Interleukin-1 Receptor Antagonist Therapy and Induction of Anterior Chamber–Associated Immune Deviation–Type Tolerance after Corneal Transplantation

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Purpose. Topical treatment with interleukin 1 receptor antagonist (IL-1ra) can promote corneal allograft survival by suppressing induction of alldestructive immunity. The purpose of these experiments was to determine whether IL-1ra could also promote induction of allospecific tolerogenic pathways, including anterior chamber–associated immune deviation (ACAID), which has been shown to participate in long-term survival of corneal transplants.

Methods. Corneal buttons from BALB/c (syngeneic) or C57BL/6 (fully mismatched allogeneic) mice were orthotopically grafted onto BALB/c recipients. Topical IL-1ra or vehicle alone was applied to grafts three times daily. Donor-specific ACAID was measured in allogeneic grafted mice at 4 and 8 weeks after transplantation by ear-challenging grafted hosts with donor-derived splenocytes 1 week after SC immunization. In separate experiments, grafted mice were treated for 4 weeks before injecting ovalbumin (OVA) into their anterior chambers to determine their capacity to induce antigen-specific ACAID.

Results. Treatment with IL-1ra did not promote, or inhibit, induction of donor-specific ACAID compared with vehicle-treated controls at either the early or late time points studied. However, IL-1ra treatment after transplantation led to significantly earlier restoration of the grafted eyes’ capacity for inducing ACAID to soluble antigen (OVA).

Conclusions. Promotion of OVA-specific ACAID by IL-1ra suggests that suppression of IL-1–mediated mechanisms contributes to recovery of the anterior segment’s immunosuppressive microenvironment at least 1 month earlier than would otherwise be seen after corneal transplantation. However, IL-1ra treatment does not alter induction of donor-specific ACAID after transplantation, suggesting that its anti-inflammatory activities do not lead to an ACAID-inducing signal per se. This suggests that IL-1ra promotes graft survival almost exclusively by virtue of suppressing inflammation and not by directly promoting tolerance or antigen-specific regulatory pathways.

Corneal transplantation has emerged as the most common and successful form of tissue transplantation. The extraordinary success of corneal transplants has been related to its immune privileged status in the ocular microenvironment, where orthotopic corneal allografts can experience prolonged survival even without immunosuppressive treatment. This is all the more remarkable given that corneal graft recipients universally become sensitized to donor-derived antigens after transplantation regardless of the final outcome of the transplanted tissue. However, in spite of this universal allosensitization, it still remains unknown why some grafts get rejected and others survive indefinitely. It has been proposed that this may be related to acquisition of tolerance by some hosts, as evidenced by the observation in laboratory animals that long-term acceptance of corneal grafts is associated with a form of donor-specific tolerance known as allo-specific anterior chamber–associated immune deviation (ACAID). Moreover, induction of donor-specific ACAID has been shown to promote graft survival, suggesting that induction of this form of allo-specific tolerance may play a critical role in long-term corneal transplant survival.

Interestingly, the time course for induction of alloreactive responses in experimental corneal transplantation does not perfectly coincide with that for induction of tolerogenic signals—a fact that may explain the observation that the preponderance of corneal graft rejections (whether in humans or rodents) occur in the relatively early period after transplantation. For example, in the mouse model, although induction of alldestructive delayed-type hypersensitivity (DTH) occurs very early after transplantation, induction of tolerogenic ACAID–promoting signals takes at least 8 weeks after orthotopic grafting. The exact reason for the observed delay in induction of tolerance remains unknown, but it has been...
demonstrated that the procedure of transplantation itself (even in the syngeneic setting) is sufficient to perturb the ocular microenvironment and abrogate the eye’s, or aqueous humor’s, normal capacity to support ACAID induction for several months.\(^6\)\(^\text{7}\) It has been postulated that surgical manipulation of eyes may induce the overexpression of a wide array of inflammatory cytokines including interleukin (IL)-1, which potentiates immune responsiveness and thereby suppresses ACAID induction.\(^6\)\(^\text{6}\)\(^\text{8}\)

IL-1 has a wide range of activities that promote immunoinflammatory responses, including critical mediation of the acute-phase response, chemotaxis, activation of inflammatory and antigen-presenting cells, upregulation of adhesion and costimulatory factors on cells, and stimulation of neoangiogenesis.\(^9\) IL-1 receptor antagonist (IL-1ra) is a naturally occurring IL-1 isof orm with high-affinity binding to IL-1 receptors but with no agonistic activity even at high concentrations, and hence is capable of profound downregulation of IL-1–mediated responses in both humans and rodents.\(^9\) There are at least two separate, not necessarily mutually exclusive, mechanisms that can explain IL-1ra’s suppression of alloimmunity and graft rejection: (a) active suppression of sensitization (i.e., priming of allodestructive Th1 cells), and/or (b) promotion of tolerance to donor antigens. Because topical IL-1ra can prevent DTH-type sensitization to corneal grafts by downmodulating antigen-presenting cell function and local inflammation,\(^3\)\(^\text{4}\)\(^\text{10}\) we performed the experiments described herein to determine whether topical treatment with IL-1ra can similarly promote the early induction of tolerogenic ACAID in grafted eyes. Two models were studied\(^1\): recovery of the grafted eye’s immune privileged microenvironment and capacity to support induction of ACAID to intracameraly injected soluble antigen, and\(^2\) the rapidity with which the host can acquire donor-specific ACAID.

**MATERIALS AND METHODS**

**Mice**

Eight- to 10-week-old male mice were purchased (Taconic, NY). Animals with dystrophic/degenerative corneal calcific deposits were excluded from study. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. BALB/c (H-2\(^b\)) strain mice were used as recipients and C57BL/6 (MHC and minor alloanigenic) strain mice were used for positive controls denoted ACAID.

**Orthotopic Corneal Transplantation**

Our usual method for corneal transplantation has been described in detail elsewhere.\(^3\)\(^\text{4}\)\(^\text{10}\) Briefly, all animals were deeply anesthetized before surgical procedures. The central 2-mm of the donor cornea was excised and secured in recipient graft beds with eight interrupted 11-0 nylon sutures (Sharpoint; Vanguard, Houston, TX). Antibiotic ointment was applied to the corneal surface, and the lids were closed for 24 hours with an 8-0 nylon tarsorrhaphy. All grafted eyes were examined after 72 hours; grafts with technical difficulties (lymphema, infection, or loss of anterior chamber) were excluded from further consideration. Transplant sutures were removed in all cases on day 7.

**Pharmacological Strategy and Assessment of Donor-Specific ACAID Induction**

Topical preparations were applied to recipient mice three times daily from the day of grafting until the appropriate time point as detailed below. The study medication was composed of 2% human recombinant IL-1ra in 0.2% sodium hyaluronate in PBS (Amgen, Thousand Oaks, CA). Controls received the vehicle 0.2% sodium hyaluronate alone. Allografted, positive, and ACAID control mice were immunized by SC injection of 10\(^\times\)10\(^6\) C57BL/6 splenocytes. ACAID control mice in addition received anterior chamber injection of 5\(^\times\)10\(^5\) C57BL/6 spleen cells 1 week before SC immunization.\(^7\) Neither the positive or ACAID controls were grafted. Seven days after immunization, 1\(^\times\)10\(^6\) irradiated (2000 rad) C57BL/6 splenocytes in 10 \(\mu\)l Hanks’ balanced salt solution (HBSS) were injected into the right ear pinnae. At 24 and 48 hours after ear challenge, ear thickness was measured with a low-pressure micrometer (Mitutoyo, MTI Corporation, Paramus, NJ). Ear swelling was expressed as follows: specific ear swelling = (24-hour measurement of right ear − 0-hour measurement of right ear) − (24-hour measurement of left ear − 0-hour measurement of left ear) \times 10\(^{-3}\) mm. Ear swelling responses at 24 hours after injection are presented as a group mean ± SEM measurement.

**Assay for the Ability of an Eye to Support ACAID Induction**

Animals received 50 \(\mu\)g ovalbumin (OVA) in 3 \(\mu\)l HBSS into the anterior chamber of syngeneically grafted eyes. Anterior chamber inoculations of ungrafted normal eyes served as ACAID controls. Seven days later these animals and positive controls (not receiving intracameral normal eyes) were immunized with OVA (100 \(\mu\)g) emulsified 1:1 in CFA in a total volume of 100 \(\mu\)l injected SC into the nape of the neck. In antigen-specificity control experiments some animals were immunized with bovine serum albumin (BSA) emulsified in CFA. Seven days after SC immunization, mice received intradermal inoculation of OVA or BSA (200 \(\mu\)g/10 \(\mu\)l of HBSS) into the right ear pinnae, and the antigen-specific ear swelling response was assessed as described above. For all experiments, DTH responses after antigenic challenge that were significantly lower than that observed in positive controls denoted ACAID.

**Statistical Methods**

Statistical analyses were performed by using the Student’s \(t\)-test for comparison of DTH responses. Second, we constructed Kaplan–Meier survival curves and used the Breslow–Gehan–Wilcoxon test to compare the probability of corneal graft survival. All \(P\) values < 0.05 were deemed significant.

**RESULTS**

**Induction of Allospecific ACAID after Corneal Transplantation**

As reported previously,\(^10\) IL-1ra treatment (\(n = 15\)) led to significant reduction in the rate of allograft rejection (Fig. 1A) compared with controls (\(n = 10\)) treated with vehicle alone (\(P = 0.05\)). Because we have previously shown that IL-1ra can suppress induction of donor-specific DTH,\(^1\) in the current experiments we aimed to determine whether IL-1ra could similarly affect the induction of tolerogenic mechanisms such
Injection of OVA into eyes bearing corneal grafts and treated immunized with OVA in adjuvant and ear-challenged (Fig. 2). That had not received intracameral antigen, were subsequently control mice were those receiving intracameral injection of alloreactivity, the data indicate that the acceptors, regardless of for one host, long-term corneal graft acceptors, regardless of treatment regimen, exhibit donor-specific tolerance compared with primed positive controls; \(P < 0.001\). Similar results were obtained at 48 hours.

allospecific ACAID, which is normally observed 8 weeks after transplantation in accepted grafts.\(^5\) At the 8-week time point, all mice, except for negative controls, were SC immunized with donor splenocytes and subsequently challenged with donor cells to assay for donor-specific tolerance (Fig. 1B). Except for one host with an accepted graft demonstrating heightened alloreactivity, the data indicate that the acceptors, regardless of their treatment regimen, exhibited suppressed allospecific responses compared with rejectors or positive controls \(P < 0.001\). There was a preponderance of grafted hosts treated with IL-1ra demonstrating allospecific DTH ear swelling responses show that except for one host, long-term corneal graft acceptors, regardless of treatment regimen, exhibit donor-specific tolerance compared with primed positive controls; \(P < 0.001\). Similar results were obtained at 48 hours.

FIGURE 1. Corneal graft survival (A) and induction of donor-specific ACAID (B) in mice treated with topical IL-1ra or vehicle for 8 weeks. Topical therapy with IL-1ra leads to significant promotion of allograft survival \(P = 0.03\) as demonstrated by Kaplan–Meier analysis (A). Donor-specific responses were measured after allospecific challenge in the ears of negative (Naive) controls, positive controls sensitized to donor splenocytes (Primed), and allografted mice either treated with IL-1ra (IL-1ra Tx) or vehicle alone (Vehicle Tx). Mean 24-hour antigen-specific DTH ear swelling responses show that except for one host, long-term corneal graft acceptors, regardless of treatment regimen, exhibit donor-specific tolerance compared with primed positive controls; \(P < 0.001\). Similar results were obtained at 48 hours.

Capacity of Grafted Eyes to Mount Antigen-Specific ACAID to Intracameral Soluble Antigens

Previous studies have shown that the capacity of the eye to support “deviant” or tolerizing immunity to intracameral injected antigens is lost for at least the first 8 weeks after transplantation, even in the syngeneic setting.\(^6\) To test whether the anti-inflammatory properties of IL-1ra could restore this capacity earlier than would otherwise be possible, the capacity of the grafted eye to induce ACAID to a nominal antigen (OVA) was tested under cover of IL-1ra treatment. Except for one host with an accepted graft demonstrating heightened alloreactivity, the data indicate that the acceptors, regardless of their treatment regimen, exhibited suppressed allospecific responses compared with rejectors or positive controls \(P < 0.001\). There was a preponderance of grafted hosts treated with IL-1ra demonstrating allospecific ACAID, but the direct association observed was between graft acceptance and ACAID and not between IL-1ra treatment per se and ACAID induction, as reflected by the hosts treated with IL-1ra that failed to exhibit donor-specific tolerance.

Possibility of Early Induction of Allospecific ACAID after Transplantation

Because data from the OVA experiments suggested that topical application of IL-1ra could promote swift recovery of the ACAID-supporting microenvironment of the eye, we embarked on a series of experiments to test whether IL-1ra could likewise induce ACAID to donor antigens in the early postoperative period because this could provide one possible mechanism to explain this cytokine’s promotion of corneal graft survival. Three weeks after allotransplantation, vehicle \((n = 6)\) or IL-1ra-treated \((n = 6)\) grafted (and control ungrafted BALB/c; \(n = 5)\) mice were immunized with C57BL/6 splenocytes before ear challenge at 4 weeks (Fig. 3). The data suggest that treatment with IL-1ra does not lead to early induction of allospecific ACAID. Rather, the data clearly show that SC immunization of the host to donor antigens, early after transplantation, leads to a robust allospecific response regardless of the treatment rendered during the engraftment period.

Necessity of Continuous Treatment with IL-1ra for Promoting Graft Survival

The data above suggest that although IL-1ra can restore the ocular microenvironment sufficiently to induce ACAID to sol-
result of IL-1ra treatment is antigen-specific and limited to antigens introduced to the specific eye thus treated. In the first series of experiments we wanted to determine whether the observed suppression of DTH reactivity to graft antigens as a result of IL-1ra therapy also extended to unrelated (third-party) antigens (Fig. 5A). BALB/c hosts (n = 12) were grafted with C57BL/6 corneas and were randomized to receive either IL-1ra or vehicle for 3 weeks, after which the grafted eyes were enucleated. One week later animals were immunized to third-party C3H/HeN antigens by SC injection, followed 1 week later by ear challenge to C3H/HeN antigens. Positive controls (n = 4) were treated with vehicle only and were immunized to C3H/HeN antigens before ear challenge; negative controls (n = 4) were treated with IL-1ra and were not immunized before ear challenge. Our results showed that ocular treatment with IL-1ra did not suppress generation of C3H-specific DTH (Fig. 5A).

In a second set of experiments we determined whether the ACAID-promoting effect of IL-1ra on grafted eyes also extended to irrelevant antigens. BALB/c animals (n = 12) were syngeneically grafted and treated with IL-1ra for 4 weeks at which point the grafted eyes were intracamerally injected with OVA. One week later they were immunized SC to an unrelated antigen, BSA, followed by ear challenge to BSA. Positive controls (n = 4) did not receive any intracameral antigen but were immunized to BSA before ear challenge; negative controls (n = 4) did not receive either intracameral antigen or SC immunization before ear challenge. As summarized in Figure 5B, the generation of ACAID response to intracameral OVA that we had observed in grafted eyes treated with IL-1ra (Fig. 2) did not extend to an irrelevant antigen BSA, suggesting that suppression of Th1-type immunity to ocular antigens as a result of treatment with IL-1ra did not extend to antigens presented to the host at nonocular sites. Finally, in another set of experiments we determined whether application of IL-1ra to one eye can modulate the immune response to antigens presented to the fellow eye. Syngeneic grafts were performed in the right eyes of animals (n = 12), whereas their unmanipulated left eyes of animals (n = 12), whereas their unmanipulated left eyes were repeated at least once.

Antigen and Site Specificity of IL-1ra Modulation of Immune Responses to Ocular Antigens

Three separate series of experiments were performed to test whether the modulation of immunity to ocular antigens as a
allospecific ACAID to ocularly delivered antigens is considered an important facet of ocular immune privilege. However, the precise mechanisms that underlie this phenomenon are not fully understood.

The experiments described in this report were conducted to test the effect of IL-1ra treatment on corneal graft survival in a model of allogeneic orthotopic transplantation (Fig. 2). Our results suggest that IL-1ra treatment can promote the restoration of the eye's capacity to support induction of ACAID-type tolerance to soluble and donor-derived antigens. The control experiments that have been performed (Fig. 5), evaluating the effect of IL-1ra on irrelevant or third-party antigens presented to the host at nonocular sites or to untreated eyes, demonstrate that the effect of topical IL-1ra on modulating immunity to ocular antigens is both antigen- and site-specific. Our results suggest that although IL-1ra treatment can promote the restoration of the eye's capacity to support induction of ACAID to intracamerally delivered soluble antigens at least 1 month earlier than would otherwise occur after corneal transplantation (Fig. 2), it does not promote (Fig. 3) or abolish (Fig. 1B) the graft's capacity to induce allospecific tolerance. Hence, the greater propensity for donor-specific tolerance seen in IL-1ra-treated animals, when evaluated as a group, appears to be more a function of the increased rate of acceptance in hosts treated with this cytokine than a function of the active generation of tolerogenic signals per se. This conclusion is further supported by our results demonstrating that early termination of IL-1ra therapy leads to subsequent graft failure (Fig. 4), because induction of tolerance should have a more long-lasting effect on transplant survival.

It is important to discuss the limitations of our study. Although we and others have shown that ocular antigen presentation and priming of DTH responses can be potentiated by IL-1, the evidence presented in this report suggests that IL-1ra does not suppress or promote induction of allospecific ACAID. Our data do not address the reasons why early restoration of the ocular microenvironment's capacity to induce ACAID to soluble antigens cannot be replicated for alloantigens. It is unlikely that IL-1ra's failure to generate early allospecific tolerance is dose-dependent. We have already established that significantly lower doses of topical IL-1ra than that used in this study can have comparable efficacy in suppressing anterior segment inflammation. Hence, it is unlikely that higher doses of IL-1ra can promote early allospecific ACAID. However, several other possibilities exist that may explain IL-1ra's failure to directly generate donor-specific tolerance, including different mechanisms that may regulate immunity to soluble compared with allogeneic antigens. For example, it is known that generation of a deviant form of immunity to intraocular antigens is critically dependent on the functional presence of a cameralosplenic axis responsible for presentation of antigen by APCs of the iris and ciliary body in the context of immunomodulatory factors (e.g., TGF-β, IL-10) that allow for generation of regulatory cells in the spleen. Although it has been proposed that tolerance to graft antigens may also involve generation of regulatory cells in the spleen, in addition, fundamental differences in the nature of soluble compared with cell-associated transplantation antigens, which can affect how these antigens are processed and presented, may affect the capacity of IL-1ra to modulate the immune response generated to a specific antigen.

Finally, it is possible that allotransplantation leads to more inflammation than syngeneic transplantation; hence, the same
anti-inflammatory effect of IL-1ra that can restore ACAID to soluble antigens in the syngeneically grafted eyes may fail to do so in the allografted eyes. Theoretically, this could be tested by evaluating induction of ACAID to soluble antigens in allogeneically grafted eyes. Practically, however, it is not feasible to use an allografted eye to test for ACAID to an irrelevant antigen (e.g., OVA); allograft rejection would confound the data derived from the OVA experiments because we know that rejecting eyes cannot support ACAID because of the significant inflammation generated during rejection. Moreover, other data from our laboratory do not support the hypothesis that the failure of IL-1ra to directly induce allospecific is due to increased inflammation in the allograft setting. IL-1ra-mediated restoration of ACAID-inducing ability to soluble antigens has been documented even in highly inflamed eyes with corneal neovascularization. Second, even in the absence of strong allogeneic DTH-inducing inflammation the acquisition of donor-specific ACAID takes a long time. Hence, it may be that the shedding of antigen from corneal grafts and their processing by intraocular ACAID-generating APCs takes a long time regardless of the cytokine milieu of the anterior segment.

Our data suggest that IL-1ra’s promotion of graft acceptance is largely by virtue of suppressing sensitization and not by promoting antigen-specific regulatory pathways per se. Because in the first month after transplantation IL-1ra can suppress generation of allospecific DTH, and yet generation of donor-specific ACAID (with or without IL-1ra) requires at least 2 months, our data would suggest that an effective way of maximally promoting graft survival with this cytokine short of its indefinite use is to use IL-1ra sufficiently long to allow for the normal generation of allospecific tolerogenic signals. In fact, we have data (not shown here) to support this prediction. Long-term (>16 weeks) data from our laboratory on animals treated for 8 weeks with IL-1ra and then followed without any treatment reveal that allograft rejection beyond 8 weeks (the standard follow-up period) is in fact rare. From a clinical application standpoint, the failure of IL-1ra to promote allospecific tolerance would mean that coverage with IL-1ra for the first several months after surgery may be required to prevent most cases of graft rejection.

References