines type 1 cytokines were measured by ELISPOT. Alloresponses were measured using spleen T cells collected from naïve C3H mice (no Tx) and from C3H mice transplanted (d10 POD) with either conventional (non-vascularized, NV-skin) or primarily vascularized (V-
skin) fully allogeneic B6 skin allografts. The data are expressed as number of spots per million T cells ± SD measured in 5-8 mice tested individually in each group.

**Panel C:** To assess the donor-specificity of the alloresponse, T cells collected from C3H mice transplanted with either non-vascularized (grey bars) or vascularized (white bars) were restimulated *in vitro* with either medium alone or different syngeneic (C3H) or allogeneic (B6 donor or BALB/c third-party) stimulators as indicated on the X axis. The results are expressed as numbers of γIFN-spots per million T cells ± SD measured in 3-5 mice tested individually in each group.

**Supplemental Figure 4. Indirect alloresponses to donor-derived peptides**

Indirect alloresponses by T cells were measured in naïve C3H mice (No Tx) and C3H mice 10 days after transplantation with B6 skin grafts treated or not with MR1. To test this, spleen T cells were isolated and incubated with medium alone (white bars) or stimulated in vitro for 48 hours with a donor-derived MHC class I K<sup>b</sup> 61-80 peptide (solid bars) or a control HEL 46-61 peptide (striated columns). The results are expressed as numbers of γIFN-spots per million T cells ± SD measured in 3 mice tested individually in each group.
Kant et al. Supplemental Figure 2
Kant et al. Supplemental Figure 3

A: Direct pathway

B: Indirect pathway

C: SKIN TRANSPLANT

- Conventional skin graft
- Vascularized skin graft

Stimulators (APCs)