MHC-Matched Corneal Allograft Rejection in an IFN-γ/IL-17–Independent Manner in C57BL/6 Mice

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Purpose. It has been widely accepted that Th1- and IFN-γ-mediated immune responses are indispensable for corneal allograft rejection in BALB/c hosts. The present study was designed to determine the role of IFN-γ and IL-17 in the rejection by C57BL/6 hosts, which display high rejection rates.

Methods. MHC-matched or mismatched corneal allografts were grafted onto IFN-γ–knockout (GKO), IFN-γ–receptor-knockout (GRKO), IL-17–knockout (IL-17KO), or wild-type (WT) C57BL/6 hosts. Graft fates were assessed clinically and histologically. At appropriate time intervals after allografting, RNA was isolated from corneal graft parenchymal and stromal tissues and cervical lymph nodes. The cytokine mRNA levels of Th1, -2, and -17 type were analyzed by real-time PCR.

Results. No significantly prolonged allograft survival was observed in any combinations. The rejected MHC-mismatched corneas in GKO elicited intensive infiltration of eosinophils, CD11b+ macrophages, and B cells, but few Gr1+CD11c+ neutrophils. In contrast, rejected MHC-matched corneas in GKO hosts, as well as GRKO and WT hosts, elicited intensive infiltration of CD11b+ macrophages and Gr1+CD11c+ neutrophils, but no B220+ B cells and eosinophils. At 1 week after MHC-matched allografting, mRNA levels of IL-6 and IL-17A in the lymph node were extensively upregulated in GKO hosts. It is of interest that anti–IFN-γ treatment did not improve the allograft survival in IL-17KO hosts.

Conclusions. IFN-γ and IL-17 play no critical role in the development of minor-specific allograft rejection in C57BL/6 mice. This indicates the presence of sophisticated rejection mechanisms that are still elusive and cannot be ascribed simply to Th1, -2, or -17.
**Materials and Methods**

**Animals**

Genetically IFN-γ-deficient C57BL/6 (GKO) mice were provided by Toshiaki Ohteki (Akita University, Akita, Japan) and were maintained at Kyoto Prefectural University of Medicine. Homozygous IFN-γ-receptor-1-chain–disrupted mice (GRKO) on a C57BL/6 background were kindly provided by Tadatsugu Taniguchi (Tokyo University, Tokyo, Japan) with the permission of Michel Aguet (University of Zurich, Zurich, Switzerland). IL-17KO mice on C57BL/6 backgrounds were established as reported and were maintained in Kochi Medical School. Male BALB/c (H-2b), C57BL/6 (H-2b), 129 (H-2b) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) at 7 to 10 weeks of age. Sex-matched 8- to 12-week-old mice were used for all experiments. All research adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were approved by the Committee for Animal Research at Kyoto Prefectural University of Medicine.

**Corneal Transplantation and Treatment with Abs**

The corneal transplantation technique has been described elsewhere. Briefly, the central 2-mm of the donor corneas were excised and secured in recipient graft beds with eight interrupted 11-0 nylon sutures. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture. Examination of all grafted eyes after 72 hours confirmed the absence of complications, such as hyphema, infection, or loss of the suture.

In the several sets of experiment, the recipient mice were treated by intraperitoneal (IP) injection three times a week of 1 mg of purified anti–IFN-γ (RA4 to 6A2), or control rat IgG (MP Biomedicals, Solon, OH) from 1 day before transplantation.

**Histologic Analysis**

For immunohistologic evaluation, more than five eyes from recipient mice were randomly selected, enucleated, frozen, embedded, and sectioned. They were stained with antibodies to CD4, CD8, CD11b, CD11c, Gr-1, B220 (PharMingen, San Diego, CA), then with goat–anti-rat Alexa 488 or goat–anti-hamster Cy3 as secondary antibodies and 4′,6-diamino-2-phenylindole (DAPI) or propidium iodide (PI) for staining the nucleus. For histologic assessment, enucleated eyes were fixed in 10% neutral-buffered formalin, and 5-μm sections were stained with hematoxylin and eosin (HE) or Giemsa. Infiltrating cells in the central corneas of the entire section were counted by two observers given blinded samples. The data are presented as averages ± SEM of all the mice examined.

**Quantitative Real-Time RT-PCR**

At the appropriate time period after surgery, the corneas and cervical lymph nodes (LNs) were collected. The transplanted corneas were excised into donor and recipient parts. Then, the corneal epithelium was removed by incubating in phosphate-buffered saline containing 20 μM EDTA for 15 minutes at 37°C. The control samples were collected from age-matched normal mice. RNA from the corneal epithelium and endothelial-stroma of both donor and recipient origins, and recipient lymph nodes were purified using RNA isolation reagent (Trizol; Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Total RNA from each tissue sample was incubated with M-MuLV reverse transcriptase (RT; Invitrogen Life Technologies) using random primers. Quantitative real-time PCR was performed with a sequence detection system (Prism 7000; Applied Biosystems [ABI], Foster City, CA). Probe and primer sequences for IL-12p35, -12p40, -15, -18, -10, -23p19, and -17A; IFN-γ; TGF-β1 and -β2; and β-actin were obtained from Takara Bio, Inc. (Otsu, Japan). Each reaction was performed in 25 μL with 50% real-time PCR premix (SYBR Green 2× PCR Master Mix; ABI), 100 nM each of the forward and reverse primer, and 200 nM of probe. Conditions for PCR were 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles, each consisting of 15 seconds at 95°C, and 1 minute at 60°C. Arbitrary cDNA values of individual samples were determined by using standard curves obtained from a fourfold serial dilution of a reference cDNA sample. Relative expression values were normalized by the expression level of β-actin and calculated as induction (x-fold) compared to normal mice.

**Assessment of DTH**

Three weeks after surgery, the mice were injected in the right ear pinnae with 1 × 10⁶ irradiated (20 Gy) donor spleen cells in 10 μL Hank’s balanced salt solution. At 24 and 48 hours after ear challenge, the ear thickness was measured with a low-pressure micrometer (Mitutoyo, MTI Corp., Paramus, NJ).

**ELISA and Proliferation Assays**

ELISA and proliferation assay were performed as described previously. Briefly, lymphocytes from cervical lymph nodes or spleens of recipient mice were resuspended in 96-well plates (2.5 × 10⁶) and stimulated with irradiated (20 Gy) splenocytes (2.5 × 10⁶) in serum-free medium. The cultures were incubated for 48, 72, or 96 hours, pulsed with tritiated thymidine (0.5 μCi/well) during the final 16 hours, and harvested with a cell harvester (MicroMate 196; Packard Instrument Company, Meriden, CT). Similar cultures were incubated, and supernatants were collected at the same time points and analyzed for their IFN-γ, IL-10, and IL-17A contents using ELISA kits (PharMingen).

**Statistical Methods**

All experiments were repeated more than three times. For the RT-PCR, ELISA, MLR, and DTH assay, a total of five samples (one sample each from five individual mice) were collected and tested. We constructed Kaplan-Meier survival curves and used the Breslow-Gehan-Wilcoxon test to compare the probabilities of allograft survival. Student’s t-test was used to compare proliferation responses, secreting cytokines, and DTH responses. P < 0.05 was considered significant.

**Results**

**The Fate of Corneal Allografts in C57BL/6 IFN-γ–Deficient Mice**

Previously, it has been reported that GKO BALB/c mice did not reject corneal allografts from MHCMatched minor H–disparate B10.D2 mice. First, to confirm the fate of MHCMatched allografts in C57BL/6 recipients, corneal grafts were prepared from normal 129 mice that share the same MHC molecule with C57BL/6 recipients and placed orthotopically in eyes of GKO
C57BL/6 mice and wild-type (WT) C57BL/6 controls. GKO C57BL/6 mice rejected 95% of the 129 allografts with a tempo and an incidence identical with the rejection in WT C57BL/6 mice. To avoid the influence of donor APC–derived IFN-γ/H9253, we also investigated GRKO C57BL/6 mice as the recipient. Similar to GKO mice, GRKO C57BL/6 mice rejected 129 allografts (Fig. 1B). These results imply that MHC-matched, minor H–only disparate corneal allografts can be rejected, even in the absence of IFN-γ, in C57BL/6 hosts with apparently normal vigor, in sharp contrast with the indefinite acceptance of MHC-matched minor H–only disparate corneal allografts in GKO BALB/c hosts.2

**Histopathologic Features of Rejected Corneas in GKO or GRKO C57BL/6 Hosts**

Histopathologic examination of the 129 rejected corneal allografts in GKO C57BL/6 revealed massive inflammatory infiltrates. The infiltrates were composed of many CD4⁺ T cells, CD11b⁺Mps, Gr-1⁺CD11c⁺Neu, and a few Gr-1⁺CD11c⁺dendritic cells (DCs). By contrast, almost none of the B220⁺B cells or Eos were observed (Fig. 2A). Similar infiltrates were observed in the 129 rejected corneas in the WT C57BL/6 (Fig. 2B) and GRKO C57BL/6 hosts (data not shown).
MHC + Minor H–Disparate Allograft Rejection by Eos in GKO C57BL/6 Mice

It has been reported that GKO BALB/c mice rejected MHC-disparate corneal allografts followed by Eos infiltration. To examine whether Eos preponderance in rejection is present in GKO C57BL/6 mice, we transplanted corneal grafts from normal BALB/c mice to GKO or WT C57BL/6 hosts (MHC minor H disparate). GKO C57BL/6 mice showed significantly prolonged allograft survival, but rejected 95% of BALB/c allografts (Fig. 3). Inflammatory infiltrates in the rejected corneas were characterized by a preponderance of Eos and B cells (Fig. 4A). All the grafts were rejected, followed by Eos infiltration in GRKO C57BL/6 mice as well (data not shown). By contrast, BALB/c allografts that were rejected in WT C57BL/6 hosts were infiltrated with mononuclear cells (MNCs) and Neu without any B cells or Eos (Fig. 4B).

Th1-, Th2-, and Th17-Type Gene Expression after Corneal Allograft

Corneal allograft rejection is thought to be mainly mediated by the Th1-type immune response. GKO hosts of both BALB/c and C57BL/6 backgrounds rejected MHC-disparate corneas by Th2-like immune response with massive Eos infiltration. However, the minor H–directed rejection by GKO C57BL/6 hosts was not followed by Eos infiltration, but was followed by Gr-1⁺CD11c⁺Neu and CD11b⁺Mps infiltration. Th17 responses reportedly induce the vigorous infiltration of Neu into tissue inflammatory sites. To gain insight into the participation of distinct CD4⁺ helper T subsets, Th1-, Th2-, and Th17-type cytokine gene expression was investigated 7 days after corneal grafting. In the case of 129 corneal allografting in WT C57BL/6 mice, Th1-type (IL-12p35 and IFN-γ) and Th17-type (IL-6, -23p19, and -17A) cytokine gene expression was upregulated in the absence of IL-10 upregulation (Fig. 5A). In GKO

Figure 3. The effect of IFN-γ deficiency on MHC + minor H–disparate corneal allograft survival. BALB/c allografts were placed onto normal eyes of IFN-γKO (□, n = 20) or WT C57BL/6 (■, n = 20) mice.

Figure 4. Histologic examination of rejected MHC + minor H–disparate donor cornea. Rejected BALB/c donor corneas transplanted in GKO C57BL/6 (A) and WT C57BL/6 (B) hosts were stained immunohistologically with antibodies to CD4, CD8, CD11b, Gr1⁺CD11c, B220, or Giemsa and HE.
recipient mice, upregulation of Th17-type cytokines (IL-6, -23p19, and -17A) was kept at a level comparable with that in WT hosts. Of note, IL-6 and -17A expressions in cervical LN were more than threefold higher than those of the WT control mice. This finding raised the concern that the Th17-type immune response may participate in the MHC-matched minor H–disparate corneal allograft rejection under IFN-γ deficiency.

It is of note that Th1 and Th17-related cytokines were more highly expressed in corneal stroma than in corneal epithelium. It is conceivable that the high expression of those cytokines is primarily due to the more extensive number of infiltrates that were observed at the rejected corneal stroma (Fig. 4), whereas the reported difference of immunogenicity among the different layers of the cornea21 may reflect mostly the difference of antigenicity of each layer.

Lymphocytes from the cervical LN of GKO and WT C57BL/6 mice that received the 129 allograft for 3 weeks were cocultured with irradiated 129- or C57BL/6 splenocytes for 3 days. Thymidine uptake was evaluated during the final 16 hours. (C) Lymphocytes from GKO or WT mice that received 129 corneas 3 weeks before (□ or ■), immunized by SC injection of 129 splenocytes 1 week before (○ or ◆), and naive mice (△ or ▲) were cultured with irradiated 129 splenocytes for 3, 4, and 5 days. IFN-γ, IL-10, and IL-17A concentrations in culture supernatants were measured by ELISA. Bars, SE.
projected 129 allografts at the same tempo and frequency as WT C57BL/6 mice (Fig. 6A). Histologic examination showed the infiltration of Gr-1⁺CD11c⁻ Neu and CD11b⁺ Mps, but not Eos (data not shown). To eliminate possible complementation by IFN-γ in IL-17KO C57BL/6, IL-17KO mice were treated with either a neutralizing anti–IFN-γ monoclonal antibody or isotype-matched control IgG. The anti–IFN-γ-treated IL-17KO mice did not show any prolonged allograft survival, similar to the control animals (Fig. 6B). The rejected cornea in anti–IFN-γ-treated IL-17KO mice displayed the infiltration of Gr-1⁺CD11c⁻ Neu and CD11b⁺ Mps, comparable with GKO or IL-17KO C57BL/6, but no detectable level of Eos infiltration (data not shown).

**Donor-Reactive DTH in GKO C57BL/6 Hosts**

We confirmed the acquisition of the donor Ag-specific DTH response in GKO and WT C57BL/6 mice that received the 129 allograft for 3 weeks. After the irradiated 129 splenocytes were challenged, all allografted GKO mice, as well as WT mice, acquired the DTH response, similar to the mice immunized with 129 splenocytes subcutaneously 2 weeks before (Fig. 7).

**DISCUSSION**

Current results have shown that MHC-compatible, minor H–only disparate corneal allografts in C57BL/6 mice are rejected, accompanied by Neu and Mps, but not Eos (Table 2), infiltrates in the absence of IFN-γ and/or IL-17. The allograft rejection is closely correlated with alloAg-specific DTH. Given that GKO C57BL/6 mice acquired the DTH response 3 weeks after surgery, the presence of the Th1-like immune response, even in the absence of IFN-γ signaling, might be plausible. IL-4 induces eosinophilia, inflammation, and exacerbates a rejection response. Eos have been implicated as effector cells in IFN-γ-independent allograft rejection. Beauregard et al. proposed synergistic Th1/Th2 collaboration in rejecting the allograft. Contrary to the well-accepted Th1-inducing activity leading to the tissue destruction, IFN-γ has recently become recognized as a crucial factor regulating immune responses in a protective direction. IFN-γ has potentially dichotomous effects on organ allograft survival, which raises the possibility that the deficiency in IFN-γ provokes the unregulated immunologic eruption. Some of these phenomena may be responsible to the observed graft rejection directed to minor H. There is now compelling evidence indicating that IFN-γ plays a role as a master regulator in the development of operational tolerance to donor alloAg by regulating the development and functions of CD4⁺CD25⁺ Tregs. Recently, there has been a remarkable evolution in explaining the regulation of tissue damage defined by DTH. A pathway named Th17 has recently been credited for causing tissue damage in immune-mediated tissue injuries. The drivers of Th17 differentiation are Treg-derived TGF-β and DC-derived IL-6. Several investigators have claimed that there are reciprocal interactions between IFN-γ and IL-17. Because IL-6 and IL-17A genes were upregulated in the LN of the GKO C57BL/6 host (Fig. 5A), it is tempting to presume the participation of Th17 cells in the observed rejection in GKO C57BL/6 mice. Contrary to this expectation, minor H–only disparate allografts were rejected in IL-17KO mice, even in combination with IFN-γ blocking. IL-17KO mice exhibited diminished DTH in some rodent models. However, we confirmed the comparable level of alloAg-specific DTH in IL-17KO C57BL/6 mice. Since the Th17 response may still remain in IL-17A-deficient mice, and since Th17 cells are known to be highly heterogeneous, additional intensive studies are needed to elucidate the underlying mechanism in the rejection of minor H–only disparate corneal allografts. Th1 and Th17 cells differ in migratory behavior specific to certain tissue sites, indicating the presence of the spatially and temporally distinct work by these cytokines.

The term tissue damage represents a spectrum of diseases involving more than CD4⁺Th1/2 or Th17 cells, and involves Neu, Mps, and Dcs, even in the presence of DTH. The process of graft rejection is very much an inflammatory one infiltrated with massive MNCs. The ocular infiltrates in GKO-deficient mice under the pathologic progression of experimen-
tal autoimmune uveitis was dominated by Neu and Eos, suggesting that Th1-associated chemokines play a pivotal role in the attraction of MNCs to the eyes in the presence of IFN-γ, whereas in the absence of IFN-γ, Th2-, and Th17-related chemokines may be the key elements for the influx of granulocytes.37,42 Minor H–only disparate corneal graft rejection in GKO mice was reportedly characterized by an intensive Eos infiltration compared with a preponderant MNC infiltration in WT BALB/c mice.2 This finding is in contrast with the recent findings of Flynn et al.43 that the increased rate of allograft rejection was attributable to aggravated local inflammation rather than to Th2 responses. Many Eos infiltrates were observed in the MHC-disparate corneal allograft in GKO C57BL/6 mice, indicating a Th2-type immune rejection. In contrast, regardless of the upregulated IL-6 and -17 gene expression, no histologic difference was observed in the minor H–only disparate allografts from GKO and WT C57BL/6 hosts. The Neu and Mps infiltrates in rejected grafts were evident in both hosts. It is remarkable that almost no significant infiltration of Eos was detected. The present results coincide well with the previous finding that pointed to a critical role for IFN-γ in regulating Neu infiltration of allografts.44,45 To clarify the role of IFN-γ in this rejection, the influence on the chemokine expression should be examined. In cardiac allograft transplantation, it is suggested that IFN-γ–independent induction of intense Neu infiltration is accompanied by extensive graft parenchymal necrosis.46 In acute rejection of MHC-disparate skin grafts by C57BL/6 mice, infiltrated minor Neu play a direct causal role in the rejection.47 IL-17 is known to develop a Neu-rich inflammatory response48 and to mediate granulopoi-
esis.49,50,51 Our results reveal that both IFN-γ- and IL-17-deficient mice elicited similar pathologic features in the re-
jected MHC-matched allografts, indicating the presence of either an IFN-γ/IL-17–independent Th1/Th17 response or a more subtle rejection mechanism over acquired immunity.

The redundancy of rejection mechanisms explains the dif-
ficulty of inducing transplantation tolerance. To further strengthen our knowledge on the complex immune/inflammat-
ory axis during corneal allograft rejection, we must extend the scientific investigation further to include previously unconsid-
ered aspects, such as the role of functionally distinct and plastic APCs and the fibrotic responses in graft beds. DCs deficient in reactive oxygen species production induced high levels of IFN-γ and IL-17 in Ag-triggered T cells.52 Given the fact that DCs mediate IFN-γ production in an autocrine manner and that such autocrine production of IFN-γ very likely plays a divergent role in both innate and acquired immunity,53 a better understanding of the intriguing dual roles of IFN-γ-producing APCs in graft beds is vital. We have reported that allelic-gene semimatching, combined with the local induction of APCs with reduced intracellular redox potential, results in allograft survival comparable to the survival of MHC-matched grafts.52,53

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