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Relevance of the Direct Pathway of Sensitization in Corneal Transplantation Is Dictated by the Graft Bed Microenvironment

Syed Huq,* Ying Liu,* Gilles Benichou,*† and M. Reza Dana2*†

Corneal grafts were until recently considered entirely devoid of resident APCs, giving rise to the tenet that alloantigen recognition is mediated exclusively by the indirect (host APC-dependent) pathway. The recent discovery of a resident myeloid corneal dendritic cell population that is normally MHC class II− but can readily up-regulate class II expression during inflammation led us to hypothesize that under certain conditions the direct pathway of allosensitization becomes operative. To test this, corneal allotransplants were performed in either inflamed (high-risk (HR)) or uninfammed (low-risk (LR)) host beds in mice, and the frequencies of host T cells activated via the direct pathway were determined. We found that directly primed CD4+ T cells were detected in the HR but not LR setting, and these cells displayed a clear Th1 phenotype by 2 wk after grafting. Moreover, the use of MHC class II knockout donor tissue led to significantly enhanced survival of HR but not LR allografts. Finally, we show that donor corneal APC demonstrate high expression of CD40, CD80, and CD86 costimulatory molecules when derived from HR but not LR grafts. These data are the first to report that a functional donor APC-dependent direct response is elicited in corneal transplant hosts when the graft bed is inflamed and underscore the relevance of the graft microenvironment in dictating the pathway of allosensitization. The Journal of Immunology, 2004, 173: 4464–4469.

In transplantation, alloantigens expressed by donor tissues are recognized by recipient T lymphocytes that initiate allograft rejection (1, 2). Both recipient and donor-derived APCs residing in the grafted tissue at the time of transplantation can play pivotal roles in orchestrating the host alloresponse. The direct response involves activation of recipient CD4+ Th cells by intact class II molecules borne by graft-derived APC or “passenger leukocytes” that migrate out of the transplant to the host’s lymphoid organs (3, 4). In contrast, the indirect pathway is mediated by host-derived APCs that process and present graft Ags to naive host T cells in association with “self” MHC (5, 6).

Corneal transplantation is by far the most widely performed and successful form of tissue transplantation in the world, with nearly 40,000 cases performed in the U.S. annually (7). Although the survival rate of first grafts performed onto avascular and uninfammed (low-risk (LR)) host beds approaches 90% under cover of topical steroids, well over 50% of corneal grafts performed onto inflammed and vascularized (high-risk (HR)) host beds are rejected, regardless of the immunosuppressive strategy used (8). However, the exact mechanisms that mediate allograft rejection in HR corneal transplantation are incompletely understood.

Corneal transplantation provides an interesting model to study routes of allosensitization and graft failure because the character of the immune response generated is known to differ from that of other solid tissue grafts in several important ways (9). First, B cells and CTLs play virtually no important role in corneal graft rejection (10), while the Th1-type CD4+ T cells are the dominant effectors of the acute rejection process (11). Second, it has been shown that the indirect pathway is the dominant route of allosensitization for CD4+ T cells in corneal transplantation, especially when grafts are placed onto uninfammed LR host beds (6, 12). In fact, given the virtual lack of constitutive MHC class II expression in the cornea, corneal grafts were, until recently, thought to be entirely devoid of resident APC and hence incapable of eliciting a direct CD4+ T cell alloresponse (6, 13, 14).

However, the putative near-exclusive role played by the indirect pathway fails to fully account for several important observations in HR corneal transplantation, which is performed in hosts that are at HR of graft rejection because of corneal pathology present at time of transplantation surgery. For example, the vast majority of grafts placed onto HR beds in mice eyes are fulminatingly rejected within days posttransplantation — a pace of rejection not entirely consistent with that of an indirect alloresponse, which is known to be slower than the direct response and is classically thought to play a more prominent role in late acute and chronic rejection (15, 16). This is particularly the case when the response is emanating from an alymphatic and avascular tissue (cornea), as these characteristics would further retard APC trafficking to and from the graft site. Furthermore, experimental immune modulation directed at impeding host APC infiltration into corneal grafts (hence suppressing the indirect pathway) fails to completely abolish HR graft rejection (17), suggesting that the indirect alloresponse may not be the sole contributor to HR corneal allograft rejection.

Until recently, it was claimed that corneal transplants are incapable of mounting a direct alloresponse (6, 13, 14). However, the
recent discovery that the cornea does in fact possess resident myeloid CD11c+ dendritic cells (DCs) has provided new insights into the immunobiology of corneal transplantation (18), and revived the possibility for direct alloimmunity in corneal grafts. Indeed, our work has shown that while these corneal DC are universally class II+ in their constitutive state, they are capable of expressing class II Ag and migrating out of the graft posttransplantation (19), and are hence potentially capable of directly priming T cells. In this study, we have used the highly sensitive ELISPOT to characterize the T cell alloresponse in both LR and HR corneal transplantation, and we formally establish for the first time the functional relevance of the direct pathway in the HR setting.

Materials and Methods

Mice

Six- to 8-wk-old BALB/c (H-2a) and C57BL/6 (H-2b) mice were purchased from Taconic Farms (Germantown, New York). MHC class II knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Orthotopic corneal transplantation

Two models of orthotopic corneal transplantation were used. C57BL/6 donor grafts transplanted onto normal uninfamed BALB/c host beds served as the model of LR grafting. C57BL/6 grafts placed onto inflamed and neovascularized BALB/c host beds served as a model of HR grafting. The validation of these models of corneal transplantation has been established previously (12, 19). To create inflamed HR host beds, three intratarsal 11-0 sutures were placed aseptically in the central cornea of the right eye of recipient mice 14 days before surgery. Mice demonstrating a robust corneal neovascular response were selected as HR graft recipients.

The method of orthotopic corneal transplantation has been described previously (20). Briefly, on day 0, a 2-mm corneal button was excised from the donor animal and grafted onto a 1.5-mm recipient bed. Eight 11-0 interrupted nylon sutures (Sharpoint; Vanguard, Houston, TX) were placed to secure the graft. Grafts were examined for signs of acute rejection two to three times per week by slit lamp biomicroscopy. A standardized graft opacity scoring system was used to evaluate rejection (20). Grafts were defined as rejected when they became opaque and the iris details could not be recognized clearly.

Preparation of responder cells and stimulator APC

Cells were harvested from draining cervical lymph nodes (LN) ipsilateral to the grafted eyes of transplant recipients (n = 5) at days 3 and 14 posttransplantation. Cells harvested from LNs of ungrafted animals served as controls. The cells were washed once in AIM-V medium (Invitrogen Life Technologies, Grand Island, New York), and resuspended in AIM-V medium containing 1% FBS at a concentration of 5.0 × 10^7 cells/ml for further use. Irradiated splenocytes from donor and recipient naive (ungrafted) mice served as allogeneic and syngeneic APCs, respectively. Single-cell suspensions of splenocytes were prepared in AIM-V medium containing 1% FBS at a concentration of 5.0 × 10^7 cells/ml for further use. Irradiated splenocytes from donor and recipient naive (ungrafted) mice served as allogeneic and syngeneic APCs, respectively. Single-cell suspensions of splenocytes were prepared in AIM-V medium containing 1% FBS at a concentration of 5.0 × 10^7 cells/ml for further use. Irradiated splenocytes from donor and recipient naive (ungrafted) mice served as allogeneic and syngeneic APCs, respectively. Single-cell suspensions of splenocytes were prepared in AIM-V medium containing 1% FBS at a concentration of 5.0 × 10^7 cells/ml for further use. Irradiated splenocytes from donor and recipient naive (ungrafted) mice served as allogeneic and syngeneic APCs, respectively. Single-cell suspensions of splenocytes were prepared in AIM-V medium containing 1% FBS at a concentration of 5.0 × 10^7 cells/ml for further use. Irradiated splenocytes from donor and recipient naive (ungrafted) mice served as allogeneic and syngeneic APCs, respectively. Single-cell suspensions of splenocytes were prepared in AIM-V medium containing 1% FBS at a concentration of 5.0 × 10^7 cells/ml for further use.

T cells and T cell subset isolation

Contamination of host APCs in the responder cell suspension was circumvented by isolating T cells using a commercially available puriﬁcation column according to the manufacturer’s instructions (Cedarlane Laboratories, Hornby, Ontario, Canada). Purified T cells were washed and resuspended in AIM-V medium supplemented with 1% FBS, and subsequently loaded at a concentration of 5 × 10^7 cells/well onto ELISPOT plates. To isolate the CD4+ and CD8-mediated response, commercially available rat anti-mouse CD4 ( GK 1.5) and CD8 (53-6.72) mAbs (BD Pharmingen, San Diego, CA) were used at concentrations ranging from 0 to 10 μg/ml, based on our previously established protocol (6).

ELISPOT assays

Ninety-six-well ELISPOT plates (Polyfritronics, Rockland, MA) were coated with capture Abs (mAb) in sterile PBS overnight. Anti-IL-2, IFN-γ, IL-4, and IL-5 capture mAb (BD Pharmingen) were used at 3, 4, 2, and 5 μg/ml, respectively. On the day of the experiment, the plates were washed twice with sterile PBS, blocked with PBS containing 1% BSA for 1.5 h, and then washed three times with sterile PBS. Purified responder T cells were then loaded into wells preloaded with intact donor splenocytes (direct response). Cells were subsequently incubated for varying periods depending on the cytokine measured: 20 h for IL-2 and IL-4, and 42 h for IL-5 and IFN-γ, based on a previously optimized protocol (21). After incubation, the plates were washed three times with PBS and four times with PBS containing 0.025% Tween 20. Biotinylated anti-lymphokine detection mAbs (BD Pharmingen) were added at a concentration of 2 μg/ml and the plates were incubated overnight at 4°C. The following day, the plates were washed three times with PBS containing 0.025% Tween 20, and subsequently were incubated for 1.5 h with avidin-HRP (25 μg/ml). Next, four washes with PBS were performed and the spots were revealed using a developing solution consisting of 800 μl of 3-aminon-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO; 10 mg dissolved in 1 ml of dimethylformamide/dehydrate) in 24 ml of 0.1 M sodium acetate (pH 5.0), catalyzed by 12 μl H2O2. The resulting spots were then counted and analyzed on a computer-assisted ELISPOT analyzer (Cellular Technology Limited, Cleveland, OH).

Flow cytometry

Cells were harvested and pooled from ipsilateral cervical LN of LR and HR BALB/c allograft recipients (n = 5, each group) at 1, 3, and 7 days posttransplantation. Upon blockade by anti-FcR mAb (CD16/CD32), cells were colabeled with cytochrome-c-conjugated mouse anti-Iaα and PE-conjugated rat anti-mouse CD40, or FITC-conjugated CD80 or CD86 (BD Pharmingen). For isotype controls, cells were labeled with respective fluorochrome-conjugated mouse IgG2a and rat anti-mouse IgG2a (BD Pharmingen). Cells were subsequently washed and analyzed using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). The proportion of donor-derived Iaα-positive cells that were also CD40, CD80, and CD86 positive was quantified. Percent positivity for each costimulatory factor was calculated with respect to isotype control staining (<2% staining in each case). Mean values for each group were compared between HR and LR recipients via the Mann-Whitney statistic. The p values < 0.05 are considered significant.

Results

A direct response is generated as early as 72 h after HR keratoplasty

Cervical LNs draining the transplanted eyes were chosen for study based on the recent finding that these LNs play a critical role in corneal immunity, with complete and universal abrogation of graft rejection following cervical lymphadenectomy (22). To investigate the presence of the direct alloresponse, host cervical LNs were harvested 72 h after surgery and assayed as described in Materials and Methods. Medium alone or syngeneic APC were used in lieu of allogeneic APC as controls. T cells harvested from naive ungrafted mice served as the primary MLR equivalent to develop the background direct alloresponse. In the HR setting, a significant (p < 0.01) increase in the frequency of activated alloreactive T cells producing IL-2 was observed as compared with the ungrafted controls (Fig. 1A). However, the number of directly primed T cells secreting IFN-γ was not appreciably different in the transplanted and control mice (Fig. 1B). These results demonstrate that in HR transplantation, directly primed IL-2-secreting T cells are detected as early as 72 h posttransplantation.

A potent type 1 direct response is detected 2 wk after HR transplantation

To determine the persistence and phenotype of the direct alloresponse, we investigated the frequency of IL-2- and IFN-γ-producing cells 14 days after surgery (a time point just preceding the manifestation of clinical rejection in the murine model). As seen in Fig. 2, significant IL-2 (A) and IFN-γ (B) responses were generated by directly primed responder T cells of HR graft recipients when compared with both LR recipients and ungrafted controls (p < 0.01). In the LR setting, the frequency of T cells producing...
these cytokines did not significantly differ from the naive background signals.

T cells sensitized through the direct pathway may adopt a Th1, Th2, or mixed phenotype, as seen in skin transplantation (21). To determine the frequency of Th1/Th2 biased alloreactive T cells, we performed ELISPOTs using IL-4 and IL-5 (archetypical Th2 cytokines) capture mAbs. Neither IL-4 nor IL-5 was produced at levels above background by T cell responders of any group at any of the time points studied (data not shown). Hence the direct response generated after HR corneal transplantation is of the Th1 type.

Directly primed cells that produce IL-2 in the HR setting are CD4/HLA-DR, while the IFN-γ response is generated by both CD4/HLA-DR and CD8/HLA-DR cells

We next investigated the phenotype of the directly primed cells by blocking the activity of the corresponding T cells in the assay as described previously (6). IL-2 production in HR BALB/c graft recipients was profoundly inhibited in a dose-dependent manner by anti-CD4 mAbs, and only modestly by anti-CD8 mAbs (Fig. 3A). Similar studies were performed to phenotype the directly primed IFN-γ response. IFN-γ production was found to be nearly equally inhibited in a dose-dependent fashion by addition of either anti-CD4 or anti-CD8 Abs, with maximal inhibition of the IFN-γ response with use of both anti-CD4 and anti-CD8 (Fig. 3B). These data suggest that while the phenotype of IL-2-producing T cells is principally CD4⁺, the IFN-γ-producing cells are comprised of both CD4⁺ and CD8⁺ T cells.

Elimination of the direct alloresponse has no impact on LR corneal allograft survival

To assess the actual functional contribution of the direct alloresponse to the rejection process, we used C57BL/6 MHC class II KO mice as donors. The absence of class II on donor cells results, by definition, in lack of a CD4 direct alloresponse. Wild-type (WT) C57BL/6 donor corneas grafted onto BALB/c recipients served as controls. As seen in Fig. 4A, no difference was noted in...
the timing or frequency of rejection of LR grafts in the absence of donor MHC class II on the cornea, further confirming that the direct pathway is not materially involved in the rejection of LR transplants.

**Elimination of the direct pathway of T cell sensitization significantly improves corneal allograft survival in vascularized HR beds**

To test the effect of eliminating the donor class II-mediated direct response in HR grafting, MHC class II-deficient C57BL/6 donor grafts were placed onto inflamed HR beds of BALB/c recipient mice. WT C57BL/6 corneas grafted onto HR BALB/c beds served as controls. All WT grafts were swiftly and uniformly rejected by 2 wk (Fig. 4B). By contrast, grafts obtained from class II KO mice demonstrated both delayed and a lower frequency of rejection, with a significant improvement in median survival (21 days vs 11 days in controls \( p < 0.01 \)), suggesting that the direct priming of host T cells that occurs in HR corneal transplantation is functionally relevant and contributes significantly to the acute and universal rejection seen in HR transplants.

**High proportion of donor APCs draining from HR grafts express costimulatory molecules CD40, CD80, and CD86**

Previous data from our laboratory have shown that donor-derived APCs migrate to the draining cervical LNs in both LR and HR transplantation, but that the trafficking is significantly enhanced from HR grafts (19). However, the data as summarized above show the induction of a direct response solely in HR corneal transplantation. Can this be explained by sheer volume of APC traffic, or do the APCs derived from HR grafts have a distinct phenotype that would make them more capable of priming? To investigate these issues, we examined the phenotype of donor-derived DCs...
that migrate to the draining LN from LR and HR transplant recipients using flow cytometry, and measured the proportion of APCs staining positively with donor class II and the CD40, CD80, and CD86 costimulatory molecules. Fig. 5 demonstrates expression of these molecules at 1, 3, and 7 days posttransplantation. Expression of CD40, CD80, and CD86 is distinctly higher on donor APCs trafficking to LNs of HR graft recipients compared with that of LR recipients at every time point tested. These data suggest that the donor DCs undergo significant phenotypic changes depending on the presence or absence of inflammation in the host bed.

Discussion
It has been firmly established that the indirect pathway plays a critical role in corneal transplantation, especially when grafts are placed onto uninfamed host beds. In the context of minimal inflammation, the absence of constitutive expression of MHC class II by normal corneal tissues (13, 14) has been implicated in the apparently minor role of the direct pathway in LR transplantation. Indeed, Boisgerault and coworkers (6), and others (12), have convincingly demonstrated that indirectly primed allospecific CD4+ T cells generated against minor transplantation Ags are vital in driving the alloimmune response in LR corneal transplantation. Our data lend further support to the concept that the direct pathway is not materially operative in rejection of LR allografts. Using LN responder T cells isolated from transplanted hosts, we were unable to detect any direct response in LR graft recipients. Similarly, elimination of the class II-mediated direct response by use of class II KO donor tissue had no effect on the survival of these LR grafts.

However, from a clinical standpoint, the main problem in corneal transplantation is not survival of LR grafts (which enjoy very high survival rates under cover of topical steroids), but rather the prognosis of grafts placed onto inflamed beds that are vascularized or have been sites of previous graft failures (7, 12, 19). Indeed, with nearly 40,000 corneal transplants performed annually in the U.S. alone, the burden of HR transplantation is increasing given the almost uniformly poor outcome of these grafts (8). Importantly, while data suggest that the indirect pathway is functional in the HR setting (12, 17), inhibiting it (e.g., via blockade of host APC infiltration into grafts) consistently fails to completely prevent graft rejection (17).

The data presented herein formally demonstrate the existence of directly primed CD4+ Th1 cells in HR transplantation. This is important because in both LR and HR grafted hosts, CD4+ T cells are known to be the chief mediators of acute corneal graft rejection (10, 11, 23). The role CD8+ T cells play in corneal alloimmunity is incompletely understood. Multiple laboratories have shown (via depleting CD8 T cells and/or CTL activity) that acute corneal graft rejection is nearly exclusively mediated by CD4+ cells, and, while CD8+ cells are often primed, their function remains speculative (10, 11, 23). In terms of timing of the direct allosresponse, our data demonstrate that direct priming can be detected as early as 72 h after HR transplantation, and the T cells develop a distinct Th1 phenotype, secreting both IL-2 and IFN-γ, by 2 wk after grafting. The differences in the cytokines generated (IL-2 early vs IFN-γ late) suggest that primed T cells produce principally proliferative signals at the early time points, while the IFN-γ-secreting cells that have been associated with effecting graft destruction (24) manifest later, and correlate with acquisition of donor-specific delayed-type hypersensitivity at 2 wk. These data correspond to the results of King et al. (25), who similarly found that IFN-γ expression is delayed until the onset of clinical rejection in the rat model of corneal transplantation. Although not statistically significant, it may be interesting to note that the Allo-LR response at 2 wk was somewhat lower than the naïve-Allo controls. This observation may be consistent with a form of tolerogenic immune response generated to alloantigens when grafted into LR recipients (9, 26). In the absence of statistical significance, however, it is difficult to interpret the importance of this finding. Moreover, our in vivo studies (Fig. 4) using corneas from class II K0 donors grafted onto LR hosts showed that these transplants do not fare better or worse, hence the role of a putative tolerogenic direct response (vs the well-established tolerogenic or “deviant” response generated by the indirect pathway in response to corneal minor histocompatibility Ags) does not seem to materially affect the fate of LR grafts.

Our data demonstrate that a direct response is detected solely in the HR setting and is conspicuously absent in LR graft recipients, while donor APCs are known to traffic to local nodes in both LR and HR transplant recipients (although donor cell traffic is more pronounced after HR transplantation) (19). To explain this finding, we studied the phenotype of donor-derived APCs harvested from both LR and HR transplant recipients and found that donor-derived LN-trafficking cells from HR grafts have markedly higher expression of CD40, CD80, and CD86 compared with APCs harvested from LR recipients. The cornea is endowed by other cells (including non-DC monocytes) that are also capable of expressing MHC class II molecules, but our studies to date have identified DC to be the principal cell type that migrate as class II+ cells to the T cell-rich parafollicular areas of draining cervical LN (19). The HR transplantation setting involves migration of passenger leukocytes through a “hostile” and inflamed graft bed microenvironment, a process that appears to significantly alter their phenotype. Our previous work has already shown the critical dependence of alloimmunization on CD40 ligation (27), and hence in the context of our current data, we hypothesize that the heightened acquisition of CD40, along with the B7 molecules, permits the DC’s capacity to incite a robust direct alloimmune response in the HR setting.

The functional role played by directly primed T cells assayed in vitro was confirmed in vivo. In the absence of MHC class II expression, corneal graft survival in HR beds was significantly enhanced, suggesting that the direct allosresponse by CD4+ T cells does indeed contribute significantly to the HR phenotype. It is important to emphasize that while there was a marked reduction in
the tempo and frequency of rejection among class II KO grafts in the HR setting, still, the overall graft survival (25%; Fig. 4B) was lower than that observed in LR grafts (50%; Fig. 4A). This implies that the intensity of the alloimmune response in HR grafting cannot be entirely accounted for by the direct pathway. One possible explanation for this is that the kinetics and potency of the indirect pathway may also be accelerated in HR keratoplasty. Indeed, our previous data demonstrating that HR corneal grafts can also acquire large numbers of host Langerhans cells (28), strongly suggest that the HR environment may also facilitate the indirect pathway. Hence, the observed fulminancy of HR corneal graft rejection is likely the result of the effect of both a direct as well as heightened indirect alloimmune response.

These data may have significant clinical implications. The Collaborative Corneal Transplantation Studies (CCTS) were performed to assess the contribution of HLA matching to HR corneal graft survival in humans (7). Interestingly, when the results were published no such benefit was seen despite many reports to the contrary emanating from Europe (29, 30). The CCTS data were subsequently interpreted as further evidence for lack of a direct pathway in corneal transplantation (20), and led, in almost all cases, to cessation of tissue HLA matching in the U.S. However, even simultaneous with the published results of the CCTS were claims that the very high dose of postoperative steroids used in the CCTS protocol had biased the results toward the null hypothesis by blunting donor MHC expression (31). Our data provide proof that direct priming does indeed occur, and is relevant to survival of HR grafts; raising the question once again of the utility of HLA matching in HR corneal transplantation.

Corneal transplantation represents the only recourse for millions of people worldwide suffering from corneal blindness. HR corneal beds are encountered frequently, with regrafting, chronic inflammation, and burns being common indications. However, graft survival rates in this group rarely exceed 50% despite intensive immunosuppressive therapy (8). Our study is the first to directly test and affirm the contribution of direct alloreactivity to the rejection of HR grafts. These findings shift the focus of the conventional paradigm in corneal transplantation from exclusively host-mediated alloimmunity to the active role played by the graft tissue in orchestrating the immune response.

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