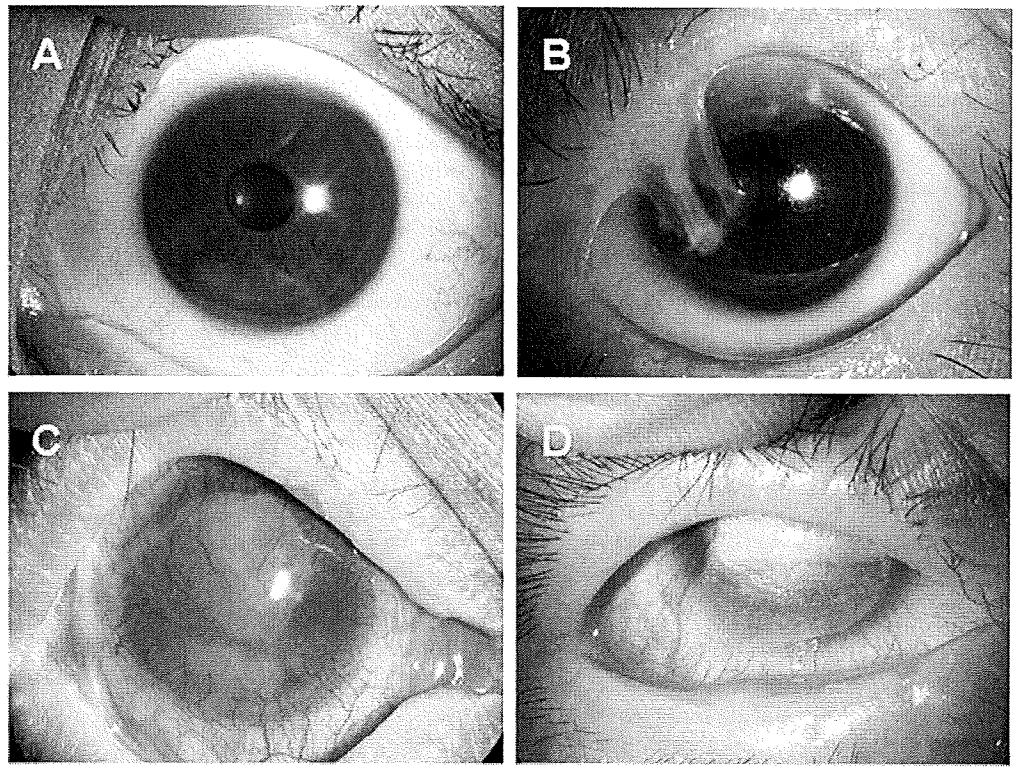


**Figure 1.** Photographs showing representative ocular manifestations at the chronic stage, with corresponding visual acuity. A, Clear cornea and best-corrected visual acuity of 20/20 or better: 34 eyes (18.3%). B, Moderate conjunctivalization and visual acuity worse than 20/20 and up to and including 20/200: 55 eyes (29.6%). C, Severe conjunctivalization and neovascularization and visual acuity worse than 20/200 and up to and including 20/2000: 53 eyes (28.5%). D, Keratinization, severe opacification, and visual acuity worse than 20/2000: 44 eyes (23.7%).

### Diagnosis at the Acute Stage

Eleven patients were diagnosed with acute conjunctivitis by ophthalmologists before the development of systemic eruptions. An additional 12 patients were misdiagnosed as having measles (n = 4), chickenpox (n = 2), herpetic infection (n = 2), rubella (n = 1), or other diseases by physicians in other fields.

Among 94 patients, only 37 patients were diagnosed as having SJS or TEN at disease onset. Seven patients were diagnosed properly at several weeks (range, 2–8 weeks) after the onset, and surprisingly, 6 patients obtained the diagnosis at 2 to 45 years after the onset. For the remaining patients, when they received a proper diagnosis could not be ascertained.

Table 1. Symptoms and Mucosal Involvements of the 94 Patients at the Acute Stage

| Symptoms | Experienced | Did Not Experience | Unknown |
|---|---|---|---|
| Prodromal common cold-like symptoms | 75 | 17 | 2 |
| Extremely high fever (>39° C) | 86 | 1 | 7 |
| Ocular involvement | 94 | 0 | 0 |
| Oral involvement | 82 | 0 | 12 |
| Genital involvement | 46 | 18 | 30 |
| Fingernail loss or deformation | 94 | 0 | 0 |

## Discussion

Stevens-Johnson syndrome and TEN are rare but potentially fatal skin disorders. Ocular involvement is common and often results in long-term complications such as serious visual impairment with ocular discomforts.[13,28] Although much has been learned over the past 50 years about the management of SJS and TEN, the following 3 important problems still remain: (1) the difficulty of obtaining a prompt and accurate diagnosis of SJS or TEN at disease onset, (2) ocular involvement often is overlooked easily because of the serious general symptoms and high lethality of these 2 diseases, and (3) a universally accepted treatment regimen for SJS and TEN has yet to be adopted and treatment with corticosteroids remains controversial.[10,28–30] There is also no standardized ophthalmologic treatment for the prevention of ocular complications.

In this study, 12 patients were misdiagnosed as having chickenpox, measles, herpetic infection, or other diseases. For early diagnosis, the clinical pictures of SJS and TEN need to be well understood, and to that end, the results of this study provided new and important data. Common cold-like symptoms (general malaise, slight fever, sore throat, etc.) preceded skin eruptions in 82% of the cases, and in all but 1 patient, the disease was accompanied by very high fever (more than 39° C) at the onset. It should be emphasized that acute conjunctivitis occurred before or simulta-

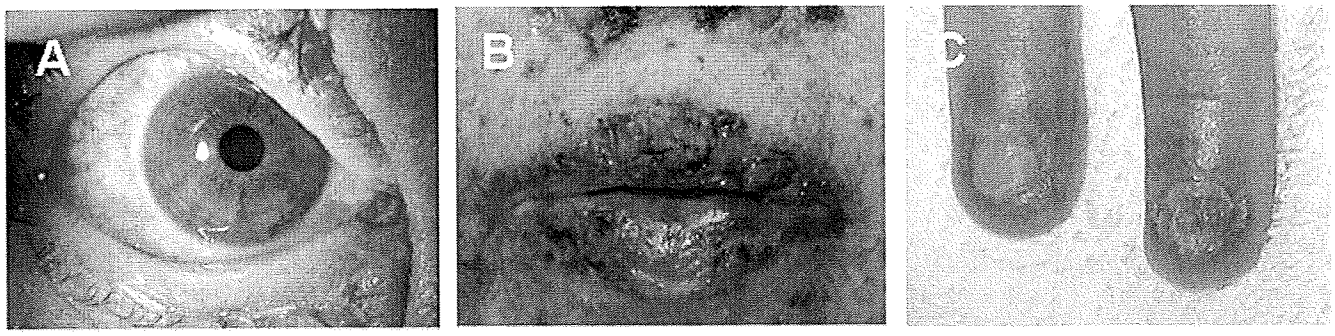


**Figure 2.** Representative photographs showing Stevens-Johnson syndrome/toxic epidermal necrolysis-associated ocular and oral involvement and fingernail loss at the acute stage. A, Conjunctivitis, which was accompanied by extensive loss of bulbar conjunctival epithelium. B, Swollen and crusted lips. C, Fingernail loss and deformation with paronychia.

neously with skin eruptions and that the involvement of oral mucosa was observed in 100% of the cases who could remember the details. Fingernail loss at the acute stage, deformation at the time of the writing of this report, or both also occurred in all of the patients, suggesting that paronychia occurred in all patients at the acute phase.

Visual outcomes were significantly better in the patients who received treatment with topical steroids during the first week from disease onset compared with those of the patients who received no topical steroid treatment. However, those outcomes may be because of the presumed fact that patients who fail to receive treatment with topical steroids are highly likely not going to receive systemic steroids as well. Thus, treatment with topical steroids, systemic steroids, or both at the early stage of the disease helps to decrease the incidence of chronic ocular complications. At the onset of the diseases, both necrotic changes of the skin and the destruction of the ocular surface progress rapidly. Prompt use of topical steroids, and presumably systemic steroids, from disease onset may prove to be important for preventing the loss of corneal epithelial stem cells. Unfortunately, a detailed history concerning the systemic therapy during the acute stage could not be obtained in most instances. Additional studies are needed to confirm the safety and efficacy of those medications.

Of the 94 patients, the mean duration of the illness was 16.1 years, and more than 50% of the eyes manifested visual acuity worse than 20/200. Considering the fact that patients with SJS or TEN experience ocular complications for an extended period, it is vital that strict attention be paid to any ocular involvement. When dermatologists, physicians, and healthcare professionals suspect SJS or TEN, prompt referral to an ophthalmologist is vital for the prevention of permanent loss of vision. Ophthalmologists have to find distinctive appearances such as pseudomembrane formation and corneal or conjunctival epithelial defects, or both.

Table 2. Order of Conjunctivitis and Skin Eruptions of the 94 Patients at Disease Onset

| Conjunctivitis | Period Preceding Eruption | No. of Patients |
|---|---|---|
| Occurred before skin eruption | 4 days | 1 |
| | 3 days | 3 |
| | 2 days | 11 |
| | 1 day | 12 |
| | Several hours | 9 |
| | Unknown | 6 |
| | | Total = 42 |
| Occurred simultaneously | | 21 |
| Occurred later | | 1 |
| Unknown | | 30 |
| Total | | 94 |

In the first report by Stevens and Johnson, 2 boys reported eye pain before skin eruptions and manifested a purulent conjunctivitis. Visual prognosis was total blindness in one case and severe corneal scarring in the other case. Both cases had the typical clinical picture clarified in the present study.[1] If their eyes had been treated with topical steroids from disease onset, the visual outcomes might have been different.

To date, the pathophysiologic mechanisms underlying the onset of SJS and TEN have yet to be fully elucidated. The rarity of these diseases has led us to speculate that patients with SJS or TEN genetically are susceptible to specific environmental precipitants. A report from the United States showed an increase of human leukocyte antigen (HLA)-B12 (HLA-Bw44) antigen in white patients with SJS with ocular involvement.[31] Analyses of TEN patients in France also disclosed an association with HLA-B12 (HLA-Bw44).[32] In Han Chinese, there was a very strong association between carbamazepine-induced SJS and the HLA-B*1502 allele.[33] The authors also reported that in Japanese persons, HLA-A*0206 was strongly associated with SJS and TEN with ocular surface complications.[34,38] These findings suggest that SJS and TEN are associated with a complex genetic inheritance background.

The prodromal symptoms occurred in 82% of the cases in this study. Given the association between the onset of SJS and TEN and infections and the opportunistic infection of ocular surfaces by bacteria such as methicillin-resistant *Staphylococcus aureus* or methicillin-resistant *Staphylococcus epidermidis*,[41] it is highly possible that there is an association between SJS and TEN and a disordered innate immune response. Recently, the association of the polymorphisms in the toll-like receptor 3 gene with SJS and TEN in the Japanese population were reported.[36] Also, an association between SJS and TEN and the IL-4R gene polymorphism and combined IL-13/IL-4R signaling pathway gene polymorphism was reported.[35,39]

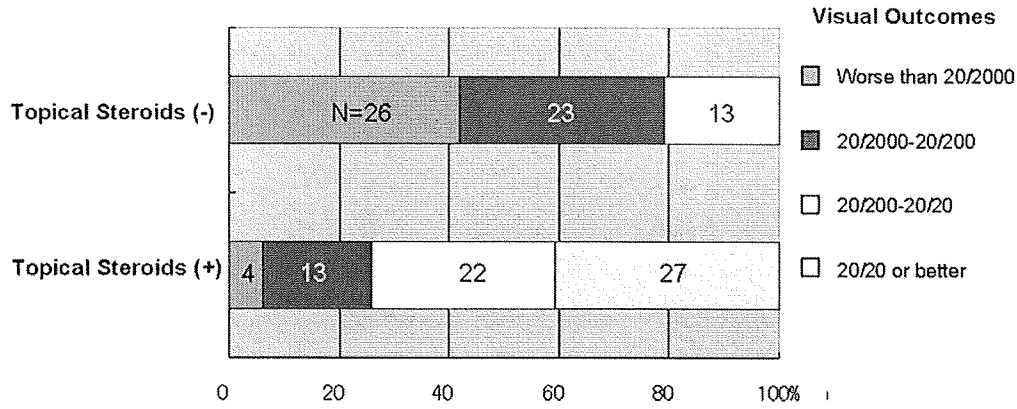


Figure 3. Graph showing the relationship between topical steroid use during the first week from disease onset and visual outcomes. Sixty-six eyes of 33 patients began topical steroid treatment during the first week from disease onset, whereas 62 eyes of 31 patients received no topical steroid treatment or any other treatment. Visual outcomes were significantly better in the group receiving topical steroids at the acute stage compared with those of the no-treatment group ($P<0.00001$).

Thus, both innate immunity and host–defense mechanisms may play a critical role in the development of SJS and TEN.

In conclusion, ocular involvement at disease onset is a helpful symptom for the diagnosis of SJS and TEN. Acute conjunctivitis before or occurring simultaneously with skin eruptions accompanied by very high fever and blisters on the mouth greatly implies the initial signs of SJS and TEN, and prodromal symptoms and genital involvements support that diagnosis. Initiating treatment with topical steroids from the onset seems to be important for the improvement of visual prognosis. A prompt and accurate diagnosis as assisted by the clinical manifestation offers a breakthrough against the historically poor visual outcomes associated with patients with SJS or TEN.

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## Footnotes and Financial Disclosures

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# Successful Treatment of Stevens-Johnson Syndrome with Steroid Pulse Therapy at Disease Onset

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• PURPOSE: To evaluate the visual prognosis of patients with Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), followed by general and topical high-dose corticosteroids administration from disease onset.

• DESIGN: Prospective, observational case series.

• METHODS: Between May 1, 2003 and June 30, 2005, we enrolled 5 patients with SJS or TEN with ocular complications at the acute stage. Intravenous pulse therapy with methylprednisolone (steroid pulse therapy; 500 or 1000 mg/day for 3 to 4 days) was initiated within 4 days from disease onset. Topically, 0.1% betamethasone was applied over 5 times daily for at least 2 weeks. Visual acuity (VA) and slit-lamp microscopic appearance 1 year from disease onset were evaluated.

• RESULTS: At the first examination, corneal or conjunctival epithelial defects and pseudomembranous conjunctivitis were present in all cases. Skin eruptions dramatically improved after steroid pulse therapy. Although ocular inflammation increased for several days, pseudomembranes disappeared and corneal and conjunctival epithelium regenerated within 6 weeks. At the chronic stage, all eyes had clear corneas with the palisades of Vogt (POV), implying the presence of corneal epithelial stem cells. Best-corrected VA was 20/20 or better in all eyes. Five eyes showed superficial punctate keratopathy. No eye had cicatricial changes except for 1 with slight fornix shortening. No significant adverse effects of steroid occurred during all clinical courses.

• CONCLUSIONS: Steroid pulse therapy at disease onset is of great therapeutic importance in preventing ocular complications. Topical betamethasone also shows great promise for preventing corneal epithelial stem cell loss in the limbal region and cicatricial changes. (Am J Ophthalmol 2009;147:1004–1011. © 2009 by Elsevier Inc. All rights reserved.)

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STEVENS-JOHNSON SYNDROME (SJS), FIRST REPORTED in 1922, is an acute inflammatory disease that predominantly affects the skin and mucosal membranes, including the ocular surface.[1] In 1956, Lyell described a clinical condition characterized by extensive epidermal loss, termed *toxic epidermal necrolysis* (TEN).[2] Recent reports suggest that SJS and TEN are the same disorder, but of different severities.[3–5] In the acute stage, these diseases predispose patients to life-threatening complications such as sepsis, respiratory dysfunction, and multiorgan failure. Mucosal sites including the ocular surface and oral membrane commonly are involved at the onset of acute fever and skin eruptions. Although skin usually heals without dysfunction, severe corneal opacity and dry eye often persist during the chronic stage. Patients with SJS or TEN require life-long management for ocular discomfort and morbidity.[6–9] Recently, it was reported that amniotic membrane transplantation (AMT) onto the ocular surface is effective for reducing the destructive inflammation in acute SJS or TEN and for preventing cicatricial change.[10–12] However, safe and effective medical treatment for the prevention of ocular complications has yet to be established.

Although the pathogeneses of SJS and TEN has not been elucidated fully, it has been indicated that soluble FasL-mediated apoptosis plays a crucial role in the pathogeneses of SJS and TEN.[13] Drug-specific cytotoxic CD8+ T lymphocytes were detected in blister fluids of SJS and TEN patients and in cytotoxic lymphocyte cytolytic pathways, including major histocompatibility complex class I.[14] It also has been reported that tumor necrosis factor and interferon-γ also are involved in the mechanisms of epidermal necrosis.[15] Therefore, it is highly possible that medication at the acute stage to downregulate such immunologic reactions is useful for the treatment of SJS and TEN.

The use of systemic corticosteroids for the care of patients with acute SJS and TEN is controversial.[16,17] Although the beneficial effects of corticosteroid therapy during the acute stage has been reported,[18–20] high mortality rates in patients receiving corticosteroids has been shown.[21,22] The timing, dose, formulation, and route of administration of the steroid differ in these reports. At disease onset, skin involvement and ocular involvement progress rapidly, and facial manifestation and general condition became worse from morning to evening. Considering the pathogenesis described above and the rapid progression of SJS and TEN at disease onset, we hypoth-





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esized that the timing and dose of the administered steroid are both key to obtaining beneficial effects.

In patients with SJS- or TEN-induced chronic ocular complications, the total loss of the palisades of Vogt (POV) commonly is observed.[23] POV in the limbal area implies the presence of corneal epithelial stem cells.[24] At the acute stage, corneal epithelial defect or corneal ulceration occur in more than 50% of the patients with SJS or TEN.[25] In cases with limbal stem cell loss, conjunctivalization and neovascularization of the cornea progress, leading to severe visual impairment or blindness.[6–9] Loss of the POV occurs during the acute stage of SJS and TEN and can be accompanied by severe inflammation. The administration of high-dose general and topical corticosteroids from disease onset may downregulate the immunologic reactions described above and may prevent the loss of corneal epithelial stem cells.

The aim of this study was to evaluate the ophthalmic efficacy of high-dose corticosteroid therapy at the acute stage of SJS or TEN. All patients in this study were administered high-dose systemic methylprednisolone (steroid pulse therapy) and topical betamethasone for SJS or TEN with ocular involvement from the onset of the disease. Side effects of the steroids were monitored carefully over the duration of this study, and a great amount of attention was paid to systemic and ophthalmic infections. We then evaluated visual acuity (VA) and the slit-lamp microscopic appearance in these patients at the chronic stage.

## METHODS

BETWEEN MAY 1, 2003 AND JUNE 30, 2005, WE ENROLLED 5 consecutive patients (2 males and 3 females, 23 to 49 years of age at disease onset; mean age, 32.8 years) referred to us during the first 4 days from the onset of SJS or TEN accompanied by ocular complications (ocular surface epithelial defects, pseudomembranous formation, or both). The diagnosis of SJS or TEN was confirmed by dermatologists based on clinical and histopathologic classification.[26,27] Prior informed consent to participate in the study was obtained in written form from all patients, their families, or both.

We initiated therapy with intravenous high-dose methylprednisolone and intensive topical betamethasone immediately after the dermatologic and ophthalmologic diagnosis. For initial treatment, the protocol used in this study was as follows: intravenous methylprednisolone at a dosage of 500 to 1000 mg/day was used for 3 to 4 days (steroid pulse therapy) and 0.1% betamethasone was applied topically more than 5 times daily for at least 2 weeks. The topical antimicrobial agent was applied prophylactically.

Signs of systemic and ophthalmic infection were monitored by the culture of blood, conjunctival swab, and the swab of other mucous membranes. The body temperature and the patient's symptoms and biochemical parameters also were monitored carefully.

Patient-related ocular findings and the complications during the acute stage were recorded fully until the remission of the ocular surface inflammation. As for ocular complications, corneal complications (superficial punctate keratopathy [SPK], epithelial defect, loss of the POV, conjunctivalization, neovascularization, opacification, and keratinization), conjunctival complications (hyperemia and symblepharon formation), and eyelid complications (trichiasis, mucocutaneous junction involvement, meibomian gland involvement, and punctal damage) were recorded according to a new grading system for SJS that we previously reported.[23] Tear secretion was assessed by the Schirmer 1 test, and meibomian gland morphologic features were evaluated using meibography.[28,29] VA and ocular findings at the chronic stage were evaluated after 1 year from the onset of the disease.

## RESULTS

• OCULAR FINDINGS AND THERAPY DURING THE ACUTE STAGE: Five patients were referred to us within 4 days (0 to 4 days; mean, 1.2 days) from the onset (the initiation of skin eruptions accompanying mucocutaneous illness) of the disease (Table). All patients had rapidly progressing skin eruptions, mucous membrane erosions, and a very high fever (more than 39 C) at presentation. Those symptoms were preceded by common cold-like symptoms (general malaise, fever, sore throat, or a combination thereof) in 4 patients. The causative drugs were cold remedies (Cases 1 and 5), antibiotics (Cases 3 and 5), and nonsteroidal anti-inflammatory drugs (Cases 3, 4, and 5). In 1 patient, high fever and erythematous macules developed after vaccination for measles (Case 2). All 10 eyes had pseudomembranous conjunctivitis. Corneal or conjunctival epithelial defects were present in all cases. Corneal epithelial defects existed in 5 eyes, and severe SPK was present in the other 5 eyes. Conjunctival epithelial defects were observed in 6 eyes (Figures 1 and 2). Skin biopsy specimens of the erythematous macules from all patients showed necrotic keratinocytes and liquefaction degeneration that were consistent with the diagnosis of SJS or TEN (Figure 3).

In all patients, steroid pulse therapy was initiated immediately after confirming ocular involvement, except 1 case (Case 5) in which steroid pulse therapy already had been initiated previously by a dermatologist. Thereafter, systemic steroids were changed to prednisolone or betamethasone (Table). Topically, 0.1% betamethasone (0.1% betamethasone solution, 0.1% betamethasone eye ointment, or both; 5 to 8 times daily) was used from the day we confirmed ocular involvement. An ophthalmic fluoroquinolone solution (0.3% gatifloxacin or 0.3% ofloxacin; 4 times daily) was used for the prevention of ocular infec-

**TABLE.** Dosage and Duration of Systemic Corticosteroid Administration during the Acute Stage of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis

| | | | | Steroid Pulse Therapy (Methylprednisolone) | | | Steroid Administration after Pulse Therapy (Prednisolone Equivalent) | | |
|---|---|---|---|---|---|---|---|---|---|
| Case | Diagnosis | Age (yrs) | Gender | Elapsed Time from Onset to Initiation of Therapy (days) | Steroid Dose (mg/day) | Duration (days) | Initial Dose (mg/day) | Total Duration (days) | Total Amount (mg) |
| 1 | SJS | 23 | M | 1 | 500 | 3 | 40 | 85 | 1045 |
| 2 | SJS | 27 | F | 0 | 1000 | 3 | 40 | 35 | 510 |
| 3 | SJS | 31 | F | 4 | 500 | 3 | 60 | 20 | 425 |
| 4 | SJS | 34 | F | 1 | 1000 | 3 | 40 | 72 | 570 |
| 5 | TEN | 49 | M | 0 | 500 | 4 | 60 | 9 | 420 |
| Mean | | 32.8 | | 1.2 | | | | 44 | 594 |

F = female; M = male; SJS = Stevens-Johnson syndrome; TEN = toxic epidermal necrolysis; yrs = years.

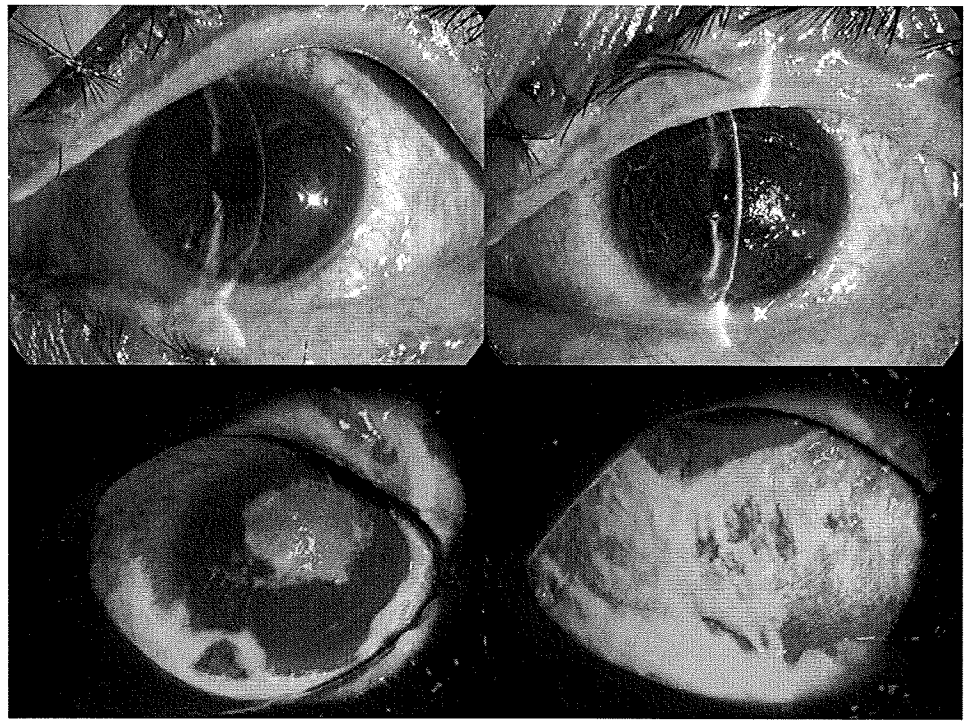

FIGURE 1. Images demonstrated Stevens-Johnson syndrome (SJS) at the acute stage (Case 1). (Top left) Two days after disease onset, pseudomembranous conjunctivitis with conjunctival epithelial defect was present around the limbus. The cornea was clear without defect and superficial punctate keratopathy. (Top right) At the most inflamed phase, 9 days after disease onset and 8 days after the start of steroid pulse therapy, the ocular surface was most inflamed with increased eye discharge, and the pseudomembrane and cilia fell out partially in the lower eyelid. (Bottom left) Corneal epithelial defect. (Bottom right) Conjunctival epithelial defect extending to almost the entire bulbar and palpebral conjunctiva.

tions. Prophylactic systemic antibiotics were not used, because all 5 cases were associated with drug reactions.

Skin eruptions dramatically improved after initiation of the steroid pulse therapy (Figure 4). Despite intensive use of systemic and topical corticosteroids, pseudomembranous formation increased and epithelial defects enlarged during the first several days, peaking at 1 to 9 days (mean, 4.0 days) from their onset. Thereafter, corneal epithelial defects improved day by day and disappeared within 2 to 13 days (mean, 5.2 days). Conjunctival epithelium regenerated completely within 1 to 38 days (mean, 13.0 days).

The administration of systemic steroids was tapered off gradually according to the patient's general and ophthalmic conditions. Whereas cutaneous involvement was quickly eliminated after initiation of steroid pulse therapy, ocular surface inflammation tended to persist longer than cutaneous inflammation. The total amount of steroids was 420 to 1045 mg (changed to prednisolone) during 9 to 85

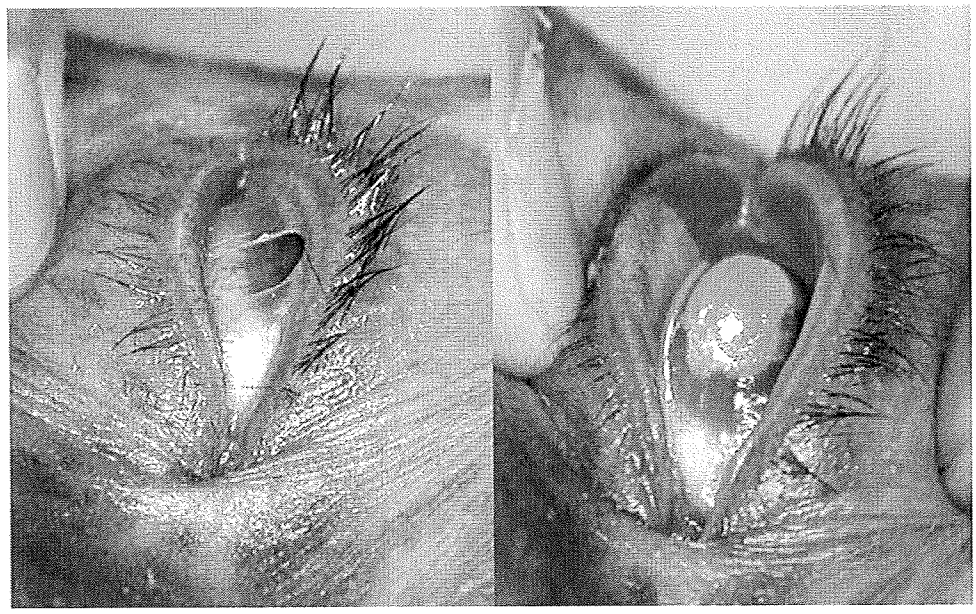

FIGURE 2. Images showing toxic epidermal necrolysis at the acute stage (Case 5). Because the general condition was still critical, the patient was examined on his bed. (Left) Pseudomembrane was present between the upper and lower eyelids. (Right) After removal of the pseudomembrane, corneal epithelial defect was observed.

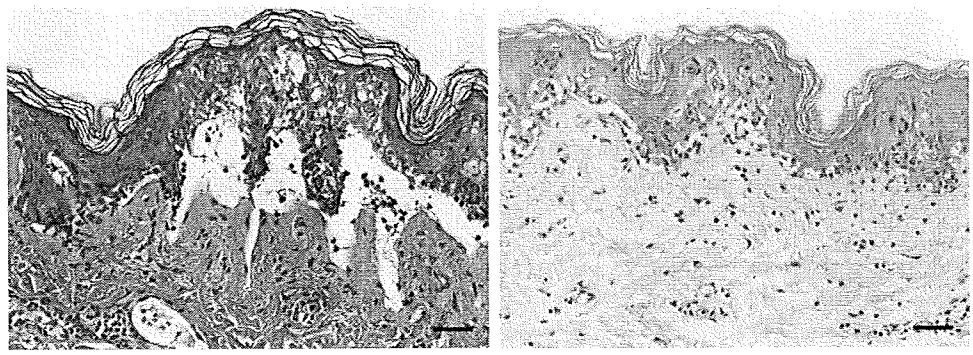

FIGURE 3. Photomicrographs showing sections from a skin lesion of SJS or toxic epidermal necrolysis at the acute stage. (Left) Case 1 with SJS (Right) Case 5 with toxic epidermal necrolysis. These sections show liquefaction degeneration producing a subepidermal cleft. The epidermis contains numerous necrotic keratinocytes with vacuolated cytoplasm or pyknotic nucleus (hematoxylin and eosin, bars = 100 mm).

days from disease onset (Table). One patient with TEN received plasmapheresis[30,31] for 3 days after steroid pulse therapy. Topical 0.1% betamethasone was used for a total of 40 to 165 days (mean, 91.4 days), then switched to 0.1% fluorometholone.

We observed no significant adverse effects from steroid pulse therapy, such as sepsis, pneumonia, or other infections. No cardiac arrhythmia or kidney or liver dysfunction occurred. We continued the culture of the conjunctival swabs during the use of topical or systemic steroids, or both. Methicillin-resistant *Staphylococcus aureus* was detected from the culture of the conjunctival swab in 2 eyes of 1 case at 1.5 months from disease onset, and coagulase-negative *Staphylococci* was observed in 2 eyes of another case at 10 days from disease onset. However, both cases showed no infectious ocular manifestations.

• VISUAL OUTCOMES AND OCULAR FINDINGS AT THE CHRONIC STAGE: In all eyes, best-corrected VA at 1 year from disease onset was 20/20 or better. No eyes had the appearance of an epithelial defect, the loss of the POV, conjunctivalization, neovascularization, opacification, or keratinization. As for corneal complications, only mild SPK was present in 5 eyes. As for conjunctival complications, fornix shortening with mild symblepharon was present only in 1 eye (Case 4). In contrast, all eyes manifested mild lid complications and mild irregularity of the mucocutaneous junction (Figure 5). All patients ex-

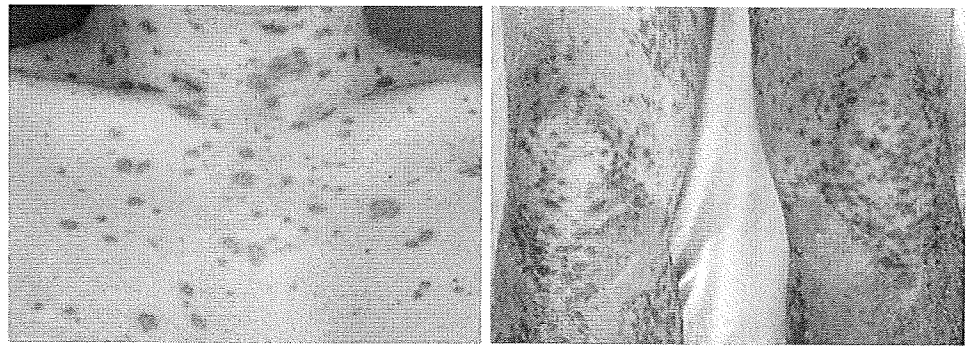

FIGURE 4. Photographs showing skin eruptions of SJS or toxic epidermal necrolysis after steroid pulse therapy. (Left) Case 1 with SJS. (Right) Case 5 with toxic epidermal necrolysis.

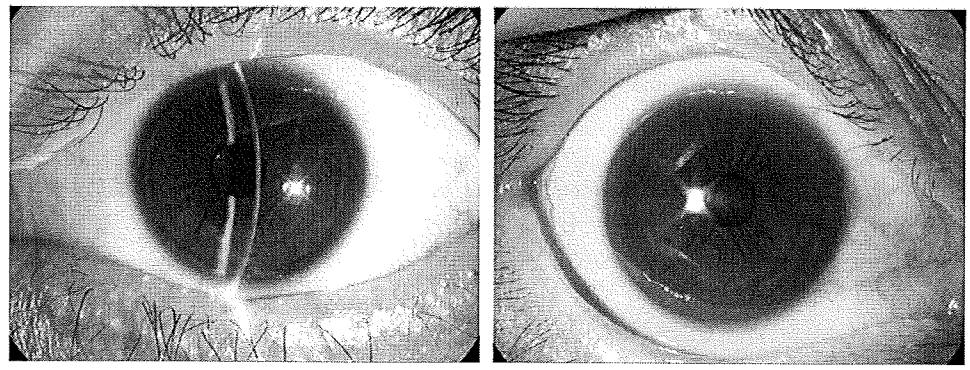

FIGURE 5. Photographs showing the ocular appearances of SJS or toxic epidermal necrolysis at the chronic stage. (Left) Case 1 with SJS. (Right) Case 5 with toxic epidermal necrolysis. The corneas were clear and the ocular surfaces were not inflamed 1 year after disease onset. Mucocutaneous junction involvements were mild.

perienced slight discomfort from irritation to their ocular surface, thus requiring the instillation of artificial tears. The Schirmer 1 test measured less than 5 mm in 4 eyes of 3 cases and 5 mm or more in the other eyes. There was no punctal damage in all eyes. Meibomian gland morphologic features were normal in 8 eyes and mild to moderately dropout in 2 eyes. No increase of intraocular pressure and no infectious keratitis occurred during all clinical courses.

• CASE 1: A healthy 23-year-old man (Case 1) presented to our hospital on October 14, 2004. He had erythematous skin eruptions on the trunk and extremities after taking cold remedies. The body temperature increased to more than 39 C and the erythematous macules increased rapidly and became blisters. Extensive hemorrhagic erosion on the lips and oral mucosa were also observed when he visited our hospital. He was aware of bilateral red eyes just before the skin eruptions. Eye discharge appeared with skin rashes and markedly increased as the skin and oral site worsened. At first examination, both eyelids were edematous, and pseudomembranous conjunctivitis was noted. The tarsal and bulbar conjunctivae were affected severely, and extensive epithelial defect was observed in both eyes. Lid margins also were ulcerated with the partial loss of cilia (Figure 1). A skin biopsy was performed and histopathologic findings were compatible with the clinical diagnosis of SJS (Figure 3).

Immediately after the diagnosis, steroid pulse therapy (methylprednisolone; 500 mg/day for 3 days) was initiated. Topically, betamethasone was instilled 8 times daily (eye drop and ointment each administered 4 times daily). The improvement was dramatic. First, the development of new lesions stopped after the initiation of steroid pulse therapy. Thereafter, skin eruptions decreased and systemic conditions improved day by day (Figure 4). However, ocular inflammation increased with pseudomembranous formation, bilateral epithelial defects in the center of the cornea, and large conjunctival epithelial defects extending to nearly the entire bulbar and palpebral conjunctiva. After the peak of ocular surface inflammation at 9 days from disease onset, corneal and conjunctival epithelium began to regenerate.

Steroid pulse therapy was switched to intravenous betamethasone at a dosage of 4 mg/day for 5 days and then gradually tapered off. The total amount of systemic steroid was 1045 mg of a prednisolone equivalent, administered for a total of 85 days. Topically, betamethasone was initially administered for 31 days, with a total duration of 165 days. The pseudomembrane was removed daily and

symblepharon was separated with a glass rod for 15 days. Although skin eruptions diminished without recurrence, pseudomembranous conjunctivitis recurred after the reduction of systemic or topical steroids at 2 to 4 weeks from disease onset. Both systemic and topical steroids were tapered off carefully according to the ocular surface appearance. Corneal epithelial defects healed within 3 days from their appearance. Large conjunctival epithelial defects needed 21 and 38 days for epithelization, respectfully. Ocular inflammation gradually subsided over a 5-month period.

At the chronic stage, 1 year from disease onset, VA was 20/20 in both eyes. Both corneas were clear with POV and there existed no symblepharon, trichiasis, conjunctivalization, or neovascularization. Only the mucocutaneous junction showed slight cicatricial changes with mild irregularity (Figure 5). The Schirmer 1 test showed 1 mm in the right eye and 2 mm in the left eye. Meibomian gland morphologic features were normal in both eyes. The patient used topical artificial tears, and mild SPKs existed. He reported either no or slight discomfort on the ocular surface.

## DISCUSSION

AT THE BEGINNING OF THIS STUDY, WE HYPOTHESIZED that it is important to start steroid pulse therapy at the acute stage as soon as possible. Because the destruction of the ocular surface epithelium, especially corneal epithelial stem cells, at the acute stage progresses rapidly, the initiation time of corticosteroid therapy may be the key to obtaining a good prognosis. Based on this hypothesis, we prospectively used high-dose methylprednisolone and intensive topical betamethasone immediately after the dermatologic and ophthalmologic diagnosis in cases with ocular involvement.

Five cases of SJS, including 1 case of TEN, were enrolled in this study. We initiated steroid pulse therapy with methylprednisolone 0 to 4 days (mean, 1.2 days) from disease onset. Simultaneously, topical betamethasone treatment was initiated immediately. One year after disease onset, VA was 20/20 or better in all 10 eyes.

It is noteworthy that the POV were maintained completely in all eyes in this study, suggesting the survival of corneal epithelial stem cells. Although it is uncertain whether stem cell loss is the primary or secondary damage of SJS and TEN, it is probable that steroid pulse therapy at the onset of the disease protected corneal epithelial stem cells from depletion. Intriguingly, antioxidative agents reportedly restore the reconstitutive capacity of hematopoietic stem cells.[32] Corneal epithelial stem cells may be protected via the reduction of oxidative stress by high-dose corticosteroid instillation.

Previously, we reported that the most severely affected ocular components in SJS and TEN at the chronic phase were loss of the POV (82.6%) and meibomian gland involvement (73.9%).[23] In the cases reported in this study, there existed no cicatricial changes on the cornea, and only 1 eye showed fornix shortening. It is highly possible that intensive steroid therapy also prevented the destruction of the meibomian gland, thus resulting in much fewer lid-related complications. In 1 case (Case 1), pseudomembranous conjunctivitis recurred after the reduction of systemic or topical steroids. A careful reduction of general and topical steroids is necessary to prevent cicatricial changes of the ocular surface. Both the systemic and topical application of steroids is needed to suppress the ocular surface inflammation effectively, which sometimes persists for a longer period than the cutaneous inflammation.

Although this study was not a randomized trial, we also compared the patients with SJS and TEN at the chronic stage during the period of this research. Six patients (3 males and 3 females; 6 to 67 years of age at disease onset; mean age, 32.7 years) without systemic and topical steroids at the acute stage showed severe cicatricial changes of the ocular surface. Their VA was between hand movements and 40/200. Additional studies are needed to compare the systemic and ophthalmologic prognosis of SJS and TEN patients with or without the early administration of steroids.

Several reports have suggested the advantage of using cryopreserved AMT for the treatment of acute SJS and TEN.[10–12] The similarity between these reports and ours is the intervention of the treatment at the acute stage. It is noted that both treatments demonstrated the beneficial effects of reducing ocular surface inflammation at the acute stage, as well as positive results in preventing cicatricial changes at the chronic stage. It seems probable that early intervention limited the cicatricial changes later. Although the intensive steroid therapy in this study was started within 4 days from disease onset, the timing of AMT in the previous reports was later than the initiation of treatment shown in our study.[12] In addition, the use of systemic and topical steroids was not described in the previous reports. For these reasons, we are unable to compare the effect of AMT and steroid pulse therapy on the ocular complications in SJS and TEN. Further studies are needed to compare and elucidate the effects, indication, and complications associated with treatment by surgery vs medical intervention.

It should be emphasized that in this study, steroid pulse therapy was initiated within 4 days from disease onset. If extensive mucocutaneous damage already has progressed, the risks of general and local infections increase. Although all 5 cases in this study were associated with drug reactions, not all cases of SJS and TEN are caused by drugs. A fraction of cases are caused by viral (such as herpes simplex virus) or mycoplasma infection. It is important to confirm that there are no signs of infectious activity before the administration of steroid pulse therapy by monitoring vital signs and by detecting potential pathogens by serum

antibody titer, blood culture, and the polymerase chain reaction method. Intensive management by a medical team consisting of at least 1 dermatologist, 1 ophthalmologist, 1 physician, and 1 infection control doctor is needed to obtain the best results.

Our recent reports, and those of others, have indicated the participation of genetic endowment in SJS and TEN.[33–41] For instance, there are statistically significant differences in single nucleotide polymorphisms of toll-like receptor 3 interleukin 4R/interleukin 13, and FasL in SJS and TEN; thus, genetic screening may help to deliver a more rapid diagnosis or prevention of SJS and TEN in the future. At present, however, prompt diagnosis and early treatment with high-dose steroids is a vital aspect of preventing general and ophthalmic complications.

In conclusion, steroid pulse therapy at the disease onset is of great therapeutic importance in preventing ocular complications. Although both SJS and TEN are self-limiting diseases, appropriate intervention during the acute stage holds great promise for the prevention of corneal epithelial stem-cell loss and corneal and conjunctival cicatricial changes. An appropriate and prompt diagnosis followed by the administration of high-dose corticosteroids may improve the visual prognosis of these 2 devastating diseases.

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## Biosketch

Dr Yayoi Araki graduated and received her medical degree from Kyoto Prefectural University of Medicine, Kyoto, Japan in 2003, and completed her residency training at the Department of Ophthalmology at Kyoto Prefectural University Hospital, Kyoto, Japan. Dr Araki currently specializes in clinical research and cornea-related diseases.

# 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 $Gr\text{-}1^+CD11c^-$ neutrophils. In contrast, rejected MHC-matched corneas in GKO hosts, as well as GRKO and WT hosts, elicited intensive infiltration of $CD11b^+$ macrophages and $Gr\text{-}1^+CD11c^-$ 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. (*Invest Ophthalmol Vis Sci.* 2009;50:2139–2146) DOI:10.1167/iovs.08-2993

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Although penetrating keratoplasty has emerged as one of the successful corneal transplantation procedures, 20% to 40% of allografts fail within 10 years. The field of allogeneic transplantation continues to strive for indefinite allograft survival and alloantigen (alloAg)-specific tolerance in the absence of chronic medical intervention.[1] Although the value of major histocompatibility antigen complex (MHC) matching in organ transplantation is well established, considerable controversy surrounds the issue of whether MHC matching reduces the risk of corneal graft rejection.[2–5] In the recent past, it is well known that minor histocompatibility (minor H) antigens (Ag) behave as the most critical initiator of alloimmunity.[6,7] Irrespective of whether the graft bed is low or high risk, minor H disparate cornea grafts are rejected at a higher rate and more vigorously than are grafts displaying only MHC-encoded alloAg.[6,8]

Corneal allograft rejection is thought to be mainly mediated by the T helper (Th)1-type immune response, which correlates with IFN-γ. Until recently, the most widely accepted paradigm was that most forms of organ graft rejection are mediated by Th1 cells and that Th2 cells protect against rejection by suppressing delayed-type hypersensitivity (DTH) and by counteracting the actions of IFN-γ.[9] In a previous report, we showed that mice manipulated to respond preferentially toward a Th2 pathway, concomitant with a sharp decrease in IFN-γ, displayed a greater than 50% reduction in corneal graft rejection.[10] Differentiated Th1 or natural killer (NK) cells can directly produce IFN-γ in response to antigen-presenting cell (APC)-derived IL-12/IL-18, and both act synergistically to enhance IFN-γ production by graft-infiltrating T cells and NK cells.[11–15]

According to the study using BALB/c-background IFN-γ–deficient mice as the hosts, minor H–only, disparate, fully MHC-matched allografts survived indefinitely, yet MHC-only mismatched allografts were rejected.[2] It may be plausible that the results observed in one strain of mice may not be applicable to all strains and species.[8,16] It is evident that polymorphic genetic factors influencing immune reactivity and allograft sensitization are distinctive among different strains, as they are between BALB/c and C57BL/6 mice. Approximately 50% of allografts survived indefinitely when C57BL/6 (MHC+minor disparate) or B10/D2 (MHC-matched) corneal allografts were placed onto BALB/c recipients, whereas more than 90% of allografts were rejected within 50 days when BALB/c (MHC + minor disparate) or BALB.B (MHC matching) corneal allografts were placed onto C57BL/6 recipients (Table 1).

We performed a series of corneal transplants in the eyes of C57BL/6 mice, instead of BALB/c mice, so as to assess the effect of IFN-γ and/or IL-17 depletion on graft survival and to define the contribution of Th1, -2, and -17, in terms of their topical gene expression. The results indicate that IFN-γ does not play a critical role in minor H–directed allograft rejection in C57BL/6 mice. MHC-matched corneal allografts are rejected, not by eosinophils (Eos) or Th2 cells, but by infiltration of neutrophils (Neu) and macrophages (Mps). These results indicate the presence of sophisticated graft rejection mechanisms still elusive and not ascribed simply to Th1, -2, or -17.

TABLE 1. The Rates of Indefinite Corneal Allograft Survivals among Distinct Donor-Recipient Combinations

| Donor | Recipient | Survival Rate (%) | Allodisparity | References |
|---|---|---|---|---|
| C57BL/6 | BALB/c | 50 | MHC + minor H | 6 |
| C3H | BALB/c | 40 | MHC + minor H | 8 |
| B10.D2 | BALB/c | 50 | Minor H only | 6 |
| BALB.B | BALB/c | 80 | MHC only | 6 |
| BALB/c | C57BL/6 | <10 | MHC + minor H | 8 |
| C3H | C57BL/6 | 0 | MHC + minor H | 8 |
| BALB.B | C57BL/6 | 0 | Minor H only | 8 |
| B10.D2 | C57BL/6 | 60 | MHC only | 8 |

## MATERIALS AND METHODS

### Animals

Genetically IFN-γ-deficient C57BL/6 (GKO) mice[17] 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)[18] 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[19] and were maintained in Kochi Medical School. Male BALB/c (H-$2^d$), C57BL/6 (H-$2^b$), 129 (H-$2^b$) 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.[6,8] 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 anterior chamber. The grafts were examined by slit lamp biomicroscopy twice a week and scored using a previously described scoring system.[6] Grafts that received an opacity score of 2+ or greater (mild, deep stromal opacity with pupil margin and iris vessels visible) at 3 weeks after transplantation were considered rejected (immunologic failure). Grafts that were scored as 3+ or greater (moderate stromal opacity with only the pupil margin visible) at 2 weeks with no improvement by 8 weeks were also regarded as having been rejected at 2 weeks.

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 mM EDTA for 15 minutes at 37°C. The control samples were collected from age-matched normal mice. RNA from the corneal epithelium and endothelium-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-MLV 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 $1 \times 10^6$ irradiated (20 Gy) donor spleen cells in 10 μL Hanks' 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.[10] Briefly, lymphocytes from cervical lymph nodes or spleens of recipient mice were resuspended in 96-well plates ($2.5 \times 10^5$) and stimulated with irradiated (20 Gy) splenocytes ($2.5 \times 10^5$) 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 MHC-matched minor H-disparate B10.D2 mice.[2] First, to confirm the fate of MHC-matched 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

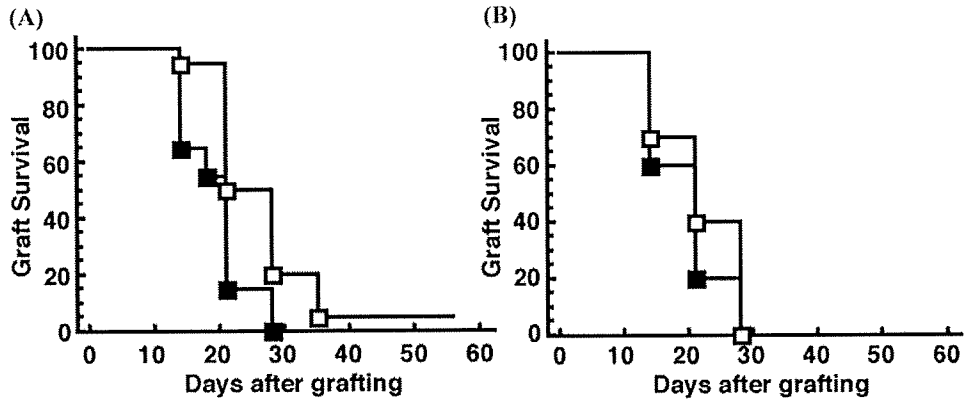


FIGURE 1. The effect of IFN-γ deficiency on MHC-matched corneal allograft survival. (**A**) Allografts ($n$ = 129) were placed on normal eyes of GKO (□; $n$ = 20) or WT C57BL/6 (■; $n$ = 20) mice; (**B**) allografts ($n$ = 129) were placed in normal eyes of GRKO (□; $n$ = 10) or WT C57BL/6 (■; $n$ = 20) mice.

C57BL/6 mice and wild-type (WT) C57BL/6 controls. GKO C57BL/6 mice rejected 95% of the129 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, 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, $Gr\text{-}1^+CD11c^-$Neu, and a few $Gr\text{-}1^+CD11c^+$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).

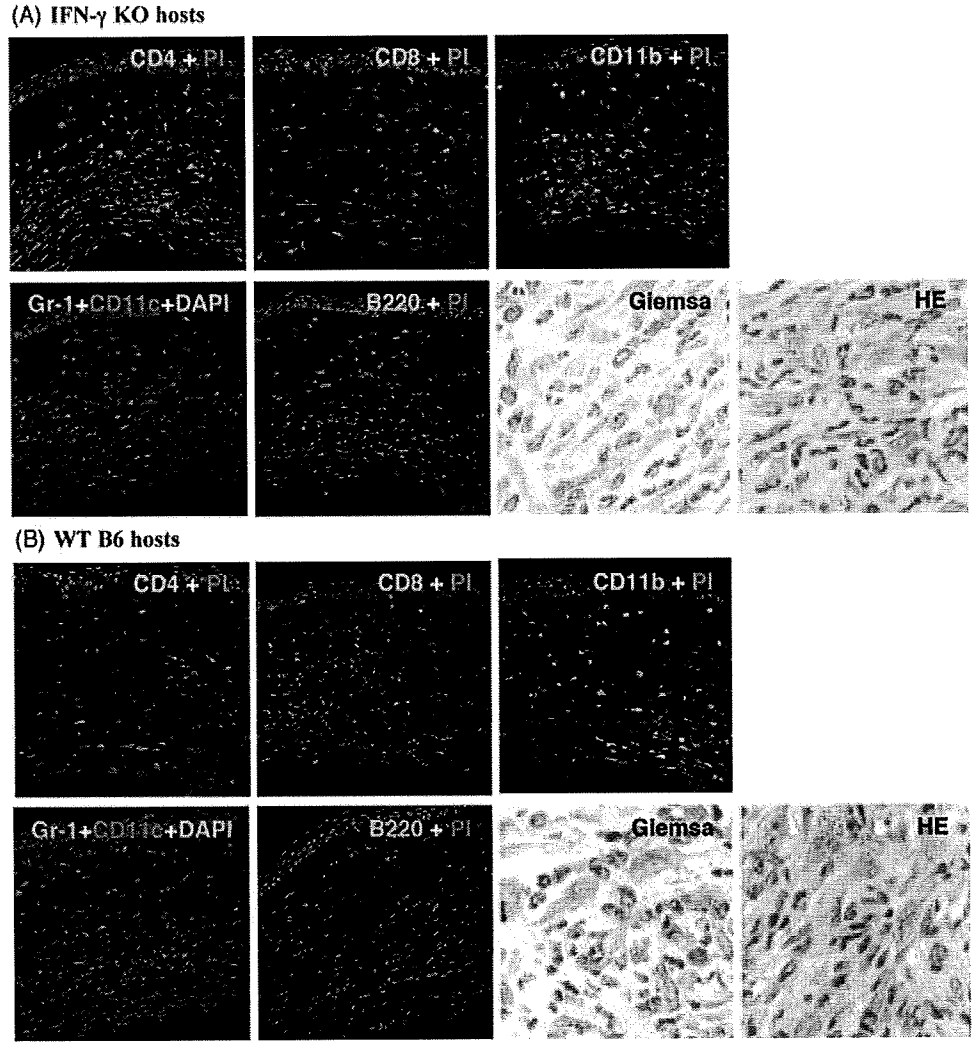


FIGURE 2. Histologic examination of rejected MHC-matched donor cornea. Rejected 129 donor corneas transplanted in GKO C57BL/6 (**A**) and WT C57BL/6 (**B**) hosts were stained immunohistologically with antibodies to CD4, CD8, CD11b, Gr-1+CD11c, B220, or Giemsa and HE.

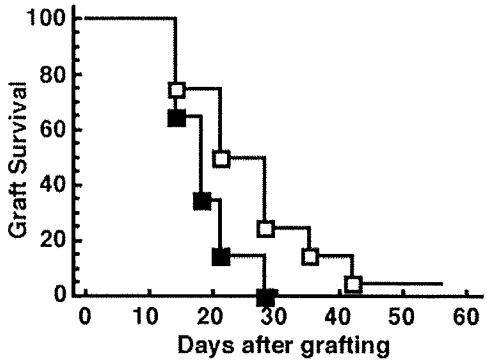


FIGURE 3. The effect of IFN-γ deficiency on MHC + minor H-disparate corneal allograft survival. BALB/c allografts were placed onto normal eyes of IFN-γKO (□; $n = 20$) or WT C57BL/6 (■; $n = 20$) mice.

## 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.[2] 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[2] 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.[20] 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

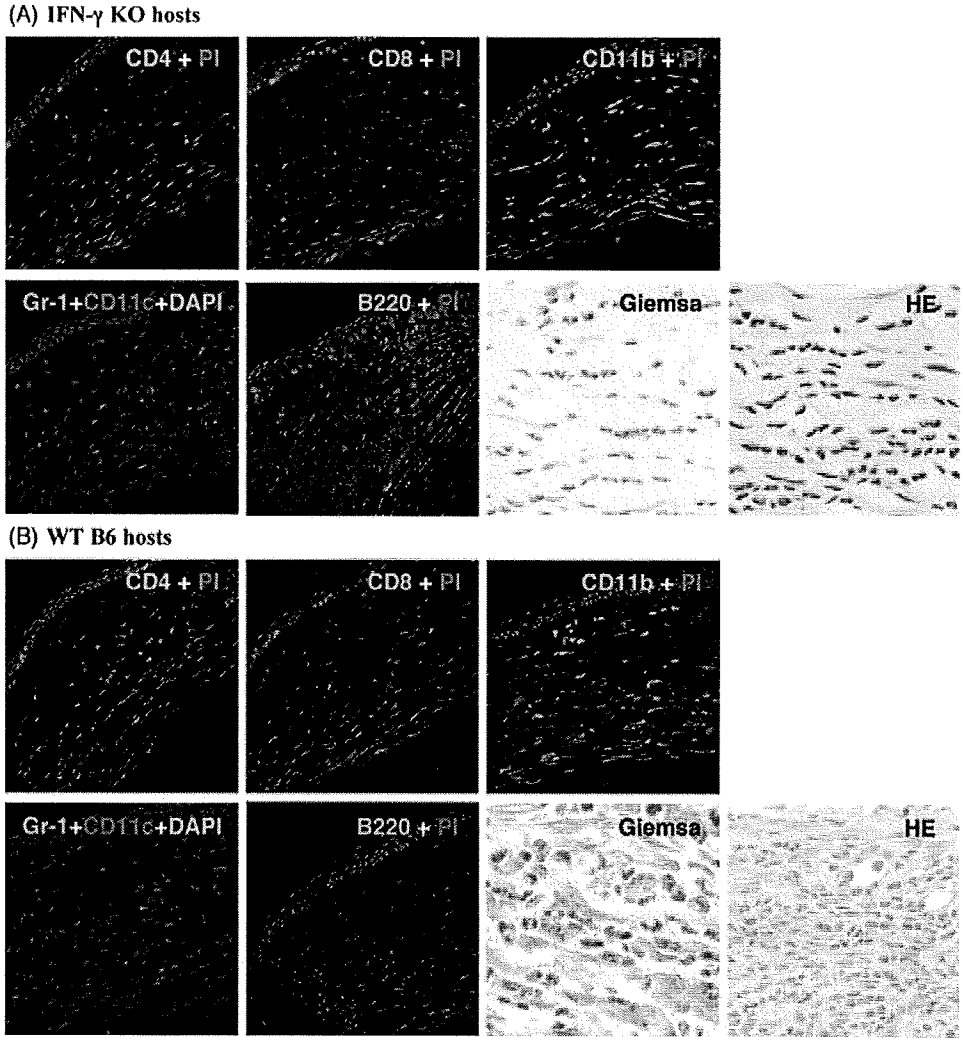


FIGURE 4. Histologic examination of rejected MHC + minor H-disparate donor cornea. Rejected BALB/c donor corneas transplanted in GKO C57BL/6 (**A**) and WT C57BL/6 (**B**) hosts were stained immunohistologically with antibodies to CD4, CD8, CD11b, Gr-1+CD11c, B220, or Giemsa and HE.

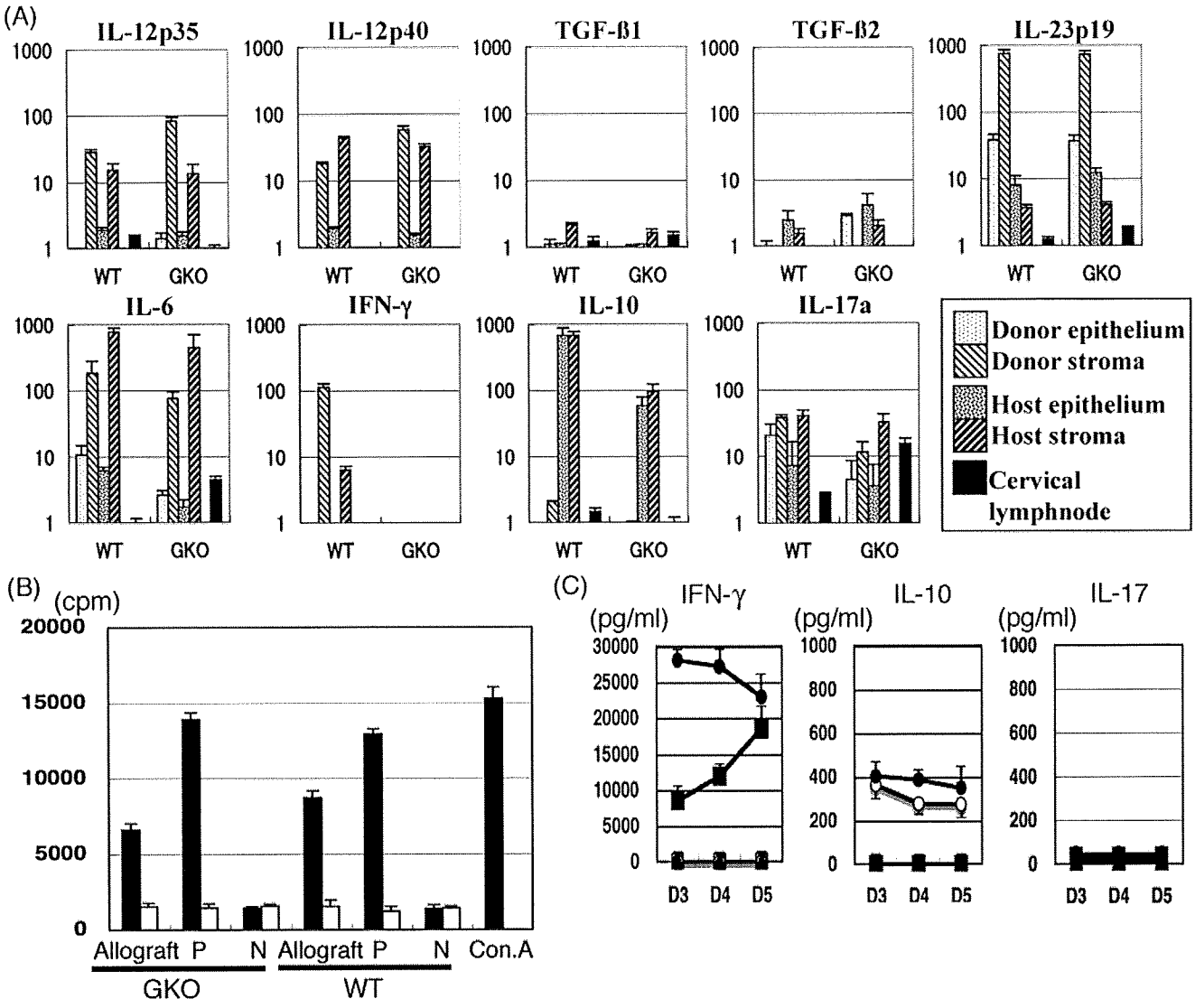


FIGURE 5. Gene upregulation (A), mixed lymphocyte reaction (B), and cytokine content in culture supernatants (C) of GKO and WT mice after MHC-matched corneal allografting. (A) One week after grafting, corneas were divided into donor-host and epithelium-stroma. Cervical LN was also collected. RNA was collected, and each gene was assessed by real-time PCR. Each gene expression was controlled by the expression of β-actin and is indicated as induction (x-fold) compared with that in naïve mice. (B) Lymphocytes from GKO or WT mice that received 129 cornea 3 weeks before (Allograft), immunized by SC injection of 129 splenocytes 1 week before (P), and naïve mice (N) were cultured 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 naïve 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.

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 cornea[21] 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. As shown in Figure 5B, 129-specific proliferation was positive in both GKO and WT control animals. IFN-γ was produced by lymphocytes from the cervical LN of allografted WT controls, but not GKO mice (Fig. 5C). IL-10 or -17A secretion by lymphocytes of allografted mice could not be detected.

## The Fate of Corneal Allografts in IL-17–Deficient C57BL/6 Mice

To address the hypothesis that C57BL/6 mice may reject MHC-matched minor H-only disparate allografts by the Th17 response, IL-17KO C57BL/6 mice were grafted with the 129 corneal allograft. The results revealed that IL-17KO mice re-

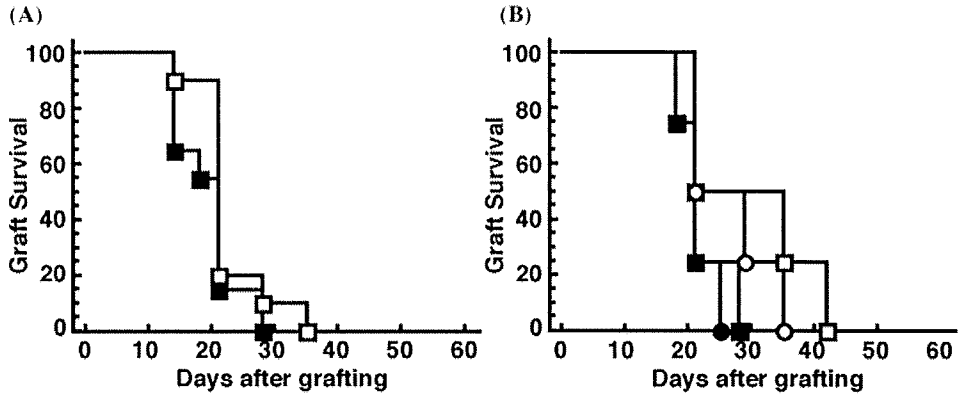


FIGURE 6. MHC-matched corneal allograft survival in IL-17KO mice. (A) Allografts ($n$ = 129) were placed onto normal eyes of IL-17KO (□; $n$ = 10) or WT (■; $n$ = 20) C57BL/6 mice. (B) Allografts ($n$ = 129) were placed in normal eyes of IL-17KO mice treated with anti-IFN-γ (□; $n$ = 4) or control IgG (■; $n$ = 4). Similarly, 129 grafted WT C57BL/6 mice were treated with anti-IFN-γ (○; $n$ = 4) or control IgG (●; $n$ = 4).

jected 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.[22] Eos have been implicated as effector cells in IFN-γ-independent allograft rejection.[2] Beauregard et al.[23] 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,[24-26] 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.[27-31]

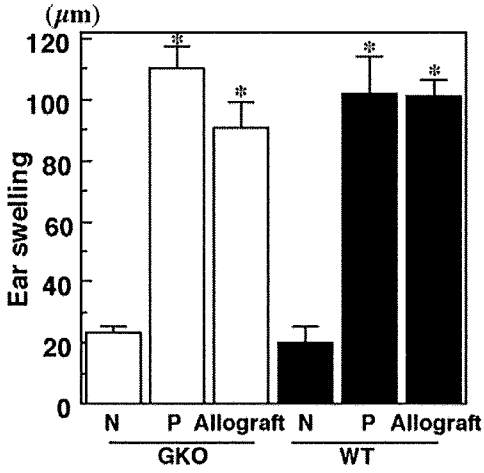


FIGURE 7. Donor-reactive DTH at 3 weeks after grafting. GKO (□) or WT (■) C57BL/6 mice received 129 corneal allografts. Three weeks later, they were ear challenged with 129 splenocytes and ear-swelling responses were assessed 24 hours later. Naïve (N) and immunized mice by SC injection of 129 splenocytes 1 week before (P) are shown as the control. *Responses significantly higher than those of the control ($P < 0.005$).

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.[32] The drivers of Th17 differentiation are Treg-derived TGF-β and DC-derived IL-6.[33] 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.[19,34] 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,[35] and since Th17 cells are known to be highly heterogeneous,[36,37] 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.[38-40]

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.[32] The process of graft rejection is very much an inflammatory one infiltrated with massive MNCs.[41] The ocular infiltrates in GKO-deficient mice under the pathologic progression of experimen-

TABLE 2. The Dominant Cellular Infiltrate in the Rejected Corneas among Distinct Donor-Recipient Combinations in C57BL/6 Hosts

| Donor | Recipient | Allodisparity | Main Cellular Infiltrate |
|---|---|---|---|
| BALB/c | wild-type C57BL/6 | MHC + minor H | Neu, Mps, T cell |
| BALB/c | IFN-γKO-C57BL/6 | MHC + minor H | Eos, B cell, Mps, T cell |
| BALB/c | IFN-γRKO-C57BL/6 | MHC + minor H | Eos, B cell, Mps, T cell |
| BALB/c | IL-17KO-C57BL/6 | MHC + minor H | Neu, Mps, T cell |
| 129 | Wild-type C57BL/6 | Minor H only | Neu, Mps, T cell |
| 129 | IFN-γKO-C57BL/6 | Minor H only | Neu, Mps, T cell |
| 129 | IFN-γRKO-C57BL/6 | Minor H only | Neu, Mps, T cell |
| 129 | IL-17KO-C57BL/6 | Minor H only | Neu, Mps, T cell |

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 response[33] and to mediate granulopoiesis.[20,48,49] Our results reveal that both IFN-γ– and IL-17–deficient mice elicited similar pathologic features in the rejected 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 difficulty of inducing transplantation tolerance. To further strengthen our knowledge on the complex immune/inflammatory axis during corneal allograft rejection, we must extend the scientific investigation further to include previously unconsidered 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.[50] 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,[51] 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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