The Critical Role of Lymph Nodes in Corneal Alloimmunization and Graft Rejection

The Critical Role of Lymph Nodes in Corneal Alloimmunization and Graft Rejection

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Purpose. To elucidate the role of draining cervical lymph nodes (CLNs) in corneal alloimmunity.

Methods. Fully mismatched orthotopic corneal transplantation was performed in BALB/c hosts that had their CLNs excised before transplantation (CLN^-). Normal hosts (CLN^+), splenectomized mice (Sp^-), and those without either CLNs or spleen (CLN^-/Sp^-) served as comparison groups. To determine the contribution of CLNs to alloimmunity more directly, CLN^- mice were reconstituted by grafting LNs from other BALB/c mice to their cervical lymphatic chains, thus deriving $CLN^{-/+}$ mice. Tetramethyl rhodamine isothiocyanate's (TRITC) flow to draining CLNs was used as a measure of afferent lymph flow. Graft survival and allospecific delayed-type hypersensitivity (DTH) were used as measures of alloreactivity.

Results. Fifty percent of normal control and 12% of Sp^- hosts accepted the allografts. In contrast, 100% of CLN^- and 88% of CLN^-/Sp^- hosts accepted allografts indefinitely ($P < 0.01$). Additionally, all CLN^- hosts failed to demonstrate allospecific DTH ($P < 0.001$). $CLN^{-/+}$ mice reconstituted with LN from naïve animals showed graft survival rates and DTH responses that were indistinguishable from those of naïve CLN^+ mice. Of particular interest, however, is that mice reconstituted with CLNs from hosts with rejected corneal grafts had swift rejection of subsequent corneal grafts and exhibited strong donor-specific DTH. In contrast, mice reconstituted with CLNs from hosts with accepted corneal grafts showed rejection of subsequent corneal grafts in a manner that was indistinguishable from rejection in naïve CLN^+ hosts.

Conclusions. Draining CLNs play a critical role in allosensitization and rejection. In contrast to the spleen, draining CLNs do not appear to play a critical role in tolerance induction in corneal transplantation. (*Invest Ophthalmol Vis Sci.* 2001;42: 1293–1298)

Corneal transplantation represents, by far, the most common form of organ allotransplantation in the world. In the United States alone, nearly 40,000 corneal grafts are performed annually.[1] Most corneal allografts performed in low-risk hosts without ocular inflammation survive indefinitely under cover of topical corticosteroid therapy. However, this therapy is commonly associated with serious side effects including glaucoma, cataracts, and opportunistic infection. Moreover, the survival of high-risk corneal transplants performed in inflamed host beds, which is an increasingly common indication, is well below 50%, even with the use of local and systemic immunosuppressive therapy.[2,3] Therefore, determining the mechanisms of corneal transplant rejection is a priority in ocular immunology research.

Cervical lymph nodes (CLNs) drain the head and facial region, including the eyes,[4,5] and have been implicated in both mucosal antigen-specific immunization and tolerance.[5–8] In a classic series of experiments Wolvers et al.[7] showed that the induction of immunologic tolerance to intranasal antigens is fully dependent on the presence of the native cervical LNs and that this tolerance induction cannot be replaced by peripheral (noncervical) LNs transplanted to the same site. Although it is known that CLNs also receive afferent lymphatics from the ocular compartment[5] and that alloreactive cytotoxic T cells are generated in draining CLNs after high-risk corneal transplantation,[9] the functional role of CLNs in the graft rejection process has not been directly studied. Moreover, because a number of secondary lymphoid organs, including the spleen, are involved in generation of allospecific responses,[10,11] the direct contribution of CLNs to corneal alloimmunity remains to be determined. Finally, it has remained unclear whether the alloimmune response generated to corneal grafts in CLNs is primarily sensitizing or tolerance-inducing—that is, whether the overall in vivo effect of the donor-specific response generated in CLNs after transplantation promotes graft rejection or acceptance.

In the present study we investigated the role of CLNs in corneal alloimmunity, first by excising them from transplant-recipient mice (CLN^-) and comparing graft survival and generation of donor (allo)-specific delayed-type hypersensitivity (DTH) to intact hosts endowed with their native CLNs (CLN^+). In addition, CLN^- mice were reconstituted by grafting LNs from other syngeneic naïve animals or from syngeneic mice that had previously been corneally graft recipients, to determine whether the functional role of the CLNs could be reestablished and whether allospecific (graft-destructive or tolerance-inducing) immunity could be adoptively transferred. In the aggregate, our data strongly suggest that CLNs, in contrast to the spleen, which plays a principal role in tolerance induction, play a critical and necessary role in allosensitization and corneal graft rejection.

Methods

Animals

Male BALB/c ($H\text{-}2^d$) mice (Taconic Farm, Germantown, NY) were used as recipients, and male C57BL/6 (B6, $H\text{-}2^b$) mice (major histocompatibility complex [MHC] and multiple minor histocompatibility [H] disparate) were used as donors for corneal transplantation. All LN transplants were syngeneic, and the transplantations were performed in a BALB/c-BALB/c combination. Animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

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Orthotopic Corneal Transplantation and Definition of Graft Rejection

Orthotopic penetrating keratoplasty was performed as described previously in one (right) eye of each mouse.[12] Briefly, after induction of mydriasis, the recipient host bed was marked with a trephine and excised with microscissors to a size of 1.5 mm. The donor cornea was excised with a 2.0-mm trephine (Storz, St. Louis, MO) and transplanted into the host corneal bed with interrupted 11-0 nylon sutures (Sharpoint, Vanguard, TX). The corneal sutures were removed 7 days after surgery. Eyes with the complication of postoperative cataract, infection, or anterior synechiae formation were excluded from the study. The corneal grafts were observed weekly by slit lamp biomicroscopy. Grafts were defined as rejected when they became opaque and the iris details could not be recognized clearly using a standardized opacity-grading scheme. Kaplan–Meier analysis was adopted to construct survival curves, and the log-rank test was used to compare the probability of corneal graft survival in different settings. $P < 0.05$ was considered significant.

Surgical Removal of CLNs and Spleen

After induction of deep anesthesia, a small incision was made in the neck skin under the operating microscope, and the superficial cervical and facial LNs (usually two to three each) were removed bilaterally for generation of LN-deficient CLN^- mice. The incision was then closed with several 8-0 nylon sutures. Splenectomy was performed similarly, and the skin wound closed with 8-0 nylon sutures. Concurrent removal of both spleen and CLNs (CLN^-/Sp^-) or splenectomy alone (Sp^-) was performed in some ($n = 8$, each group) mice. In separate experiments not involving transplantation, CLN^- mice were examined at various time points (2 to 8 weeks after LN excision) to ensure that the technique had led to indefinite loss of lymphatic flow to the cervical area. For the experiments described herein, unless otherwise noted, CLN^- mice were used for corneal transplantation at least 4 weeks after LN excision.

Assay for DTH

To evaluate the allospecific cell-mediated immune response in CLN^- mice, DTH responses were determined by an ear-swelling assay. Five weeks after grafting, splenocytes from donor B6 mice were irradiated (30 Gy), resuspended at a concentration of 1×10^6 in 10 μl phosphate-buffered saline (PBS), and injected into the right ear pinnae of hosts. PBS was injected as a control in the left ear pinnae. After 24 hours, ear thickness was measured with a low-pressure micrometer (Mitsutoyo, Tokyo, Japan). DTH-dependent ear swelling was calculated according to the following formula: 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. Similar measurements were made at 48 hours. All measurements were performed in a masked fashion, and all experiments were repeated at least once. The unpaired *t*-test was used to compare the DTH responses. $P < 0.05$ was considered significant.

Transplantation of LNs to the Cervical Lymphatic Chain and Analysis of Lymph Flow

Cervical and facial LNs, or popliteal and inguinal LNs, were harvested from BALB/c mice for transplantation to the cervical lymphatic chains of CLN^- mice to derive reconstituted $CLN^{-/+}$ mice, as previously described.[7] In summary, donor LNs were aseptically collected by use of an operating microscope and placed on chilled sterile RPMI 1640 until transfer to another mouse. After the mice were anesthetized and the bilateral CLNs removed, as detailed earlier, to derive CLN^- mice, mice were reconstituted by placing either orthotopic (CLN) or heterotopic (inguinal or popliteal) LNs onto to each side of the trachea of CLN^- mice, thereby deriving $CLN^{-/+}$ mice. In all cases, one LN was placed on either side of the cervical lymphatic chain, as previously described.[7]

To determine whether there is afferent lymphatic flow from the eye to the grafted LNs, 5 μl (25 μg/5 μl) tetramethyl rhodamine isothiocyanate (TRITC; Sigma, St. Louis, MO) was injected into the subconjunctival space of one (right) eye of reconstituted $CLN^{-/+}$ mice at 2, 4, and 8 weeks after LN transplantation. One hour after TRITC injection, the (native or grafted) CLNs were removed and embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN). Frozen specimens were sectioned at 9 μm by cryostat. The tissues were mounted on glass slides and observed for fluorescence by a fluorescence microscope. LNs of mice that received subconjunctival injections of PBS instead of TRITC were used for negative control animals, and naïve CLN^+ mice served as positive control animals ($n = 5$ LNs per group). The amount of TRITC in the LNs, as a measure of afferent lymph flow, was graded in a masked fashion and without knowledge of the source of the LNs in each of 10 sections through the center of the node per time point studied. The number of $TRITC^+$ cells per $\times 200$ field were averaged for each time point.

Harvesting of CLNs for Transplantation into BALB/c Mice from Syngeneic Corneal Graft Recipients

Once we established how long it took (4 weeks, as detailed later) to re-establish normal lymphatic flow from the eye to grafted LNs in CLN^- mice, we sought to determine the effect of grafting LNs from hosts of either rejected or accepted corneal grafts to syngeneic CLN^- mice to create reconstituted $CLN^{-/+}$ hosts that subsequently received allogeneic corneal grafts. Accordingly, BALB/c mice that received either accepted or rejected C57BL/6 corneal grafts were killed 8 weeks after corneal transplantation, for harvesting of their CLNs. The harvested LNs were then transplanted into naïve CLN^- mice, as detailed earlier. After 4 weeks, allogeneic C57BL/6-derived corneas were orthotopically grafted to the reconstituted $CLN^{-/+}$ hosts and observed for 12 weeks for graft survival.

Results

Clinical Course of Host Allograft Recipients with or without CLN

Figure 1 shows the survival curve of corneal allografts in naïve CLN^+ (control), LN-deficient (CLN^-), splenectomized (Sp^-), and LN-deficient and splenectomized (CLN^-/Sp^-) hosts. Of the control mice with native CLNs, 50% showed rejection of the corneal grafts by 6 weeks after transplantation, whereas 50% had grafts that remained transparent without any sign of rejection by 12 weeks after surgery. In contrast, none of the transplants grafted onto CLN^-_{hosts} ($n = 12$) showed rejection ($P < 0.001$). Because induction of tolerance has been related to the functional presence of the oculosplenic axis,[13,14] graft survival was also assessed in splenectomized hosts to contrast with graft survival results among CLN^- hosts. Fifty percent of Sp^- hosts ($n = 8$) showed swift rejection of the allograft by 2 weeks after transplantation and 88% by 5 weeks, significantly exceeding rejection rates in normal control animals ($P < 0.01$). By contrast, only one of eight allografts in splenectomized LN-deficient (CLN^-/Sp^-) hosts showed rejection ($P < 0.05$), suggesting that from a graft-survival standpoint, the beneficial effect of lymph node deficiency offset the detrimental effect of splenectomy on graft survival.

Allospecific DTH Assay

To compare the induction of allospecific immunity among the various (CLN^+, CLN^-, CLN^-/Sp^-) hosts, irradiated donor splenocytes were injected into the ear pinnae of grafted mice to elicit donor-specific swelling. Naïve non–graft-recipient mice were used as negative control animals, and BALB/c mice subcutaneously (SC) immunized to B6 splenocytes were used

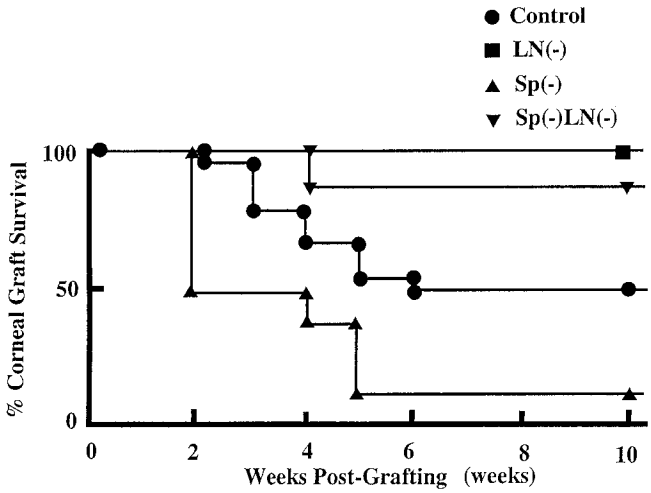

FIGURE 1. Corneal graft survival was enhanced in LN-deficient hosts. Kaplan–Meier survival curves for corneal allografts performed in naïve CLN^+ ($n = 24$), LN-deficient ($n = 12$), splenectomized (Sp^-; $n = 8$), or LN-deficient and splenectomized (CLN^-/Sp^-; $n = 8$) hosts. Fifty percent of naïve mice with native CLNs showed rejection of their corneal allografts. CLN^- hosts demonstrated universal and indefinite graft survival ($P < 0.001$); similarly, only 12.5% of CLN^-/Sp^- hosts had rejection of their grafts ($P < 0.05$). In contrast, grafts were rejected in 87.5% of Sp^- hosts with normal CLNs by 5 weeks, far exceeding those in either CLN^- or control hosts ($P < 0.01$). Control, naïve BALB/c host group; LN(−), LN-deficient host group; SP(−), splenectomized host group; Sp(−)LN(−), splenectomized LN-deficient host group.

as positive control animals ($n = 5$ animals per group in each protocol). Grafted CLN^+, but not naïve, mice exhibited strong DTH alloreactivity to donor alloantigens in the fifth week after transplantation (Fig. 2A, $P < 0.05$). In contrast, the allospecific DTH response among CLN^- mice (Fig. 2B) was profoundly lower than in positive control animals ($P < 0.001$) and was indistinguishable from that in naïve control animals, suggesting failure in induction of allosensitization in the LN-deficient mice. The response among CLN^-/Sp^- mice was indistinguishable from that seen in CLN^- mice with intact spleens (data not shown), similarly showing failure of generation of donor-specific DTH associated with loss of CLN.

Analysis of Lymphatic Flow to CLN

To evaluate lymphatic flow to grafted LNs, TRITC was injected into the subconjunctiva of one eye of each $CLN^{-/+}$ animal. At specified time points, the grafted LNs were harvested and assayed for fluorescence. Injection of TRITC into eyes of naïve animals with native LNs served as a control treatment. One hour after injection of TRITC, easily appreciable fluorescence (mean ± SD: 28 ± 8 $TRITC^+$ cells per field) was easily detected in sections through the ipsilateral (but not contralateral) CLNs harvested from control animals with their native CLNs. In contrast, CLNs harvested from reconstituted $CLN^{-/+}$ mice exhibited only trace fluorescence (9 ± 4 $TRITC^+$ cells per field; $P < 0.002$ compared with control animals) by 2 weeks after LN transplantation, suggesting that normal functional lymphatic flow had not yet been established at that time point. However, by 4 weeks after LN transplantation, normal fluorescence (30 ± 9 $TRITC^+$ cells per field) was again readily detectable in the grafted CLNs at levels indistinguishable from that seen in control animals with native CLNs. Similar results (34 ± 7 $TRITC^+$ cells) were observed at 8 weeks after LN transplantation.

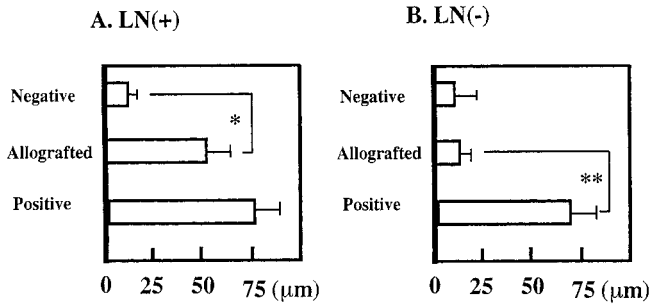

FIGURE 2. LN-deficient hosts failed to generate donor-specific DTH. Allospecific DTH in mice, with, or without CLNs, 5 weeks after corneal transplantation ($n = 5$ per group in each bar). (**A**) Mice with intact native lymph nodes (CLN^+), similar to positive control animals, exhibited significant DTH responses to donor alloantigens compared with negative control animals (*$P = 0.04$). (**B**) Allospecific DTH response in CLN^- hosts, similar to naïve control animals, was markedly suppressed compared with that in positive control animals (**$P < 0.001$).

Clinical Course of Corneal Allografts in Reconstituted Mice

The data presented demonstrate the association between failure in induction of allospecific DTH and enhanced graft survival in CLN^- mice and the reestablishment of normal lymphatic flow to LNs grafted to the cervical chain. To confirm that it is the presence of LNs per se that promotes alloreactivity, reconstituted ($CLN^{-/+}$) mice were derived by grafting syngeneic LNs to the cervical chain of CLN^- mice. Moreover, to determine whether the contribution of CLNs to alloimmunity is site specific, both orthotopic (cervical) and heterotopic (inguinal–popliteal) nodes were harvested for transfer as detailed. Because we had established that it takes 4 weeks for reestablishment of lymphatic flow to the grafted LNs, $CLN^{-/+}$ mice became hosts to C57BL/6 corneal allografts 4 weeks after LN transplantation (Fig. 3). Kaplan–Meier survival analysis revealed that reconstituted $CLN^{-/+}$ mice experienced rejection of their corneal allografts in a manner indistinguishable from that in intact CLN^+ control animals, irrespective of the origin of the grafted LNs. Accordingly, allografts were rejected in 5

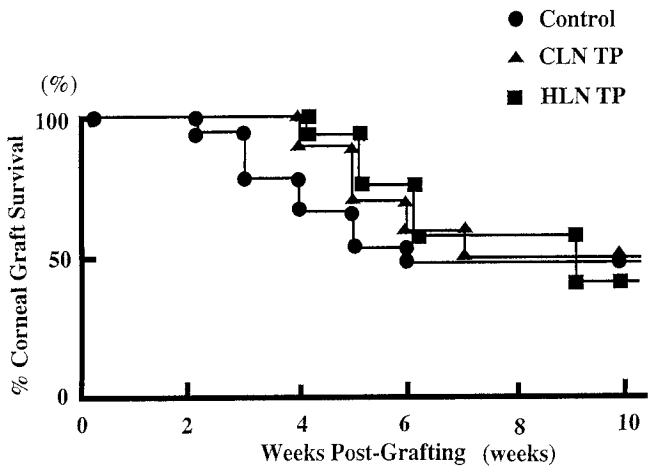

FIGURE 3. Graft survival in reconstituted host recipients of transplanted syngeneic naïve LNs. Kaplan–Meier survival curves for corneal allografts performed in reconstituted $CLN^{-/+}$ hosts with grafted nodes that originated either orthotopically (CLN; $n = 10$) or heterotopically (inguinal/popliteal; $n = 12$). Graft survival rates were 50% in the orthotopic and 42% in the heterotopic group, consistent with that seen in normal CLN^+ hosts ($P > 0.6$) and significantly lower than that observed in CLN^- hosts (Fig. 1). Control, naïve CLN^+ hosts; CLN TP, $CLN^{-/+}$ mice with orthotopically grafted CLN; HLN TP, $CLN^{-/+}$ mice with heterotopically grafted LNs.

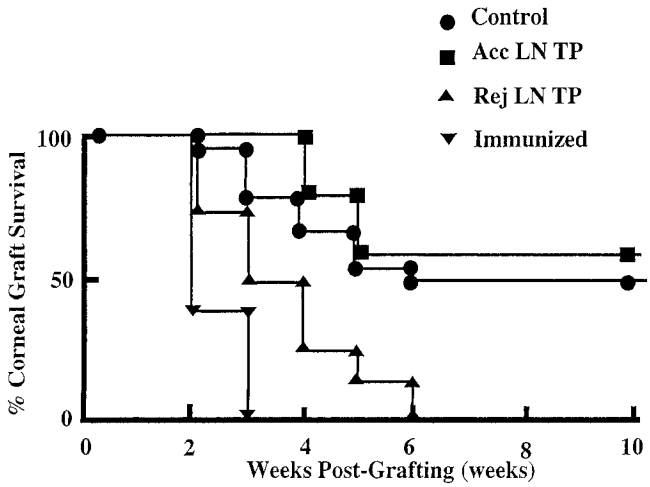

FIGURE 4. Graft survival in reconstituted host recipients of transplanted LNs derived from corneal graft recipients. Kaplan–Meier survival analysis of C57BL/6 corneal transplants in the following BALB/c hosts: Control, naïve control animals; Acc LN TP, immunized to donor splenocytes; Rej LN TP, recipients of CLNs from corneal graft recipients that had had previous rejection of corneal allografts; and Immunized, recipients of CLNs from hosts in which corneal allografts were accepted. Of the hosts that were either naïve or that had received CLN transplants from mice with accepted corneal allografts, 60% had acceptance of their corneal grafts. In contrast, allografts in hosts that were either presensitized or that were reconstituted with CLNs from rejector mice underwent swift and universal rejection ($P = 0.0017$).

(50%) of 10 of the reconstituted $CLN^{-/+}$ mice receiving orthotopic LNs and in 7 (58%) of 12 CLN^{+} hosts receiving heterotopic LNs. In either case, the rapidity and rate of corneal graft rejection was identical with that seen in CLN^{+} control animals.

Having established that functional drainage to CLNs is critical in allosensitization and that CLN^{-} mice reconstituted with transplanted LNs are capable of generating alloreactivity, we tested the hypothesis that CLNs transplanted from corneal graft recipients that have had rejection of their corneal allografts are more effective in subsequent corneal graft rejection than CLNs transplanted from recipients with accepted corneal grafts. Accordingly, CLNs were harvested from mice with rejected ($n = 8$) or accepted ($n = 10$) corneal grafts 8 weeks after transplantation and grafted orthotopically into the cervical lymphatic chain of CLN^{-} mice. Four weeks later, the reconstituted $CLN^{-/+}$ mice received C57BL/6 corneal allografts. Positive control animals were normal mice that were presensitized before corneal transplantation by SC immunization with C57BL/6-derived splenocytes; naïve intact CLN^{+} mice served as negative control animals. The data show that 40% of the reconstituted $CLN^{-/+}$ mice receiving CLNs originating from mice with accepted corneal grafts had rejection of their corneal allografts—comparable to the rejection rate seen in naïve CLN^{+} mice (Fig. 4).

Thus, it is important to note that grafting CLNs from accepted corneal graft recipients did not bias subsequent graft survival among reconstituted hosts, positively or negatively. In contrast, hosts reconstituted with CLNs from mice that had had rejection of their corneal transplants had universal rejection of their corneal allografts. In fact, the rapidity of rejection in this host group was similar to that seen in hosts that were sensitized to donor splenocytes by SC immunization before corneal transplantation, reflecting the functional adoptive transfer of alloreactivity with LN transplantation.

Allospecificity of CLN Transplantation

We conducted several experiments to ensure that the response generated in reconstituted mice after LN grafting was allospecific. Naïve BALB/c animals receiving CLNs from hosts that had rejection of their B6 grafts were challenged, either with C57BL/6-derived or third-party C3H-derived splenocytes (Fig. 5). Negative control animals were naïve, and positive control animals were BALB/c mice that were SC immunized to either B6 or C3H antigens before challenge. Ear swelling was measured at 24 and 48 hours after challenge in all cases to assay for allospecific DTH. $CLN^{-/+}$ hosts reconstituted with CLNs from mice that had shown rejection of B6 corneal grafts exhibited strong allospecific DTH to a B6 splenocyte challenge similar to that seen in positive control animals (Fig. 5A, $P < 0.05$). By contrast, the DTH response of similarly reconstituted mice to third-party C3H splenocytes was indistinguishable from that seen in negative control animals and was significantly lower than in positive control animals (Fig. 5B, $P < 0.0002$).

DISCUSSION

The ocular anterior segment is considered to be an immune privileged site.[13] The anterior chamber can elicit a unique immune response, anterior chamber–associated immune deviation (ACAID), that involves generation of regulatory cells believed to be responsible in part,[14–16] but not exclusively,[17] for the prolonged survival enjoyed by many corneal grafts compared with other solid organ transplants. This tolerance-induced response, characterized by a systemic suppression of antigen-specific DTH to ocularly delivered antigens, is called deviant, because it is distinct from the immunizing response generated to antigens at non–immune-privileged sites.[13]

However, the totality of immune mechanisms involved in downmodulating the immune response to ocularly delivered antigens is still insufficient to entirely suppress the generation of T-helper 1 (Th1) cell–mediated alloimmunity.[13–15] In fact, Sonoda and Streilein[16] and Yamada et al.[18] have shown that recipients of corneal grafts universally acquire donor-specific DTH within several weeks of transplantation. It is interesting, however, that although generation of DTH alloreactivity does not necessarily lead to graft rejection,[10,14–16] several laboratories have independently shown that strategies that suppress induction of donor-specific DTH are very effective in promoting corneal graft survival.[14,18–19] These observations have led us[18,19] and others[14,15] to conclude that generation of allospecific DTH is probably a necessary, albeit insufficient, facet of

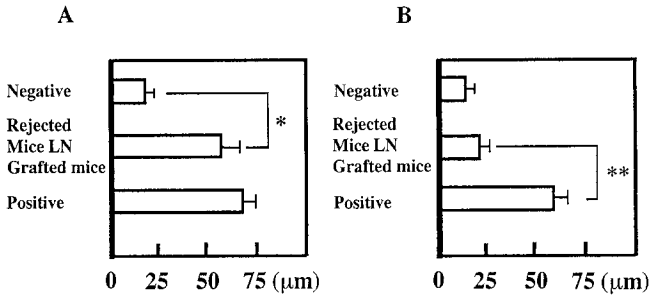

FIGURE 5. LN reconstitution led to adoptive transfer of allospecific immunity. Reconstituted $CLN^{-/+}$ mice recipients of LNs from BALB/c mice that had rejection of their C57BL/6 corneal grafts were challenged with C57BL/6 (**A**) or with C3H (**B**) splenocytes. Naïve animals served as negative control animals, and BALB/c SC immunized with C57BL/6 (**A**) or C3H (**B**) splenocytes before challenge served as positive control animals. Results demonstrate that the adoptive transfer of alloreactivity to reconstituted $CLN^{-/+}$ mice was allospecific (**A**; $^{*}P < 0.05$) and did not extend to third-party C3H antigens (**B**; $^{**}P < 0.0002$).

allorejection, because not all hosts that are sensitized eventually have rejection of their corneal grafts. Corneal transplant rejection ultimately also requires effective recruitment of primed alloreactive Th1 cells to the ocular anterior segment.[12,15] Critical facets of ocular immune privilege, however, such as generation of regulatory cells in the spleen and active CD95-mediated killing of alloreactive T cells in the ocular microenvironment can temper the allodestructive response in the host,[11,13,16,20,21] thereby offering corneal grafts a better chance of success than that of other solid organ transplants.

The classic work of Billingham et al.[22] demonstrated the critical role of draining LNs in skin alloimmunity years before the specific role of T cells in solid organ graft rejection was appreciated. It is therefore surprising that, until recently, very little attention has been paid to the role of the draining LNs in the immunity generated in response to corneal transplantation. It is known that the unique immune response generated to ocularly delivered antigens, including corneal transplants, is due, at least in part, to ocular antigens and antigen-presenting cells (APCs) bypassing the lymph nodes and gaining access to the systemic (venous) blood supply and priming tolerogenic cells in the spleen.[13,23,24] Although Ksander et al.[9] have clearly demonstrated generation of self-restricted donor minor histocompatibility (H)–reactive cytotoxic T cells in the draining LNs of high-risk murine corneal allografts, other studies have failed to demonstrate an important role for cytotoxicity in corneal graft rejection,[15,25,26] and the contribution of these observations to transplant rejection therefore remains unclear.

More recently, Kuffova et al.[27] have demonstrated a CD40-dependent dendritic cell–driven activation of host T cells after murine corneal transplantation, but the antigen specificity of these cells or their functional role in corneal graft rejection have not yet been elucidated. Therefore, although generation of an alloresponse in the draining LNs of eyes after corneal transplantation is now established, its contribution to graft rejection is still poorly understood. Our data from CLN^- mice clearly demonstrate that corneal grafts in mice devoid of draining LNs enjoyed universal and indefinite survival (Fig. 1). The enhanced survival of grafts in CLN^- hosts is associated with failure in the induction of allospecific DTH reactivity (Fig. 2). Taken together with the observation by other laboratories that DTH-mediated mechanisms are critical in the rejection of corneal allografts,[10,13–16,18,25,26] our data suggest that the generation of allospecific and graft-destructive DTH is dependent on functional lymphatic flow to the draining CLNs. It is now clearly established, by the work of several groups associated with the laboratories of Niederkorn[11,14,26] and Streilein[13,23,24,28] that regulatory allospecific graft-protective responses are generated in the spleen after corneal transplantation, and that disruption of the oculosplenic axis can have a deleterious effect on graft outcome, as reflected by our data demonstrating enhanced graft rejection rates in splenectomized hosts compared with either naïve control animals or CLN^- hosts (Fig. 1).

The strong link between draining CLNs and generation of DTH-type allodestructive immunity, and not tolerance, is supported, not only by data demonstrating universal graft survival in CLN^- mice, but also by our observation (data not shown) of an intact ACAID response to ocular antigens in CLN^- mice, suggesting that LN-deficient mice are still competent in the promotion of tolerance to intracameral antigens. Thus, we explain the enhanced survival of allografts in CLN^-/Sp^- hosts by proposing that the interruption of the normal lymphatic drainage from the eye in these hosts and thus of allosensitization, as reflected by failure in generation of donor-specific DTH, circumvents the critical need for (spleen-dependent) induction of tolerance in promoting graft survival and thereby renders CLN^-/Sp^- hosts less susceptible to any deleterious effect of splenectomy per se on transplant survival (Fig. 1).

Finally, reconstitution of CLN^- mice by transfer of LNs from hosts with corneal grafts of long-standing acceptance failed to demonstrate any added protection from corneal graft rejection compared with normal naïve hosts (Fig. 4). Conversely, reconstitution with LNs from hosts that had rejection of their corneal grafts led to swift rejection of subsequent corneal transplants, suggesting successful adoptive transfer of allodestructive, but not alloprotective, responses. As such, transfer of Th1-mediated alloreactivity by LN reconstitution is similar to the adoptive transfer of allospecific tolerance to naïve mice by transfer of ACAID-inducing splenic cells from hosts with long-standing accepted grafts as described by Streilein,[24] Niederkorn,[26] and Sano et al.[28]

Notwithstanding the evidence from our data that relates draining LNs to allosensitization, we cannot theoretically rule out the possibility that a population of regulatory T cells may still be generated in the LNs that migrate subsequently to other secondary lymphoid organs, including the spleen, for expansion and recirculation. Because we tested the effect of reconstituting mice with LNs derived from hosts with graft acceptance 8 weeks after surgery (and noted no difference in subsequent corneal graft survival compared with naïve hosts), it is possible that the functional contribution of these putative LN-derived regulatory cells could not be assayed so late after transplantation, because these cells may have already entered the systemic circulation.

We found that nodes transplanted to the cervical chain demonstrate re-established normal afferent lymph flow from the eye by 4 weeks after transplantation. This differs only slightly with the findings of Wolvers et al.,[7] who reported that it takes 3 weeks to recover normal afferent flow from the nasal mucosa to grafted CLNs. However, they determined that the cervical nodes that directly drain the nasal mucosa, but not the peripheral nodes, constitute a unique environment that favors immunologic tolerance to nasally administered antigens. Perez et al. have recently shown that there is impaired induction of Th1 response in the draining LNs of eyes intracamerally injected with soluble antigen.[29] These findings are in contrast to the data presented herein, with which we have shown that draining cervical LNs, regardless of their origin, demonstrate alloimmune responses including allospecific DTH and graft rejection.

The reasons that the draining LNs in one instance (in response to soluble antigens introduced through the nasal mucosa or anterior chamber) are related to tolerance and in the other (in response to transplantation antigens in corneal allografts) to Th1 immunization cannot be directly addressed by our data. It is possible that obvious differences between the soluble antigens (e.g., ovalbumin) tested by both Wolvers et al.[7] and Perez et al.[29] and transplantation antigens operative in our model profoundly affect the nature of the immune response generated.[30] This is not altogether unlikely: Recent studies in our laboratory[31] have shown that induction of tolerance to transplant antigens is more difficult than to soluble antigens injected into the anterior chamber.

In summary, in contrast to the extensively studied oculosplenic venous-dependent antigen and APC traffic that primes tolerance to ocular antigens, our data implicate functional flow to draining CLNs as a necessary component of alloimmunity and graft rejection in corneal transplantation. Further studies are required to better characterize the molecular facets of immunity that lead to allodestructive immunization in the case of draining LNs and transplant tolerance in the case of the spleen.

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