Differential Chemokine Gene Expression in Corneal Transplant Rejection

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Purpose. To evaluate the differential gene expression of chemokines after corneal transplantation and to determine the chemokines associated with allograft rejection.

Methods. Orthotopic mouse corneal transplantation was performed in two fully mismatched-strain combinations using C57BL/6 (H-2^b) and BALB/c (H-2^d) mice as recipients and BALB/c and C57BL/6 mice as donors. Normal nonsurgical eyes served as negative control specimens and syngeneic transplants (isografts) as control specimens for the alloimmune response. Chemokine gene expression in accepted and rejected allografts and appropriate control specimens was determined by a multiprobe RNase protection assay system.

Results. In eyes with rejected allografts, there was overexpression of regulated on activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and monocyte chemotactic protein (MCP)-1 in both C57BL/6 and BALB/c recipients. In addition, C57BL/6 eyes with rejected allografts expressed very high levels of interferon-γ–inducible protein of 10 kDa (IP-10) mRNA, in contrast to BALB/c eyes with rejected allografts, in which IP-10 expression remained very low. In contrast, lymphotactin gene expression increased only slightly in rejected allografts, and eotaxin mRNA, which was also detected in normal eyes, remained unchanged among isograft and allograft groups. T-cell activation gene (TCA)-3 mRNA was not detected in any of the assayed eyes.

Conclusions. Increased expression of mRNA for select chemokines of the CXC (α) and CC (β) families is associated with corneal allograft rejection. Significantly elevated IP-10 gene expression in high-rejector C57BL/6, but not in low-rejector BALB/c, hosts suggests that differential activation of chemokines may be related to differences in alloimmune reactivity observed among different murine strains. (*Invest Ophthalmol Vis Sci.* 1999;40:2892–2897)

Chemokines are low-molecular-weight proteins that, along with adhesion molecules, play a critical role in immune and inflammatory responses by virtue of regulating the trafficking of leukocytes in a multistep process involving arrest of rolling and firm adhesion to the vascular endothelium[1,2] and leukocyte migration to target tissues through a chemotactic gradient.[3] In addition, chemokines are involved in homeostatic noninflammatory processes such as lymphocyte homing and recirculation through secondary lymphoid organs.[4,5] Chemokines have been subdivided into families on the basis of the relative position of their cysteine residues.[3] The CXC (α) chemokine family, which includes interferon-γ–inducible protein (IP)-10, interleukin (IL)-8, and macrophage inflammatory protein (MIP)-2, has the first two cysteines separated by one amino acid residue. In the CC (β) chemokine group, the first two cysteine residues are adjacent to each other. Regulated on activation normal T-cell expressed and secreted (RANTES), eotaxin, MIP-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, and T-cell activation gene (TCA)-3 are included in the CC chemokine family. Lymphotactin (Ltn) has only one cysteine and is classified as a C chemokine.[6,7] These chemokines form a complex functional network locally and systemically in a variety of inflammatory, infectious, and immune diseases in which many CXC chemokines (e.g., IL-8 and Gro-α) mediate recruitment of neutrophils, whereas CC chemokines are primarily involved in recruitment of immune cells such as antigen-presenting cells and T cells.[3,8]

A number of molecular and anatomic features of the cornea and anterior segment are believed to contribute to the immune privilege enjoyed by orthotopic corneal transplants.[9] In spite of this privilege, corneal allografts are frequently rejected by a process characterized by leukocyte infiltration of the graft stroma and adherence of mononuclear cells to the donor corneal endothelium. Therefore, chemotactic mechanisms involved in leukocyte trafficking probably play a critical role in the alloimmune response to corneal transplants.

Upregulation in chemokine transcription or protein expression has been related to allograft rejection in a number of vascularized organ transplants.[10–14] However, to date, chemokine expression in corneal transplantation has not been char-

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acterized. We investigated the gene expression of a panel of chemokines by assaying for their mRNA using the RNase protection assay (RPA) system. We hypothesized that corneal graft rejection is associated with differential overexpression of chemokines. Specifically, because the alloreactive T-cell response to corneal grafts has been primarily associated with a T-helper (Th) 1 type phenotype,[15–17] and specific chemokines and chemokine receptors are associated with polarized Th1 and Th2 responses,[18–25] we hypothesized that chemokines associated with receptors CCR1 (e.g., MIP-1α), CCR2 (MCP-1), CCR5 (e.g., RANTES), and CXCR3 (e.g., IP-10), but not CCR3 (eotaxin), would be selectively upregulated in the process of rejection of corneal allografts because they have been associated with Th1 type immune responses. Moreover, because appreciable differences in corneal graft survival rates have been observed among high-rejecting Th1-biased C57BL/6 mice compared with low-rejecting Th2-biased BALB/c recipients,[26] we hypothesized that differential expression of chemokines in the two strains may partially account for differences in graft rejection rates in the two strains. In the aggregate, our results suggest that there is selective chemokine gene expression associated with the effector phase of corneal transplant alorejection.

Materials and Methods

Animals

Two inbred murine strain combinations were used for this study: C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (Taconic Farm, Germantown, NY) that were grafted with fully (major histocompatibility complex and multiple minor histocompatibility) disparate BALB/c or C57BL/6 corneas respectively (n = 32/strain) or with syngeneic grafts (n = 10/strain) to serve as control animals. All experiments used male mice that were 8 to 10 weeks of age. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Orthotopic Corneal Transplantation and Scoring of Grafts

Orthotopic penetrating keratoplasty was performed as described previously, with some modifications.[27] Briefly, after induction of mydriasis, the recipient cornea 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 8 to 10 interrupted 11-0 nylon sutures (Sharpoint, Vanguard, TX). The corneal sutures were removed 7 days after surgery. Eyes complicated with postoperative cataract, infection, or anterior synechiae were excluded from study.

The corneal grafts were closely observed several times each week by slit lamp biomicroscopy. Grafts were defined as rejected when they became opaque and the iris details could not be recognized clearly according to a standardized opacification grading scheme reference.[28] When approximately 50% of the allografts in each recipient strain had been rejected (3–4 weeks), eyes were enucleated and subjected to chemokine mRNA analysis.

RNA Preparation and RPA

Total RNA was extracted by the single-step method (RNA-STAT-60; Tel-Test, Friendswood, TX). Eyes were homogenized and centrifuged to remove cellular debris. The RNA pellet obtained from five eyes was resuspended in nuclease-free water and processed together as a group. Detection and quantification of murine chemokine mRNAs were accomplished with a multiprobe RPA system (PharMingen, San Diego, CA), as recommended by the supplier. Briefly, a mixture of [α-^{32}P] uridine triphosphate–labeled antisense riboprobes was generated from the chemokine template set mCK-5 (PharMingen). Twenty micrograms total RNA was used in each sample. Total RNA was hybridized overnight at 56°C with 300 pg of the ^{32}P antisense riboprobe mixture. Nuclease-protected RNA fragments were purified by ethanol precipitation. After purification, the samples were resolved on 5% polyacrylamide sequencing gels. The gels were dried and subjected to autoradiography.

Protected bands were observed after exposure of gels to x-ray film. Specific bands were identified on the basis of their individual migration patterns in comparison with the undigested probes. The bands were quantitated by densitometric analysis (Image; National Institutes of Health, Bethesda, MD) and were normalized to glyceraldehide-3-phosphate dehydrogenase (GAPDH).

Results

Chemokine Gene Expression in High-Rejecting C57BL/6 Hosts

Figures 1A and 1B show the results of the chemokine RPA autoradiograph and the quantity of chemokine mRNA normalized to GAPDH, respectively. Eotaxin mRNA was expressed constitutively in normal nonsurgical control eyes, and there was no appreciable change in this expression after syngeneic (isograft) or allogeneic grafting. In contrast, compared with the chemokine mRNA expression in isografts or accepted allografts, there was significant overexpression of RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA in rejected grafts. The expression level of IP-10 mRNA, which was undetectable in control corneas and only minimally detected in isografts, was the highest of all the chemokines studied in rejecting allografts (Figs. 1B, 1C). Ltn mRNA, which was barely detectable in accepted allografts showed only a slight increase in rejected allografts. Expression of TCA-3 mRNA was undetectable in all eyes studied. Figure 1C shows the results from assayed corneal tissue alone, demonstrating that mRNA expression in the corner correlated well with mRNA expression in whole-eye samples.

Chemokine Gene Expression in Low-Rejecting BALB/c Hosts

Figures 2A and 2B show the results of the chemokine RPA autoradiograph and densitometric quantification of chemokine mRNA, respectively. As is evident from the data, RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA were detected in eyes with rejected allografts in contrast to negative controls or isografts that showed minimal to undetectable levels. However, whereas in the case of RANTES there was a significant overexpression of mRNA in rejected compared with accepted allografts, there was only a mild to moderate increase detected in rejected grafts in the case of MIP-1α, MIP-1β, MIP-2, and MCP-1. Similar to the case in C57BL/6 hosts, Ltn mRNA was undetectable in isografts and was only barely detectable in

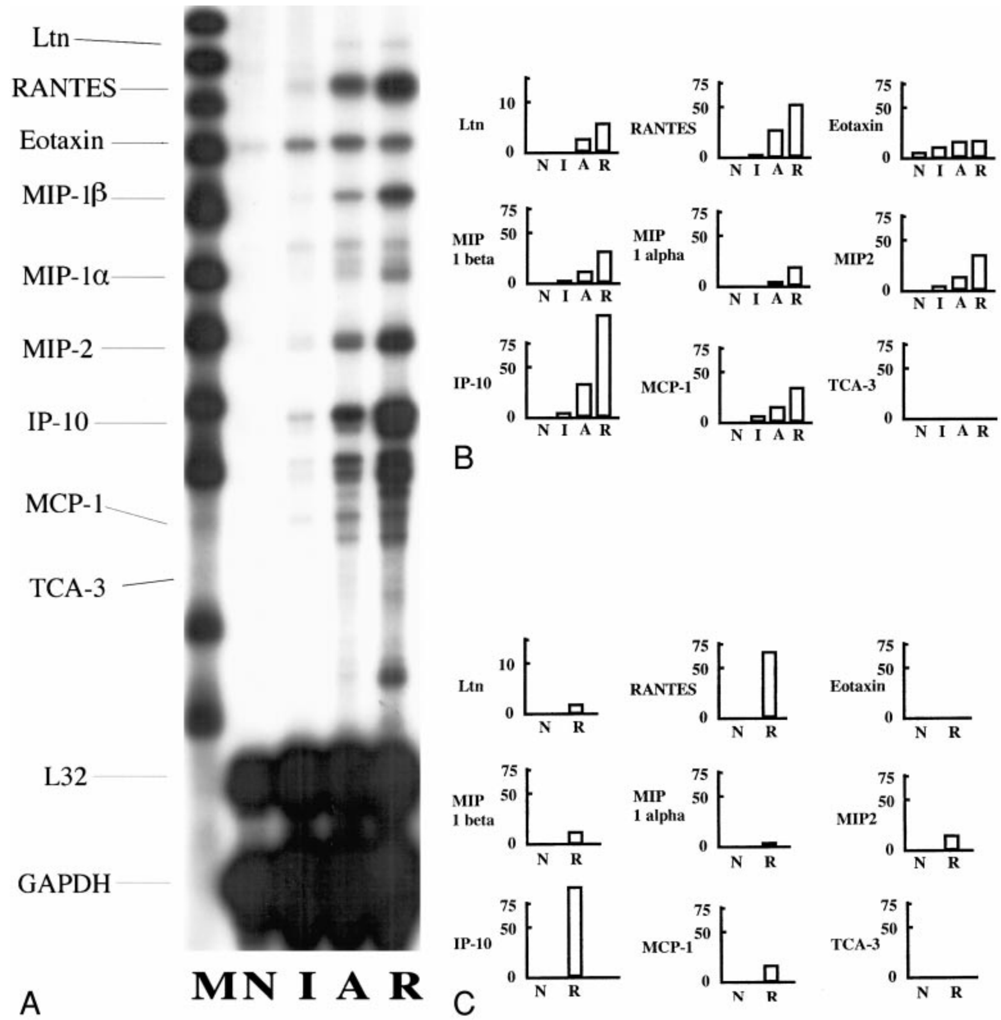

Figure 1. Chemokine gene expression after corneal transplantation in C57BL/6 hosts. Twenty micrograms total RNA was applied in each lane. Autoradiography data are shown in (**A**). On the basis of the undigested probes' migration patterns, specific bands were identified for each chemokine: *lane M*, undigested marker probe; *lane N*, naive murine eyes serving as control; *lane I*, isografts; *lane A*, accepted allografts; *lane R*, rejected allografts. Normalized densitometric analyses are shown in (**B**). Densitometric data on corneal tissue alone: *lane N*, naive corneas and *lane R*, rejected grafts are shown in (**C**). Vertical axes indicate arbitrary units based on densitometry. Whole-eye data show that in comparison with the chemokine mRNA levels in isografts and accepted allografts, RANTES, MIP-1α, MIP-1β, MIP2, MCP-1, Ltn, and IP-10 levels were significantly elevated in eyes with rejected allografts. Representative data of two experiments are shown.

accepted allografts, showing a moderate increase in expression in rejected allografts. Moreover, as in the case of C57BL/6 recipients, the eotaxin mRNA expression level was indistinguishable among the four groups, being also detectable in nongrafted eyes. However, in contrast to high-rejecting C57BL/6 hosts the level of IP-10 mRNA among BALB/c eyes was low, with minimal difference between accepted compared with rejected allografts.

Discussion

We used a multiprobe RPA system to quantify a panel of nine chemokines' mRNA levels from a single sample of total RNA. This method is highly sensitive, and allows for comparative analysis of different mRNA species from a given RNA sample. In the aggregate, we conclude from our data that there is increased expression of select chemokines, in particular RANTES, and to a lesser extent MIP-1α, MIP-1β, and MCP-1 after corneal allotransplantation regardless of the recipient host; there is marked overexpression of the Th-1-associated, interferon (IFN)-γ–induced CXC chemokine IP-10 in high-rejecting C57BL/6, but not in BALB/c, recipients. Eotaxin is constitutively expressed in normal control eyes, and its mRNA level is not appreciably affected by the alloimmune response to corneal transplantation.

RANTES and MIP-1β are known to serve as chemoattractants for activated CD4+ T lymphocytes, and MIP-1α is chemotactic for activated CD8+ T lymphocytes.[29,30] MCP-1 at-

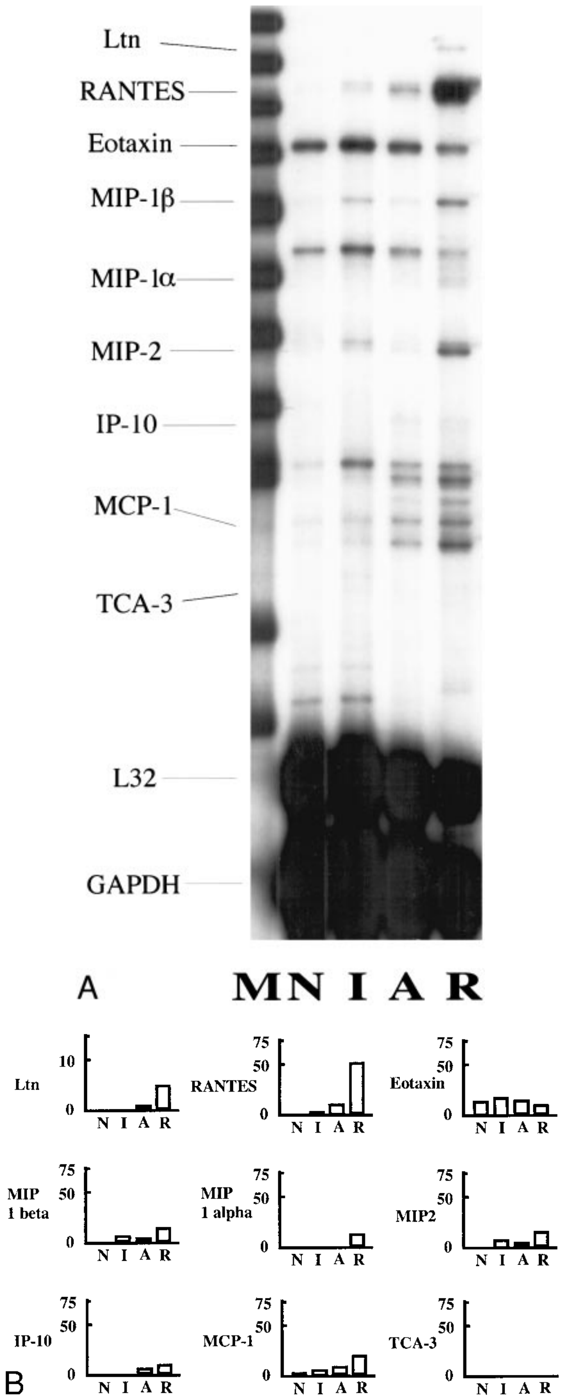

Figure 2. Chemokine gene expression after corneal transplantation in BALB/c hosts. Twenty micrograms total RNA was applied in each lane. Autoradiography data are shown in (**A**). On the basis of the undigested probes' migration patterns, specific bands were identified for each chemokine: *lane M*, undigested marker probe; *lane N*, naive murine eyes serving as control; *lane I*, isografts; *lane A*, accepted allografts; and *lane R*, rejected allografts. Normalized densitometric analyses are shown in (**B**). Vertical axes indicate arbitrary units based on densitometry. In comparison with the chemokine mRNA levels in isografts and accepted allografts, RANTES, and to a much lesser extent Ltn, MIP-1α, MIP-1β, MIP-2, and MCP-1, levels were elevated in eyes with rejected allografts. IP-10 mRNA levels are depressed in BALB/c eyes, even in those with rejected allografts. Representative data of two experiments are shown.

tracts memory T lymphocytes and monocytes.[31] Moreover, these CC chemokines not only attract natural killer (NK) cells but also enhance their cytolytic responses.[32] Because the role of NK cell activity in corneal alloimmunity remains unknown, we speculate that RANTES, MIP-1α, MIP-1β, and MCP-1 are primarily involved in corneal transplant immunity by mediating recruitment of alloreactive T cells to the anterior segment microenvironment. Ltn, known for its function as a lymphocyte-specific chemoattractant, is thought to play an important role in trafficking of resting T cells and in activated peripheral CD8+ T cells.[6,7] Our data demonstrate that Ltn mRNA expression level in eyes with rejected allografts is higher than that of accepted allografts or the undetectable levels in isografts and naive controls. However the overall Ltn mRNA level, even in rejected hosts, was uniformly low, regardless of the strain tested. This could be either a reflection of the dominant role of the CD4 compartment in corneal alloimmunity,[15–17] or because in corneal transplantation, CD8+ T cell responses may occur in the later, rather than acute, phase of allorejection,[15,33] and therefore our assay may have missed the peak level of Ltn expression.

There are significant differences in the expression of specific chemokine receptors in leukocyte subsets that are thought to serve as an important level of regulation for selective recruitment of lymphocyte subsets in different disease states. For example, the receptors CXCR3 (for IP-10), CCR1, and CCR5 (for MIP-1α, MIP-1β, and RANTES) are preferentially expressed on Th1 cells.[20–22,24] Conversely, expression of CCR4 (for TARC) and CCR3 (for eotaxin) have been linked to Th2 type activation and recruitment.[21,22,24] In this study, levels of mRNA for eotaxin, which preferentially binds CCR3 expressed on Th2 cells,[34] did not increase in rejected corneal allograft samples. These results are in accordance with previous observations suggesting Th1-, but not Th2-, dominant responses in mediating corneal allograft rejection.[15–17]

We have been interested by recent observations that fully mismatched corneal grafts are rejected more swiftly and at a higher overall rate in C57BL/6 (~90%) compared with BALB/c (~50%) recipients.[26] We were therefore intrigued by the finding that there was very high ocular mRNA expression for IP-10 in allografted C57BL/6 hosts, compared with levels in the BALB/c host group. Moreover, because draining lymph nodes are regarded as important sites for lymphocyte homing and activation after transplantation,[35] we have recently examined chemokine gene expression in these sites. Compared with that in draining lymph nodes of naive animals, high IP-10 mRNA expression has been detected in C57BL/6, but not BALB/c, hosts that rejected allografts (unpublished observations). IP-10 may very well be instrumental in corneal allograft rejection, because its receptor CXCR3 is expressed almost exclusively on T cells of the Th1 phenotype,[21,24] and its expression by interferon-treated monocytes has been shown to regulate the migration of activated CD4+ T lymphocytes.[36,37] Because C57BL/6 and BALB/c mice are thought to have preferential Th1- or Th2-polarized responses, respectively,[23,26,38] our data suggest that selective high IP-10 expression by C57BL/6 mice may be associated with the more potent alloreactivity seen in this recipient strain.[26]

It is important to address the potential limitations of this study. First, we selected for study a group of chemokines from the C, CC, and CXC families (from among the more than 40 chemokines identified to date) that are believed to be primarily involved in the recruitment of immune cells rather than neutrophils. We did not concentrate on CXC chemokines containing the NH2 terminal sequence glutamic acid-leucine-arginine that are critically relevant to recruitment of neutrophils[3] and may therefore play a significant role in the recruitment of inflammatory cells in corneal transplants. However, because we detected increased MIP-2 mRNA (MIP-2 binds the murine homologue of the IL-8 receptor), particularly in the high-rejecting C57BL/6 recipients, we cannot rule out contribution of CXC neutrophil chemoattractant chemokines to corneal transplant alloimmunity. This is especially true of the high-risk corneal transplantation setting in which we have observed neutrophilic infiltration before migration of antigen-presenting cells (unpublished data). We believe therefore that the functional role of CXC chemokines deserves further study in the high-risk corneal graft setting, particularly in the early induction phase of alloimmunity.

Second, we primarily used whole-eye homogenates for analysis of chemokine mRNA to circumvent the problems faced with the very small quantities of RNA extracted from the murine cornea, which would translate into significant increases in the number of animals used. Although admittedly this method does not allow localization of the chemokine mRNA expression (to the cornea), as may be obtained by in situ hybridization, it has the benefit of allowing simultaneous quantification of different RNA species. In addition, whereas leukocyte infiltration into the posterior compartments of the eye is not observed after corneal transplantation, effector cells involved in mediating graft rejection are commonly seen in noncorneal structures of the anterior segment such as the anterior chamber and iris, most likely a result of extravasation and recruitment at the level of the ciliary body and iris root. It is therefore very likely that noncorneal structures of the anterior segment actively contribute to leukocyte recruitment by expressing chemokines. Therefore, although analysis of whole eyes has the disadvantage of not limiting the assay to the cornea alone, it has the advantage of assaying chemokines expressed by other structures in the anterior segment that probably play a functionally relevant role in leukocyte recruitment after corneal transplantation. To confirm that the expression of specific chemokine mRNA after allograft rejection reflected in the whole-eye data are also operative in the corneal microenvironment, we analyzed C57BL/6 control and rejected corneas ($n = 12$) and were able to reproduce the whole-eye data with the exception that eotaxin, detectable in the normal whole eye, was not expressed in normal corneas (Fig. 1C).

Third, it is important to emphasize that we analyzed chemokine expression in the effector phase of the alloimmune response. The time course of chemokine expression may vary significantly from one chemokine to another. Therefore, detecting low mRNA levels for a specific chemokine (e.g., Ltn) several weeks after corneal transplantation does not mean that the chemokine is similarly minimally expressed early after transplantation in the induction phase of the alloimmune response. Fourth, because we evaluated only mRNA levels, and the biologic function of these chemotactic cytokines is dependent on ligand binding of chemokine receptors, differential levels of genetic message should not be equated with similar variations in protein expression. Finally, we emphasize that in these studies we did not evaluate the functional relevance of chemokines in corneal transplantation. Further studies, such as those involving knockout strains or specific antibodies, would be helpful in establishing the functional relevance of a chemokine or chemokine receptor system in corneal allograft survival.

Corneal transplant rejection shares with all other immune responses the fundamental process of leukocyte recruitment to the antigenic site. As such, chemokines may play a critical role in regulating not only the migration of inflammatory cells from the intravascular compartment to the graft site, but also in amplifying the alloimmune response by selectively activating and recruiting polarized Th1 phenotypic cells. In addition to demonstrating significant overexpression of RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA in rejected corneal allografts of both C57BL/6 and BALB/c host groups, our data suggest that the extremely high levels of IP-10 mRNA detected in the rejected allograft of C57BL/6 mice may explain the high rejection rate of corneal allografts in this strain. Further studies are required to evaluate the contribution of specific chemokines to corneal transplantation immunobiology.

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