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Role of CD4+ and CD8+ T Cells in Allorecognition: Lessons from Corneal Transplantation

Florence Boisgérault, Ying Liu, Natalie Anosova, Elana Ehrlich, M. Reza Dana, and Gilles Benichou

Corneal transplantation represents an interesting model to investigate the contribution of direct vs indirect Ag recognition pathways to the allosresponse. Corneal allografts are naturally devoid of MHC class II+ APCs. In addition, minor Ag-mismatched corneal grafts are more readily rejected than their MHC-mismatched counterparts. Accordingly, it has been hypothesized that these transplants do not trigger direct T cell allosresponse, but that donor Ags are presented by host APCs, i.e., in an indirect fashion. Here, we have determined the Ag specificity, frequency, and phenotype of T cells activated through direct and indirect pathways in BALB/c mice transplanted orthotopically with fully allogeneic C57BL/6 corneas. In this combination, only 60% of the corneas are rejected, while the remainder enjoy indefinite graft survival. In rejecting mice the T cell response was mediated by two T cell subsets: 1) CD4+ T cells that recognize alloantigens exclusively through indirect pathway and secrete IL-2, and 2) IFN-γ-producing CD8+ T cells recognizing donor MHC in a direct fashion. Surprisingly, CD8+ T cells activated directly were not required for graft rejection. In nonrejecting mice, no T cell responses were detected. Strikingly, peripheral sensitization to allogeneic MHC molecules in these mice induced acute rejection of corneal grafts. We conclude that only CD4+ T cells activated via indirect allorecognition have the ability to reject allogeneic corneal grafts. Although alloreactive CD8+ T cells are activated via the direct pathway, they are not fully competent and cannot contribute to the rejection unless they receive an additional signal provided by professional APCs in the periphery.


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Abstract

Recognition by recipient T lymphocytes of alloantigens expressed by donor tissues is known to initiate immune destruction of allogeneic transplants (1–3). Although the role of CD4+ and CD8+ T cells in allogeneic rejection is firmly established, the relative contribution of each of these T cell subsets to the rejection process remains controversial. Initial studies using reconstituted nude mice have supported the view that collaboration between helper and effector functional T cell subsets is required to ensure rejection. Either CD4+ or CD8+ T cells could mediate these functions (4). In several models CD4+ T cells have been shown to be necessary and sufficient for the initiation of allograft rejection (5–7). Conversely, some evidence has been provided suggesting that CD8+ T cells alone can reject MHC class I-disparate allografts (8–10). These studies underscore that the contributions of CD4+ and CD8+ T cell subsets in graft rejection are primarily dictated by intrinsic features of the transplanted tissue.

Ag presentation by both recipient- and donor-derived APCs contributes to T cell allosresponse and initiation of graft rejection. Indeed, alloantigen recognition occurs via two distinct mechanisms, referred to as the direct and indirect pathways. The direct allosresponse results from the stimulation of T cells by intact allogeneic MHC molecules displayed on donor cells. This response is dictated by the migration of donor passenger leukocytes out of the graft to the host’s lymphoid tissues (11–13). Additionally, it has been demonstrated that donor Ags are presented in peptide form during acute graft rejection (14–16). The T cell recognition of processed alloantigens in association with self-MHC molecules at the surface of recipient APCs has been called indirect allorecognition. While the direct response is polyclonal, the indirect response is oligoclonal and usually restricted to the recognition of a few immunodominant donor-derived peptides (17). Based upon this and despite the presence of both types of allosresponses at early stages after transplantation, a direct allosresponse is often viewed as the only driving force in acute rejection. In turn, presentation of alloantigens by recipient MHC class II+ APCs is thought to play a critical role in late acute and chronic graft rejection processes (18–20).

Under certain circumstances, the contribution of the direct response to acute rejection has been shown to be minimal. Abrogation of the CD4+ T cell-mediated indirect response alone could trigger the rejection of skin grafts from MHC class II-deficient mice. It is noteworthy that in this model, anti-donor cytolytic activity mediated by CD8+ T cells was thought to be the effector mechanism (22, 23). Other lines of evidence have been provided suggesting that in the absence of a direct pathway, an indirect allosresponse is sufficient to ensure rejection of allografts (5, 24). Valujskikh et al. (24) have reported that reconstitution of SCID mice with a CD4+ T cell line specific for a single donor MHC class II peptide is sufficient to ensure the rejection of skin allografts. Furthermore, nude mice bearing an MHC class I-disparate skin graft have been
showed to reject their graft after adoptive transfer of CD4⁺ T cells (5). However, in many instances, efficient destruction of MHC class I-disparate allografts requires the presence of a CD8⁺ T cell-mediated direct recognition of the alloantigens (25).

Recent studies using genetically engineered mice have shed some light on the roles of direct and indirect allorecognition in graft rejection. However, the exact contribution of each of these pathways to the alloresponse and the rejection process in normal individuals remains unclear. Corneal transplantation represents a useful model to address this issue. Corneal allografts enjoy high rates of spontaneous acceptance compared with other types of transplantation (40–50%) (26). This feature is particularly helpful to analyze the mechanisms underlying the graft rejection process. Convergent studies have shown that CD4⁺ T cells play an essential role in the rejection of orthotopic corneal allografts (7, 27, 28).

In this regard, corneal transplantation does not differ from other types of tissue transplantation (6). In turn, a number of characteristics clearly distinguish corneal transplantation from other models (29): 1) normal corneas are devoid of MHC class II⁺ APCs (30); 2) the cornea expresses low amounts of MHC class I molecules (31); and, 3) minor histocompatibility Ags are more potent than MHC Ags in mediating corneal allograft rejection (32). Based upon these observations, it has been proposed that the indirect alloresponse represents the driving force in corneal graft rejection, while the direct response does not occur (33). However, the precise nature of the T cell alloresponse and the actual contributions of direct and indirect pathways in corneal transplantation remain to be determined.

Here, we have used the sensitive ELISPOT technique to characterize the T cell alloresponse in a murine model of fully allogeneic corneal transplantation. We demonstrate that in the absence of MHC class II⁺ cells within the graft, allorecognition is mediated by two distinct T cell subsets that differ by their Ag specificities, cytokine profiles, and pathways of Ag recognition (direct vs indirect pathways). The implication of this finding in understanding the mechanisms underlying T cell allorecognition in vivo and its relationship to alloresponse and allograft rejection are discussed.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/c (H-2b), B10.D2/nSnJ (H-2b), C57BL/10 (H-2b), C57BL/6 (H-2b), and BALB.C (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained in our pathogen-free facility at the Schepens Eye Research Institute and treated according to the Association for Research in Vision and Ophthalmology resolution.

Orthotopic corneal transplantation and scoring

Donor central corneas were marked with a 2-mm diameter micropuncture, excised by vannas scissors, and placed in PBS. Recipients were anesthetized by i.p. injection of ketamine and xylazine, and the right eye was excised from a 1.5-mm diameter piece in the central cornea to prepare the graft bed. The donor cornea was placed in the recipient bed and secured with interrupted 11–0 nylon sutures (Sharpoint, Vanguard, Houston, TX). After application of antibiotic ointment, the eye lids were closed for 3 days. Seven days later, the sutures were removed. The degree of opacity as well as the degree of neovascularization were assessed using a slitlamp biomicroscopy as previously described (34). Briefly, the opacification of the graft was quantified using a scoring opacity scale from 0 to 5+. The cornea was considered in a rejecting phase for a score equal to or greater than 2+.

Skin grafts

Tail to back allografts were performed according to the technique previously described by Billingham and Medawar (35). Tail was harvested from euthanized donor mice and placed in a graft bed prepared on the left side of a recipient mouse previously anesthetized with a cocktail of ketamine and xylazine. The graft was secured using Vaseline gauze and a bandage, which were removed 9 days later.

Preparation of responder cells

Spleen cells from naive, cornea-grafted, as well as skin-grafted BALB/c mice were used as a source of responder cells to measure the total alloresponse and the direct and indirect responses. RBC were lysed for 2 min in Tris–NH₄Cl solution. Spleen cells were washed twice in AIM-V (Life Technologies, Grand Island, NY) containing 0.5% FCS and resuspended at 10⁷ cells/ml with 0.5% FCS in AIM-V for further use.

T cells and T cell subsets isolation

T cells as well as CD4⁺ or CD8⁺ T cell subsets were isolated from mouse spleen cells by negative selection using commercially available T cell purification columns according to the manufacturer’s instructions (Accurate Chemical and Scientific, Westbury, NY; R&D Systems, Minneapolis, MN). Purified T cells were washed in HBSS and used at 5 × 10⁶ cells/well in ELISPOT assays.

Preparation of APC

Mitomycin C (MMC)-treated splenocytes from donor and recipient naive mice were used as allogeneic stimulator cells or syngeneic APCs, respectively. Single-cell suspensions of splenocytes devoid of RBC were prepared in AIM-V containing 0.5% FCS and treated with MMC (50 μg/ml) for 30 min at 37°C. The cells were washed once in HBSS, incubated for 10 min at 37°C, and washed once again and finally resuspended in AIM-V/0.5% FCS at 1–3 × 10⁸ cells/ml.

Preparation of sonicates

Stimulator spleen cells were suspended at 3 × 10⁶ cells/ml in AIM-V containing 0.5% FCS and sonicated with 10 pulses of 1 s each. The resulting suspension was frozen in a dry ice/ethanol bath, thawed at room temperature, and centrifuged at 1200 rpm for 10 min to remove intact cells.

ELISPOT assays

Ninety-six-well ELISPOT plates (Polyfiltrons, Rockland, MA) were coated with a capture mAb in sterile PBS overnight. Anti-IL-2, -IFN-γ, -IL-4, and -IL-5 capture mAb were used at 3, 4, 2, and 5 μg/ml, respectively (PharMingen, San Diego, CA). On the day of the experiment, the plates were washed twice with sterile PBS, blocked for 1.5 h with PBS containing 1% BSA, then washed three times with sterile PBS. Responder cells or purified T cells were added to wells previously filled with intact donor cells (direct response) or syngeneic APCs together with donor sonicates (indirect response) as previously described (36). Cells were incubated for different periods of time depending on the cytokine measurement: 20 h for IL-2, 42 h for IFN-γ and IL-4, and 48 h for IL-5. The plates were washed three times with PBS, then four times with PBS containing 0.05% Tween. Biotinylated anti-lymphokine detection mAbs were added at 2 μg/ml (PharMingen) and incubated either for 5 h at room temperature or overnight at 4°C. After washing three times with PBS containing 0.05% Tween, avidin-horseradish peroxidase (1/2000) was added to each well for 1.5 h. Four washes with PBS were performed before the spots were revealed by the addition of the developing solution composed of 0.01 μl of 3-aminoo-9-ethylcarbazole (Sigma; 10 mg dissolved in 1 ml dimethylformamide) in 24 ml 0.1 M sodium acetate, pH 5.0, catalyzed by 12 μl H₂O₂. The resulting spots were counted and analyzed on a computer-assisted ELISPOT image analyzer (C.T.L., Cleveland, OH).

In vitro treatment with anti-CD4 and anti-CD8 mAb

Commercially available rat anti-mouse CD4 (GK1.5) and CD8 (53-672) mAbs were used in vitro blockade experiments at 10 μg/ml (PharMingen).

Results

Rejection of fully allogeneic corneal grafts is associated with an alloresponse dominated by type 1 cytokine-producing cells

Although an allospecific delayed-type hypersensitivity (DTH) response has been reported after allogeneic corneal transplantation (7, 33, 34, 37), the precise nature of the T cells activated in this model remains unclear. Here, we first investigated the pattern of cytokines produced by T cells after allogeneic corneal transplantation in mice. The total T cell response toward donor alloantigens

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3 Abbreviations used in this paper: MMC, mitomycin C; DTH, delayed-type hypersensitivity; wt, wild type.
was analyzed in rejecting and nonrejecting BALB/c mice grafted with fully allogeneic C57BL/6 (B6) corneas. In this combination, 60% of the corneal allografts were rejected 4 wk after transplantation. The rejection was estimated on the basis of the graft opacity from 2 to 5 wk post-transplantation. The frequency of IL-2−, IFN-γ−, IL-4−, and IL-5−producing splenocytes activated in response to allogeneic stimulation was measured in all cornea-transplanted mice. To test this, recipient splenocytes (BALB/c) were cultured in vitro with MMC-treated donor splenocytes (B6), a situation in which T cells can be activated via both direct and indirect allorecognition pathways. After 20–48 h (depending on the cytokine tested) the frequency of type 1 and 2 cytokine-producing cells was determined using the ELISPOT technique as previously described (36). As expected, a primary MLR, mainly characterized by the activation of IL-2 and IL-4 producers, was detected when lymphocytes from naive mice stimulated in vitro with allogeneic cells (Fig. 1). The number of IFN-γ− and IL-2−producing cells was significantly increased in mice undergoing corneal graft rejection compared with nontransplanted animals. No IL-5− and a few IL-4−producing cells were detected in the same conditions. Strikingly, in nonrejecting mice, the frequencies of cytokine producers were equivalent to those found in naive animals (data not shown). These results demonstrate that corneal transplantation elicits a vigorous type 1 response in recipients that is associated with the rejection.

**IL-2 and IFN-γ cytokines are produced by two distinct T cell subsets**

Next, we investigated the contribution of CD4+ and CD8+ T cells to the cytokine production in mice undergoing corneal graft rejection. We performed in vitro blockade experiments with mAbs against CD4 and CD8 molecules (Fig. 2). Activation of IL-2−producing T cells during primary MLR was abrogated by anti-CD4 mAb. In addition, IL-2 release by activated alloreactive T cells harvested from mice undergoing corneal transplant rejection was significantly inhibited by in vitro treatment with anti-CD4, but not anti-CD8 mAb. Conversely, the frequency of alloreactive IFN-γ−producing cells found in these mice was markedly decreased after addition of anti-CD8 mAb. In turn, anti-CD4 mAb treatment had negligible influence on IFN-γ production. We conclude that the T cell response to corneal allografts is mediated by two phenotypically and functionally distinct T cell subsets: CD4+ T cells secreting IL-2 and CD8+ T cells producing IFN-γ.

**Indirect recognition of alloantigens is mediated by CD4+ T cells**

Traditionally, the immune response to allogeneic transplants is initiated by the activation of MHC class II-restricted T cells. In corneal transplantation, since MHC class II molecules have not been detected in corneal tissues, it has been proposed that the CD4+ T cell alloresponse is mediated exclusively toward donor peptides presented by MHC class II+ recipient APCs, i.e., through the indirect pathway. However, no direct evidence has been provided in support of this hypothesis. To address this question, we measured the frequency of cytokine-producing T cells activated through the indirect pathway in cornea-grafted mice using the ELISA spot technique. This technique has been applied recently to measure the indirect response in skin-grafted mice (36). To detect indirect response in cornea-transplanted mice, T cells from rejetor animals were cultured with syngeneic APCs and donor sonicates. As shown in Fig. 3A, a potent indirect response was triggered in cornea-grafted mice. This response was mediated predominantly by IL-2−producing cells, while a significant number of IL-4−producers were also observed. We surmise that these IL-4−producing CD4+ T cells may play an essential role in the induction of DTH response after corneal transplantation, a possibility supported by a recent study from Heeger’s laboratory (38). The frequency of T cells producing IL-2 through the indirect pathway of allorecognition averaged 8 × 10^2 (Fig. 3B). In contrast, the number of IFN-γ producers was not significantly different in grafted vs nongrafted mice (Fig. 3A), a result suggesting that only CD4+ T cells were activated via indirect allorecognition.

To determine the phenotype of IL-2−producers activated through the indirect pathway, we performed in vitro blockade experiments with anti-CD4 and anti-CD8 mAbs. As shown in Fig. 4, the activation of IL-2−producing T cells was blocked by anti-CD4 mAb, while treatment with anti-CD8 mAb had marginal effect (Fig. 4A). To further demonstrate that CD4+ T cells were activated through the indirect pathway, the CD4+ T cell population was purified from graft-rejecting mice and restimulated in vitro with recipient APCs and donor sonicates. As shown in Fig. 4B, purified CD4+ T cells harvested from cornea-grafted mice elicited a potent indirect response. Together, these data show that CD4+ T cells producing IL-2 cytokine mediate indirect recognition of alloantigens in corneal transplantation.

**In cornea-transplanted mice, the indirect alloresponse is not directed toward MHC-derived peptides**

Both donor MHC and minor histocompatibility protein Ags are potential sources of peptides in indirect alloresponses. Some evidence suggests that in skin and heart transplantation models, the majority of T cells activated via indirect allorecognition recognize processed allo-MHC proteins. Here, since minor Ag-mismatched corneas are more readily rejected than their MHC-disparate counterparts, it was crucial to determine whether the same rules of immunodominance apply to cornea-grafted mice. To test this, we compared indirect T cell responses in MHC-disparate and fully allogeneic corneal transplant models. BALB/c mice were grafted either with C57BL/10 corneas (MHC- and minor histocompatibility-mismatched) or BALB.B corneas (MHC-mismatched). Mice undergoing rejection were selected (60 and 20% rejection rates, respectively). Recipient splenocytes were restimulated with syngeneic APCs and BALB.B sonicates, and the frequency of IL-2−producing cells activated against donor MHC H-2d-derived Ags
was measured. As shown in Fig. 5, we did not detect any indirect response to allogeneic MHC molecules in mice grafted with fully disparate or MHC-mismatched corneas. In contrast, vigorous indirect responses to allo-MHC were found in the same donor-recipient mouse combinations after skin grafting. We conclude that the indirect recognition of MHC Ags by CD4+ T cells does not contribute to corneal graft specific alloimmunity. It is noteworthy that in mice rejecting MHC-mismatched corneas, the frequency of IL-2 producers was comparable to that recorded in naive mice, while some alloreactive IFN-γ-producing cells were detected in some recipient mice only. Therefore, we cannot exclude that some T cell-independent mechanisms may be involved in the rejection process.

**CD8+ T cells are activated through the direct pathway of allorecognition**

Within days after transplantation, graft bone marrow-derived APCs (B cells, dendritic cells, and macrophages) migrate to the host’s lymphoid organs. Presentation of intact allogeneic MHC molecules on these so-called passenger leukocytes is known to trigger a powerful direct T cell response that plays a critical role in the rejection of all forms of tissue allografts. Corneal transplantation may not follow this rule, since at the time of transplantation corneal grafts are devoid of professional APCs. Based upon this, it has been postulated that these grafts cannot activate T cells through the direct pathway. Here, we investigated whether a direct response occurs after fully allogeneic corneal transplantation. To address this question, the T cell response to intact donor cells was measured. Recipient T cells were purified using negative selection columns and were mixed with MMC-treated donor splenocytes. As expected, some IL-2 and IFN-γ producers were activated via primary MLR. Strikingly, we detected very high numbers of IFN-γ-producing activated cells in mice experiencing corneal graft rejection (Fig. 6). Indeed, while some IL-2-producing cells were not detected in the assay shown in Fig. 6, this response was extremely low compared with that observed in skin-grafted mice. In addition, we found that these few alloreactive IL-2-producing cells were resistant to in vitro treatment with anti-CD4 mAb in cornea-grafted mice (Fig. 2; while in vitro treatment with anti-CD4 mAb completely abolished the primary MLR observed in naive mice). These observations suggest that a few T cells other than CD4+ T cells might produce some IL-2. The frequency of alloreactive IFN-γ-producing T cells averaged $10^{-3}$ in cornea-grafted mice vs $10^{-4}$ in naive animals. To determine the phenotype of these IFN-γ-producing cells, CD4+ and CD8+ T cells were purified and tested for their ability to produce IFN-γ after stimulation with intact

![FIGURE 2. Phenotype of type 1 alloreactive T cells activated in BALB/c mice rejecting C57BL/6 corneas.](http://www.jimmunol.org/)

**FIGURE 2.** Phenotype of type 1 alloreactive T cells activated in BALB/c mice rejecting C57BL/6 corneas. In vitro blockade experiments of type 1 cytokine production were performed using anti-CD4 and anti-CD8 mAbs. Spleen cells collected from cornea-grafted and naive BALB/c mice were incubated with MMC-treated B6 APCs (ratio 1:2) alone (□) or together with blocking mAbs (10 μg/ml): anti-CD4 (■), anti-CD8 (□), or anti-CD4 plus anti-CD8 mAbs (△). The frequency of IL-2 (A) and IFN-γ (B) producers is expressed as the number of spots per million splenocytes. Data are the mean ± SEM obtained from four or five mice tested individually in two independent experiments.

![FIGURE 3. The indirect alloresponse in corneal graft rejectors.](http://www.jimmunol.org/)

**FIGURE 3.** The indirect alloresponse in corneal graft rejectors. The number of IL-2, IFN-γ, and IL-4 cytokine producers (A) as well as the frequency of IL-2-producing T cells (B) activated through the indirect pathway was evaluated using ELISPOT assay. A, Spleen cells collected from cornea-grafted and naive BALB/c mice were incubated with MMC-treated BALB/c APCs (ratio 2:1) and C57BL/6 sonicates. The relative frequencies of IL-2, IL-4, and IFN-γ producers per million splenocytes were determined. The results shown are representative of one of three independent experiments. B, Spleen T cells harvested from cornea-grafted, naive (negative control), and skin-grafted BALB/c mice (positive control) were mixed with MMC-treated syngeneic BALB/c APCs (ratio 1:3) and C57BL/6 donor sonicates. The frequency of IL-2-producing T cells activated through the indirect pathway was then determined. Symbols represent data obtained from individually tested mice (duplicate). The dotted line represents the averaged number of spots found in naive mice stimulated indirectly. The black symbol represents the average frequency of IL-2-producing T cells activated through the indirect pathway in cornea-grafted mice.
corneal transplantation do not influence the fate of the allograft. Two main factors may be responsible for the apparent inability of activated CD8⁺ T cells to mediate corneal graft rejection: 1) an improper stimulation of CD8⁺ T cells by the corneal allograft, and 2) a lack of recognition of MHC class I-positive target cells in the donor cornea. To discriminate between these possibilities, we determined whether corneal graft rejection could be induced following a potential peripheral activation of T cells toward allogeneic MHC molecules. For this purpose, mice that had permanently accepted allogeneic corneas were grafted with allogeneic skin derived from the same donor. BALB/c mice were engrafted with MHC-mismatched BALB.B corneas. We selected recipient mice exhibiting no signs of rejection at 11 wk post-transplantation (80% of the mice in this combination never reject their grafts). These mice were then transplanted with BALB.B skin. Strikingly, 15 days after skin transplantation all mice underwent corneal graft rejection (Fig. 10B). No rejection of corneal graft was observed in mice that had not received a skin graft (Fig. 10A). We conclude that allogeneic MHC molecules expressed on the skin graft had activated alloreactive T cells that caused rejection of corneal allografts. Therefore, in acceptor mice, the failure to reject was not due to the lack of target recognition. Alternatively, our results support the view that in the majority of mice with MHC-mismatched corneal allografts, improper/suboptimal activation of alloreactive T cells accounted for the lack of rejection.
Discussion

In this article, we reported that mice rejecting corneal allografts mount a potent type 1 T cell response associated with the activation of IL-2-producing (A) and IFN-γ-producing (B) T cells responding to intact donor cells was evaluated using ELISPOT assay. Spleen T cells harvested from cornea-grafted, naive (negative control), and skin-grafted BALB/c mice (positive control) were mixed with MMC-treated C57BL/6 APCs (ratio 1:3). Open dark symbols represent data obtained from individually tested naive and grafted mice, respectively. The dotted line represents the averaged number of spots found in naive mice stimulated directly.

Our study demonstrates that allogeneic MHC class I molecules expressed on corneal allografts, devoid of MHC class II APCs, elicit a vigorous CD8+ T cell-mediated direct response. Auchincloss et al. (22, 23) have previously reported that the expression of allogeneic MHC class II molecules on skin allografts was not required to initiate a CD8 direct response. In this situation, Langerhans cells were shown to carry MHC class I molecules to lymphoid organs and activate alloreactive CD8+ T cells. Here, we found that MHC class II+ passenger leukocytes (i.e., dendritic cells and macrophages) are not required to prime alloreactive CD8+ T cells. Strikingly, the frequency of CD8+ T cells stimulated through the direct pathway was similar in cornea- and skin-transplanted mice (36). These observations suggest that donor APCs are not critical for the differentiation of naive CD8+ T cells into IFN-γ-producing alloreactive CD8+ T cells. Previous studies have pointed out that CD8+ T cell activation requires the presence of very few MHC class I molecules on APCs (42). It is also believed that proper costimulation delivered by professional APCs is necessary for lymphokine production and CTL activity by CD8+ T cells (41, 43). Several groups, however, have recently reported that under defined circumstances naive CD8+ T cells can be activated in the absence of professional APCs. For instance, bypass of costimulation can be achieved when the density of TCR ligands is high enough on presenting cells (44, 45). Lanzavecchia et al. (46) have demonstrated that the number of triggered TCRs required to reach the T cell activation threshold is much higher in the absence of costimulation than under normal conditions. In turn, we surmise that under physiological circumstances, costimulation is likely to be critical to achieve full T cell activation. In support of this, there is a markedly reduced Ag-primed population in people suffering from mutations in the gene encoding for CD40 ligand (47). In corneal transplantation, some evidence has been provided suggesting that the cytotoxic activity of CD8+ T cells is impaired (48). We conclude that while CD8+ T cells from cornea-grafted mice secrete IFN-γ, they apparently fail to mature into cytotoxic T cells, a phenomenon also previously observed with HIV-specific CD8+ T cells (49).

The precise role of CD8+ T cells in allograft rejection is still controversial. Activation of a CD8+ T cell response is usually sufficient, but not always necessary, to ensure rejection. This is underscored by a number of observations made in BALB/c mice.
engrafted with B6 skins devoid of MHC class II expression (5). In the absence of direct CD4 alloresponse, CD8+ T cells recognizing donor MHC class I in direct fashion mediated acute rejection. In turn, after depletion of these CD8+ T cells, the remaining CD4+ T cell indirect response alone was sufficient to ensure the rejection process. A direct CD8+ T cell response is not required to ensure the rejection of orthotopic corneal allografts. Indeed, the rejection of fully allogeneic corneas grafted in either CD8 knockout recipients or CD8+ T cell-depleted recipients is not impaired (7, 27). It is important to note that many CD8+ T cells are regularly found in the graft tissue at the time of rejection (50). This observation rules out the possibility that alloreactive T cells cannot reach their targets. The inability of CD8+ T cells to mediate corneal rejection could be related to the environment of the graft itself and/or to the incomplete maturation of these cells into effector T lymphocytes. While orthotopic corneal allografts induce a DTH response, the emergence of CTL activity has been inconsistently reported in mice (34, 48). Conversely, it is clear that corneal allografts placed heterotopically (under the skin) stimulate vigorous allospecific CTL activity (51). Therefore, corneal allograft has the intrinsic ability to stimulate CTL, but this process might not be fully achieved when the graft is placed in the eye. This presumably explains why MHC-disparate orthotopic corneas are poorly rejected. In support of this, we showed that sensitization of T cells to allogeneic MHC molecules by placing an MHC-disparate skin graft resulted in the rejection of initially accepted MHC-mismatched corneas. While an indirect recognition of allogeneic MHC molecules was observed in skin-grafted mice (Fig. 5), there was no indirect response to MHC-derived peptides in cornea-grafted mice. It is therefore unlikely that this response was necessary and sufficient to induce the rejection of corneal grafts. After skin grafting, a vigorous CTL response to donor MHC proteins is normally induced (52). In our experimental model it is likely that the induction of this response accounted for the destruction of the corneal graft.

It has been reported that mice that did not reject corneal allografts 8 wk after transplantation were tolerant to the graft due to an active suppression mechanism (53). In this article, we have described a mechanism by which tolerance can be broken and rejection induced in recipients that totally accepted their grafts. Presumably, both induction of direct and indirect CD4+ T cell responses to donor MHC accounted for the activation/differentiation of functional alloreactive CD8+ T cells. This finding may have interesting implications in corneal transplantation. First, it shows that in mice with long term graft survival, CD8+ T cells against allogeneic MHC were still available. Second, our findings demonstrate that these T cells have retained the capacity to reject the graft when provided with proper stimulation. Therefore, T cell

**FIGURE 8.** CD4+ T cells are not activated directly after corneal transplantation. CD4+ T cells were purified from splenocytes collected in cornea-grafted, naive (negative control), and skin-grafted BALB/c mice (positive control) and then mixed with MMC-treated C57BL/6 APCs (ratio 1:3). The frequency of IL-2-producing CD4+ T cells after direct stimulation was determined using ELISPOT assay. The dotted line represents the number of spots obtained with T cells from nontransplanted mice.

**FIGURE 9.** MHC class I-deficient and wt allogeneic corneal allografts are rejected in similar fashions. BALB/c mice were grafted either with wt B6 corneas (n = 15) or with β2-microglobulin knockout B6 corneas (n = 10). The graft survival was assessed at different time points post-transplantation as described in Materials and Methods.

**FIGURE 10.** Morphology of BALB.B (H-2b) corneal allografts placed onto BALB/c (H-2d) recipient mice. Aspect of the corneal graft under slitlamp bimicroscopy before peripheral sensitization with BALB.B skin allografts (A) and 15 days after (B). It is noteworthy that the formerly transparent BALB.B corneal allograft (nonrejected allograft; A) became highly vascularized and opaque after peripheral sensitization (rejected allograft; B).
tolerance to corneal allografts is not absolute and can be broken when T cells are challenged with appropriate Ag and costimulation in the peripheral host’s lymphoid organs.

Convergent studies have shown that the CD4+ T cell response is required for initiating corneal graft rejection (7, 27, 28). Corneal transplantation represents an interesting model, in that the graft is naturally devoid of MHC class II+ passenger leukocytes and cannot theoretically elicit CD4+ T cell-mediated direct alloresponse (30). In this paper we have clearly identified a CD4 indirect response, but could not detect any CD4 direct response after fully mismatched corneal transplantation. Together, these results demonstrate that the recognition of alloantigens through the indirect pathway is essential to corneal graft rejection. Since a potent DTH toward minor Ags is induced following corneal transplantation, CD4+ T cells activated indirectly could also be the provider of cytokines required for graft destruction. Even though IFN-γ is thought to be essential to mediate the rejection in this situation, it has been shown recently that CD4+ T cells deficient in IFN-γ production are still able to initiate the destruction of an allogeneic skin graft via an indirect allore cognition pathway (54).

In conclusion, we have shown that an allograft naturally devoid of MHC class II+ APCs at the time of transplantation is able to activate T cells through both indirect and direct allore cognition pathways. Each alloresponse is mediated by different T cell subsets, displaying different phenotypes and alloantigen specificities (Table I). Even if the direct CD8 response can induce graft destruction when proper costimulation is provided, the CD4 indirect response is essential in the rejection observed under normal circumstances. This supports the idea that in the absence of MHC class II on the graft and presumably in the case of MHC-matched transplants, indirect alloresponse represents the main driving force in the rejection process. This implies that in these types of transplantation, strategies designed to block the indirect alloresponse, such as donor-peptide tolerization, may be effective at preventing/delaying allograft rejection.

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References


Table I. T cells activated after allogeneic corneal transplantation: a clear dichotomy

<table>
<thead>
<tr>
<th>CD4+ T cells</th>
<th>CD8+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines produced</td>
<td>IL-2 &gt; IL-4</td>
</tr>
<tr>
<td>Allorecognition pathways</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Antigenic specificity</td>
<td>Direct</td>
</tr>
<tr>
<td>Frequency among T cells</td>
<td>8.10^3</td>
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