Analysis of Macrophage Phenotype in Rejected Corneal Allografts

Joo Youn Oh,[1,2] Hyun Ju Lee,[1,2] Ah Young Ko,[1,2] Jung Hwa Ko,[1,2] Mee Kum Kim,[1,2] and Won Ryang Wee[1,2]

[1]Department of Ophthalmology, Seoul National University Hospital, Seoul, Korea
[2]Laboratory for Ocular Regenerative Medicine and Immunology, Biomedical Research Institute, Seoul National University Hospital, Seoul, Korea

Correspondence: Joo Youn Oh, Department of Ophthalmology, Seoul National University Hospital, 101 Daehak-ro, Jongno-gu, Seoul, 110-744, Korea; jooyounoh77@gmail.com.

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Purpose. We investigated the phenotype of macrophages infiltrating rejected corneal allografts.

Methods. We performed allogeneic or syngeneic corneal transplantation in mice, and humanely killed animals at day 28 during allograft rejection when 60% of corneal allografts were rejected. We divided allografts into two groups: grafts with rejection as rejectors and grafts without rejection as nonrejectors, and analyzed for macrophage infiltration and their phenotype using immunohistochemistry. In addition, we investigated the time course of proinflammatory cytokines and chemokines by analyzing corneal grafts at days 7, 28, and 42 using real-time RT-PCR. Also, we assayed human corneal allografts with chronic graft failure.

Results. We found that a large number of $CD11b^+$, $F4/80^+$, or inducible nitrous oxide synthase cells ($iNOS^+$) infiltrated corneal allografts during rejection in mice, while the cells were found rarely in syngeneic or allogeneic grafts that were not rejected. There were rare $CD11c^+$ cells in rejectors and nonrejectors. Many mannose receptor cells (MRC^+) were present in nonrejectors, but not in rejectors. The levels of Th1 cytokines, IFN-γ, and IL-2 were highly increased in rejectors at day 28, indicating immune rejection. Also, the levels of IL-12a, IL-1β, TNF-α, CCL3, and iNOS that are produced by activated macrophages were markedly increased in rejectors at day 28, compared to syngeneic grafts or nonrejectors. Similarly, human corneal allografts with chronic graft failure had higher levels of IL-12a, IL-1β, CCL3, and iNOS than controls.

Conclusions. Increased numbers of macrophages in rejected corneal allografts implicate that these cells might contribute to the immunopathogenesis of corneal graft rejection.

Keywords: corneal transplantation, rejection, macrophages

Since monocytes/macrophages and T-lymphocytes were recognized to be the major cell types infiltrating rejecting skin grafts in 1958,[1] most studies on transplant immunology have focused predominantly on T-lymphocytes, and the involvement of monocytes/macrophages in transplant rejection remains unclear. However, an increasing number of reports demonstrate that monocytes/macrophages dominantly infiltrated allografts with acute rejection or chronic dysfunction in solid organ transplantation, such as kidney, heart, or intestines.[2–7] Similarly, in corneal allotransplantation, several studies reported the presence of monocytes/macrophages in grafted cornea, anterior chamber, or iris in rodents or humans with corneal allograft rejection.[8–11]

Macrophages are versatile effector cells that display remarkable phenotypic plasticity and functional diversity.[12,13] Hence, activated macrophages often are classified in a spectrum of polarization states. The two extremes of a continuum of macrophage activation have been designated as M1, or classically-activated macrophages, and M2, or alternatively-activated macrophages, although a variety of other functional phenotypes have been identified.[13,14] The M1 macrophages are induced by IFN-γ combined with microbial stimuli, and distinguished by their expression of high levels of inducible nitric oxide synthase 2 (iNOS).[15] Following activation, M1 macrophages produce IL-1, TNF-α, or IL-12, which are potent proinflammatory cytokines, and induce inflammation and tissue destruction. The M2 macrophages are characterized by expression of mannose receptor (MRC) and produce IL-10, an anti-inflammatory cytokine that resolves inflammation and promotes wound healing.[15]

Although a marked phenotypic and functional heterogeneity of macrophages has been studied widely in tumors or in diseases of the central nervous system,[16–19] there have been few studies on differentiation and contribution of macrophages in organ transplantation. Here, we investigated the phenotype of macrophages in rejected corneal allografts in mice and humans.

Materials and Methods

Animals and Animal Model

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute (IACUC, No. 12-0023). The study was performed in compliance with the ARVO Statement for the

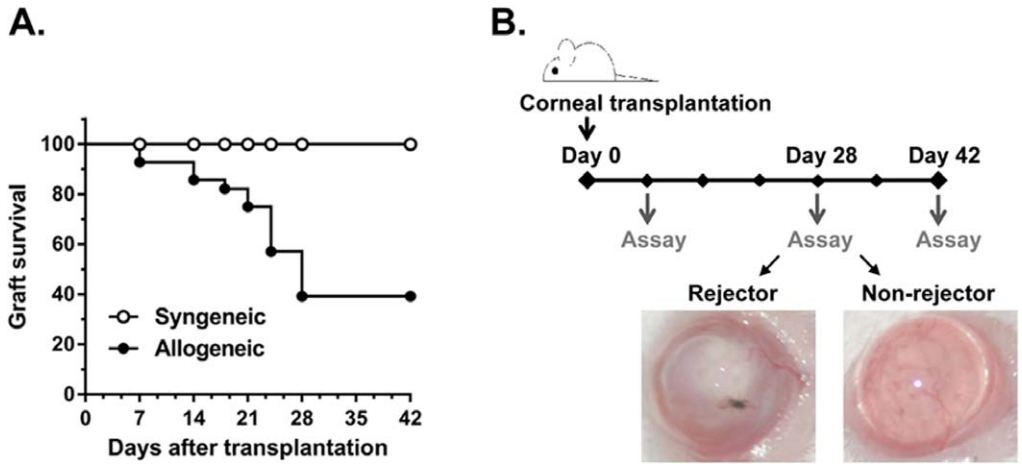

FIGURE 1. Survival of corneal allografts in mice and scheme of assays. (**A**) Kaplan-Meier survival curve showed that 17 of 28 allogeneic corneal grafts from C57BL/6J mice underwent acute rejection in BALB/c mice for a follow-up of 42 days after transplantation, while all syngeneic grafts (BALB/c-to-BALB/c, $n = 12$) survived. (**B**) The grafts with rejection (rejectors) and those without rejection (nonrejectors) were extracted either at day 28 or 42, and subjected to further analysis.

Use of Animals in Ophthalmic and Vision Research. Eight-week-old female B6 mice (C57BL/6J, H-2^b; Orient Bio, Inc., Seongnam, Korea) weighing 18 to 20 g were used as donors and BALB/c mice (BALB/cAnNCrl, H-2^d; Orient Bio, Inc.) were used as recipients.

Under anesthesia with zolazepam-tiletamine (Zoletil; Virbac, Carros, France), corneal transplantation was performed. Briefly, a central 2-mm diameter corneal button was excised from a donor using a trephine (Katena Products, Inc., Denville, NJ), and a recipient corneal bed was prepared by removing a central 2-mm diameter button in the recipient cornea with a trephine. The donor cornea was transplanted to the recipient bed with six interrupted 10–0 nylon sutures. All corneal sutures were removed at day 14 after transplantation. Syngeneic grafts (BALB/c-to-BALB/c) were performed as controls. To prevent the desiccation of transplanted cornea, the eyelids were closed with an 8-0 nylon temporal tarsorrhaphy and maintained (except for clinical evaluation) during the study period. All grafts were evaluated three times a week. Graft rejection was defined as a complete loss of graft transparency that impeded the visualization of the pupil margin and iris (Fig. 1). The mice were humanely killed at days 7, 28, and 42 after transplantation, and corneal grafts were subjected to further analysis.

Human Samples

We obtained 14 human corneal allografts that underwent chronic failure as a result of rejection from patients (10 males and four females, 58.0 ± 17.9 years) undergoing repeat penetrating keratoplasty, under the approval of the Institutional Review Board (H-1102-092-353) and with adherence to the tenets of the Declaration of Helsinki. The duration from rejection to regrafting (tissue acquisition) was 363.7 ± 72.8 days. Five corneas without a history of previous inflammation or surgery were obtained from patients undergoing penetrating keratoplasty because of advanced keratoconus (two males and three females, 26.2 ± 5.4 years) and used as controls.

Histology

The removed corneal grafts from mice or humans were cut into 4-μm sections and subjected to histologic analysis. The formalin-fixed corneal sections were deparaffinized with ethanol and antigen was retrieved using a steamer in epitope retrieval solution (IHC WORLD, Woodstock, MD). A rabbit polyclonal anti-CD4 antibody (Novus Biologicals, Littleton, CO), rabbit polyclonal Cy3-conjugated anti HLA-DR antibody (Bioss USA, Woburn, MA), rabbit polyclonal Alexa 488–conjugated anti-CD11b antibody (Bioss USA), rabbit polyclonal Alexa 647–conjugated anti-CD11c antibody (Bioss USA), rat monoclonal anti-F4/80 antibody (Novus Biologicals), rabbit monoclonal anti-iNOS antibody (Novus Biologicals), and mouse monoclonal anti-MRC (mannose receptor) antibody (Abcam, Cambridge, UK) were used as primary antibody. A 4′6-dimidino-2-phenylindole (DAPI) solution was used for counterstaining (IHC WORLD). The stained slides were observed under a laser confocal microscope (LSM700; Carl Zeiss MicroImaging GmbH, Jena, Germany) or fluorescence microscope (BX61; Olympus, Hamburg, Germany). Images were acquired at ×200 or ×400 magnification.

Real-Time RT-PCR

For RNA extraction, the whole corneal tissue, including the epithelium, stroma, and endothelium, was cut into small pieces, lysed in RNA isolation reagent (RNA Bee; Tel-Test, Inc., Friendswood, TX), and sonicated using a probe sonicator (Ultrasonic Processor; Cole Parmer Instruments, Vernon Hills, IL). Total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA), and double-stranded cDNA was synthesized by reverse transcription (SuperScript III; Invitrogen, Carlsbad, CA). Real-time amplification was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). A mouse or human GAPDH was used for normalization of gene expression. All PCR probe sets were purchased from Applied Biosystems (Taqman Gene Expression Assay kits).

Statistical Analysis

A graft survival curve was acquired using the Kaplan-Meier method by Graphpad Prism (GraphPad Software, Inc., La Jolla, CA). Parameters other than graft survival were compared using the 1-way ANOVA for three groups or 2-tailed Student's *t*-test for two groups using SPSS software (SPSS 12.0; SPSS, Inc., Chicago, IL), and presented as the mean value ± SE. Differences were considered significant at $P < 0.05$.

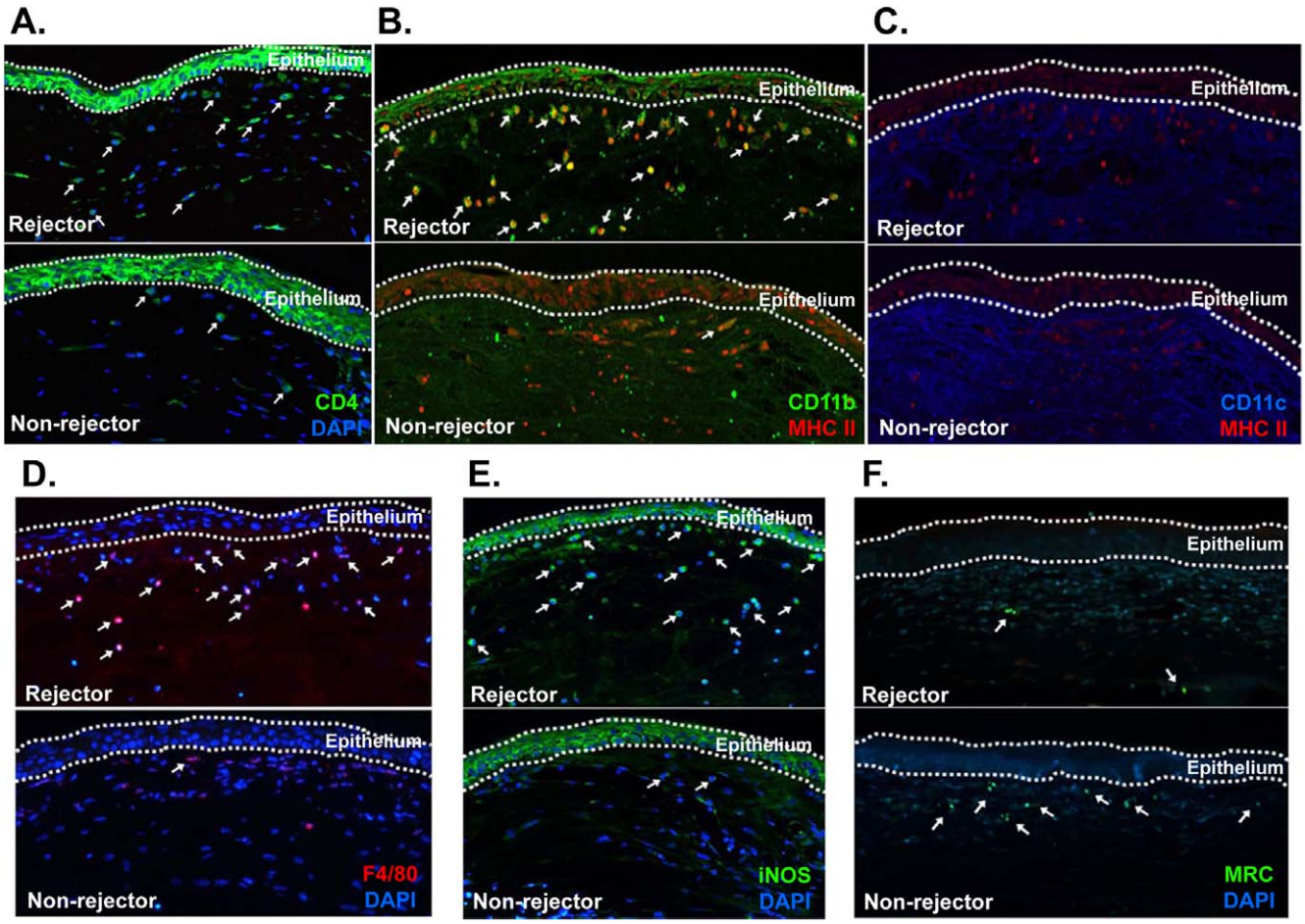

FIGURE 2. Histologic assays of corneal allografts for infiltrating cells. (**A**) Immunohistochemical staining of corneal allografts from mice revealed heavy infiltration of $CD4^+$ T-lymphocytes in rejected grafts (rejectors) indicating rejection. (**B**) Also, a large number of MHC class II^+CD11b^+ cells infiltrated the corneal stroma of rejectors, while there were few MHC class II^+CD11b^+ cells in grafts that were not rejected (nonrejectors). (**C**) However, MHC class II^+CD11c^+ cells were found rarely in rejectors and nonrejectors. (**D**, **E**) Many $F4/80^+$ and $iNOS^+$ cells were accumulated in the anterior stroma of rejectors, but not in nonrejectors. (**F**) In nonrejectors, many MRC-expressing cells were found in the stroma of the grafts. However, there were few MRC^+ cells in rejectors. Original magnification: ×200. The central parts of the cornea were shown ([**A**–**F**], $n = 4$ in each group of rejectors and nonrejectors).

RESULTS

Macrophage Infiltration in Corneal Allografts With Acute Rejection in Mice

We performed allogeneic corneal transplantation using C57BL/6 mice ($H\text{-}2^b$) as donors and BALB/c mice ($H\text{-}2^d$) as recipients, and observed corneal grafts for rejection for 42 days after transplantation. We found that approximately 60.7% (17 of 28) of grafts underwent acute rejection by day 28, while none of the syngeneic corneal allografts (BALB/c-to-BALB/c) were rejected (Fig. 1A). After humanely killing animals at day 28, we divided the grafts into two groups: grafts with rejection as rejectors and grafts without rejection as nonrejectors (Fig. 1B), and analyzed rejectors and nonrejectors for infiltration of macrophages and their phenotypes.

Immunohistochemical staining showed that many $CD4^+$ lymphocytes infiltrated rejecting corneal allografts confirming immune rejection (Fig. 2A). There were no $CD4^+$ cells in syngeneic corneal grafts or normal corneas (Supplementary Fig. S1). The MHC class II^+CD11b^+ or $F4/80^+$ cells indicating macrophages massively infiltrated the corneal stroma of rejectors, while there were few macrophages in nonrejectors, syngeneic grafts, or normal corneas (Figs. 2B, 2D, Supplementary Fig. S2). On the other hand, MHC class II^+CD11c^+ cells were found rarely in rejectors and nonrejectors (Fig. 2C). Notably, there were a large number of iNOS-expressing cells in rejectors, but not in nonrejectors, syngeneic grafts, or normal corneas (Fig. 2E, Supplementary Fig. S3). Some of $iNOS^+$ cells also expressed F4/80, indicating M1 macrophages in rejected grafts (Supplementary Fig. S4).[20] In contrast, the cells expressing MRC, a surface marker for M2 macrophages,[15,20] were detected frequently in the stroma of nonrejectors, while rejectors had few MRC^+ cells (Fig. 2F).

Time Course of Inflammation-Related Cytokines and Chemokines in Corneal Allografts in Mice

To investigate the time course of macrophage infiltration, we analyzed further corneal grafts at days 7, 28, and 42 for proinflammatory cytokines and chemokines that are produced by activated macrophages. Real-time RT-PCR demonstrated that the levels of Th1 cytokines, IFN-γ, and IL-2, started to increase in rejectors at day 7 and reached a peak at day 28, suggesting activation of immune system and rejection (Figs. 3A, 3B). Thereafter, IFN-γ and IL-2 gradually decreased until day 42. The levels of IFN-γ and IL-2 were significantly higher in rejectors compared to syngeneic grafts or nonrejectors. Similar to Th1

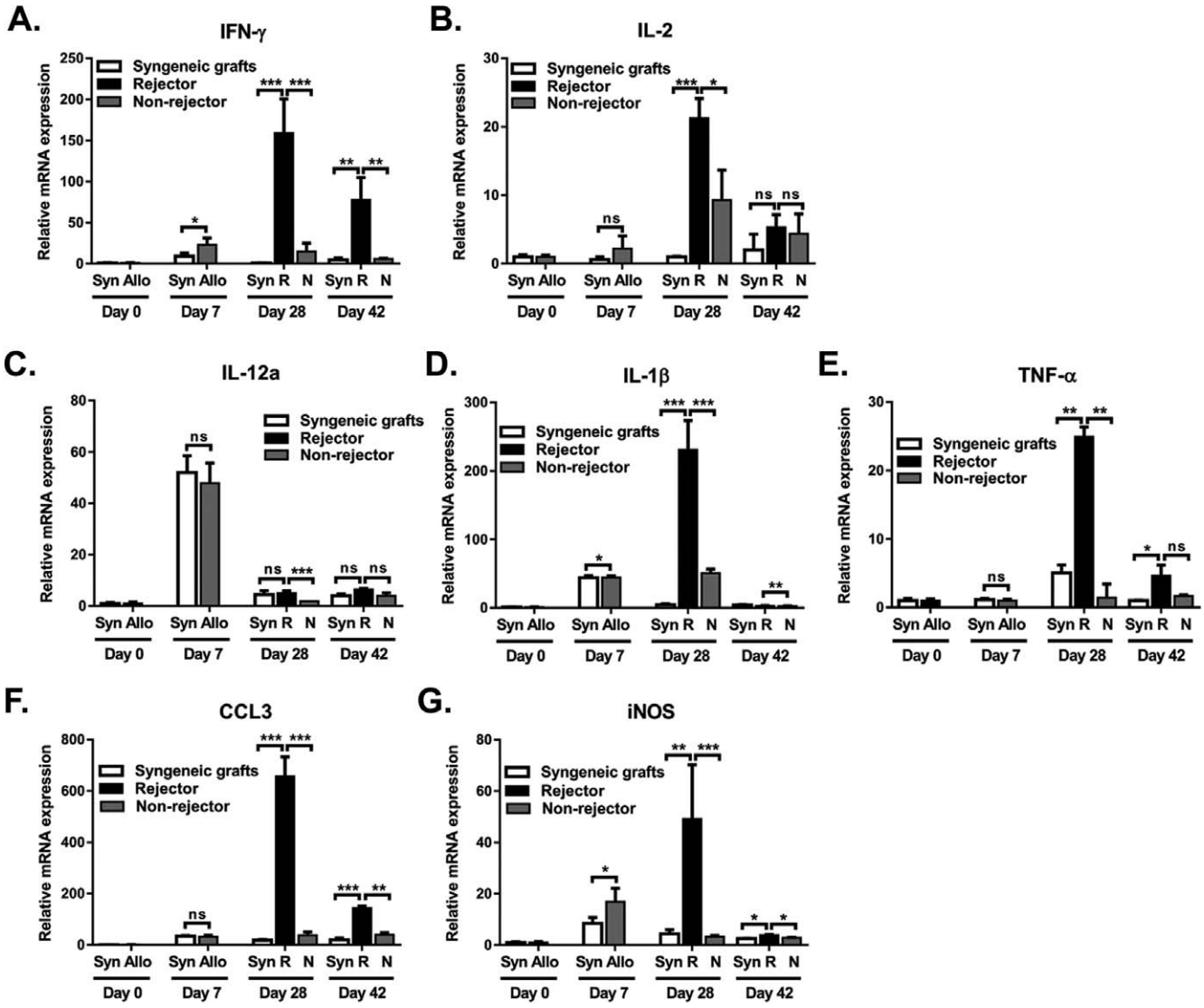

FIGURE 3. Real-time RT-PCR analysis of corneal grafts for inflammation-related cytokines in mice. The levels of transcripts for IFN-γ, IL-2, IL-12a, IL-1β, TNF-α, CCL3, and iNOS were evaluated in corneal allografts in mice using real-time RT-PCR, and compared between rejectors (*R*) and nonrejectors (*N*) or between syngeneic (*Syn*) and allogeneic grafts (*Allo*). (**A**, **B**) The levels of Th1 cytokines, IFN-γ, and IL-2 were significantly increased in rejectors up to day 28, suggesting rejection. (**C**) The IL-12a level was markedly increased in syngenic and allogeneic grafts at day 7, suggesting surgically-mediated inflammation. (**D**–**G**) The levels of IL-1β, TNF-α, CCL3, and iNOS were highly increased in rejectors until day 28, and then decreased at day 42. The levels of IL-12a, IL-1β, TNF-α, CCL3, and iNOS were significantly higher in rejectors at day 28, compared to syngeneic grafts or nonrejectors ($n = 8$ in each group of rejectors and nonrejectors, $n = 4$ in each group of syngeneic grafts). Data were presented as fold changes relative to normal corneas without transplantation (mean ± SE). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

cytokines, the levels of IL-12a, IL-1β, TNF-α, CCL3, and iNOS, which are potent proinflammatory cytokines produced by activated M1 macrophages,[15,20] were highly increased in rejectors at day 28, and then decreased until day 42 (Figs. 3C–G). The levels of IL-12a, IL-1β, TNF-α, CCL3, and iNOS were significantly higher in rejectors at day 28 than in nonrejectors (Figs. 3C–G).

Profiles of Inflammation-Related Cytokines in Human Corneal Allografts With Chronic Graft Failure

We examined 14 human corneal grafts that underwent chronic failure from immune rejection after allotransplantation. The edema and fibrosis of grafted cornea, and an increased level of IFN-γ suggested chronic allograft rejection (Fig. 4). Similar to the data in mice, the levels of IL-12a, IL-1β, CCL3, and iNOS as measured by real-time RT-PCR were significantly increased in rejected corneal allografts compared to control corneas without previous inflammation or surgery (Fig. 4C). The IL-2 and TNF-α was not detected in human corneal allografts.

DISCUSSION

Our results demonstrated that a large number of macrophages were infiltrated in corneal allografts with acute rejection. Also, along with Th1 cytokines, the expression of proinflammatory molecules that are produced largely by activated macrophages was highly increased in murine corneal allografts with acute

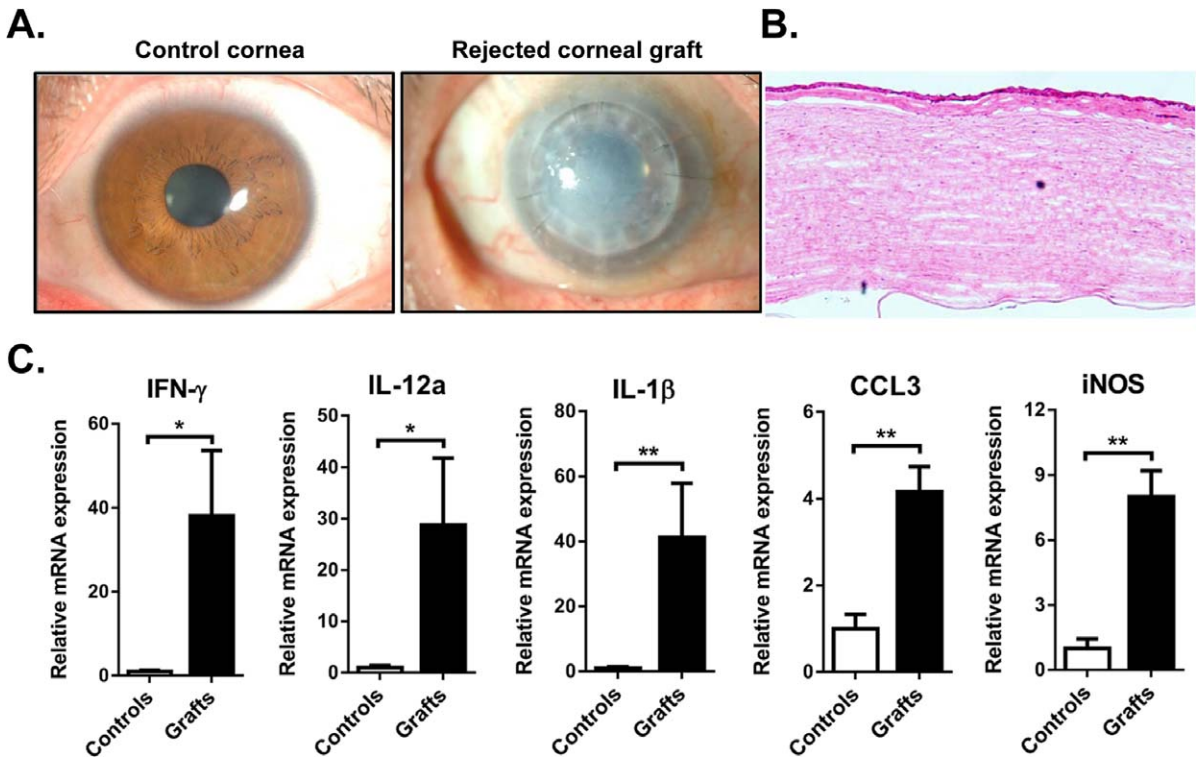

FIGURE 4. Real-time RT-PCR analysis of human corneal allografts with chronic dysfunction. (**A**) Representative corneal photographs of ungrafted control cornea and grafted human cornea with chronic graft dysfunction. (**B**) Hematoxylin-eosin staining of human corneal allograft with chronic dysfunction. Original magnification: ×100. The central part of the cornea was shown. (**C**) Real-time RT-PCR assays showed that the levels of IFN-γ, IL-12a, IL-1β, CCL3, and iNOS were significantly higher in corneal allografts with chronic failure compared to control corneas ($n = 14$ in grafts, $n = 5$ in controls). $^{**}P < 0.01$, $^{*}P < 0.05$.

rejection or in human allografts with chronic failure. Together, these findings might implicate macrophages as effector cells of tissue damage in corneal allograft rejection.

The notion that monocytes/macrophages might participate in allograft rejection has been supported largely by studies on kidney allotransplants.[2,3,20–26] In acutely rejecting kidney allografts, macrophages comprised approximately 38% to 60% of infiltrating leukocytes.[3] Also, accumulation of monocytes/macrophages during rejection was correlated clearly with poor short-term graft outcome and chronic graft dysfunction.[21–25] Furthermore, depletion of circulating monocytes after kidney allotransplantation reduced macrophage infiltration and prevented acute rejection in mice.[26,27] Similar to kidney allotransplantation, macrophage depletion using subconjunctival injection of clodronate liposome improved the graft survival in rat corneal allotransplantation,[28] although clodronate liposome was shown to eliminate $CD11c^+$ cells, as well as $F4/80^+$ and $CD11b^+$ cells.[29]

For the mechanisms of the deleterious role of M1 macrophages in allograft rejection, it is possible that activated macrophages secrete large amounts of proinflammatory cytokines IL-1, IL-12, or TNF-α as shown in our study, and these cytokines subsequently mediate tissue destruction by divergent pathways.[20,25] Also, macrophages might exert direct cytotoxicity and act as effector immune cells by producing reactive nitrogen species.[20,25] This possibility is supported by our observation that the level of iNOS and the number of iNOS-expressing cells were highly increased in corneal allografts during rejection.

However, macrophages are highly heterogeneous and plastic cells, and change their phenotype and function in response to microenvironments.[12,13] Our data demonstrated a massive accumulation of $iNOS^+$, $F4/80^+$, or $CD11b^+$ cells, and elevated levels of IL-12a, IL-1β, TNF-α, CCL3, and iNOS in corneal allografts during acute rejection. Based on these results, we assumed that M1 macrophages might contribute to the pathogenesis of corneal graft rejection. However, we could not exclude the possibility that these cytokines might have been released not only from M1 macrophages, but from other macrophages, monocytes, lymphocytes, or activated resident cells. Hence, to know the definitive role of macrophages and the relative roles of different macrophage subsets in allograft rejection and dysfunction, further studies would be necessary. Complete understanding of macrophage biology and mechanisms underlying macrophage differentiation and phenotypic maturation would provide novel therapeutic targets for preventing or treating allograft rejection in the cornea and other organs.

In conclusion, we found heavy infiltration of macrophages, and high expression of proinflammatory cytokines and chemokines in mouse corneal allografts with acute rejection, and in human corneal grafts with chronic failure. Our data implicated macrophages as one of the effectors mediating acute rejection and chronic dysfunction of corneal allografts, and further suggested as novel therapeutic targets for treating allograft rejection of the cornea or possibly other organs.

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