

# Genetic background determines susceptibility to experimental immune-mediated blepharoconjunctivitis: Comparison of Balb/c and C57BL/6 mice

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## Abstract

Balb/c and C57BL/6 mice have been reported to be biased towards Th2 and Th1 immune responses, respectively. We investigated which strain is more susceptible to the development of experimental immune-mediated blepharoconjunctivitis (EC), which is predominantly mediated by Th2 immune responses. EC was induced by three different methods in Balb/c and C57BL/6 mice using ragweed (RW) as the antigen. The mice were thus either actively immunized with RW, passively immunized by transfer of RW-primed T cells, or passively immunized by transfer of RW-specific IgE, followed by RW challenge in eye drops. Twenty-four hours after the challenge, conjunctivas, sera and spleens were harvested for histological analysis, measurement of serum IgE and assessment of cellular immune responses, respectively. The responses of the Balb/c and C57BL/6 mice were compared. In addition, to assess the involvement of IFN- $\gamma$  in the development of EC in the two strains, IFN- $\gamma$  knockout (GKO) mice of the two strains were actively immunized and evaluated as above. Regardless of the method of induction, EC, as determined by the degree of eosinophil infiltration into the conjunctiva, was more severe in Balb/c mice than in C57BL/6 mice. Moreover, more IgE was produced by actively immunized Balb/c mice than C57BL/6 mice and RW-primed splenocytes from Balb/c mice produced more IL-4 but less IFN- $\gamma$  than those from C57BL/6 mice. EC could be induced in the GKO mice of both strains. However, when their EC was compared to that in WT mice, significantly less infiltration of eosinophils was noted in the Balb/c GKO mice. Taken together, Balb/c mice are more susceptible to EC than C57BL/6 mice and this higher susceptibility might be related to the Th2 immune response bias of Balb/c mice. Furthermore, the involvement of endogenous IFN- $\gamma$  in the development of EC in these two strains differs. © 2005 Elsevier Ltd. All rights reserved.

## 1. Introduction

Genetic background has been shown to affect susceptibility to numerous types of diseases. An example is allergic diseases, as it has been shown that patients and experimental animals with particular backgrounds appear to respond in an inappropriately powerful fashion to allergens (Anderson and Cookson, 1999). How such hyper-responsiveness is induced depends on the type of disease in question and

remains poorly understood. Nevertheless, it is clear that genetic background contributes to disease susceptibility.

With regard to immune-mediated ocular diseases, uveitis has been particularly thoroughly investigated. In humans, human leukocyte antigen (HLA) has been clearly shown to be involved in the development of certain types of uveitis (Mizuki et al., 1997). Uveitis-susceptible and -resistant strains of rats and mice have also been identified, and the susceptible strains have often been used to investigate the mechanisms by which uveitis develops (Caspi, 2002). Allergic conjunctivitis is also an immune-mediated ocular disease. While the involvement of HLA in its development has not been well investigated (Barney and Foster, 1993), it is clear that genetic background affects susceptibility to it, since SWR/J mice are one of the most susceptible strains of mice to experimental allergic conjunctivitis (AC) (Magone

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et al., 1998). In addition, we have demonstrated that Brown Norway (BN) rats are more susceptible to EC (experimental immune-mediated blepharoconjunctivitis) than Lewis rats (Yoshida et al., 1999; Iwamoto et al., 2000; Fukushima et al., 2003). Our series of studies suggest that the different susceptibility to EC by BN and Lewis rats is due to a genetically determined bias in induced immune responses: BN rats tend to produce Th2 immune responses to a particular antigenic stimulus, while Lewis rats tend to generate a Th1 response to the same antigen (Yoshida et al., 1999; Iwamoto et al., 2000; Fukushima et al., 2003). Thus, the genetic background related to the Th1/Th2 balance may play a particularly important role in susceptibility to AC.

Balb/c and C57BL/6 mice have often been compared in studies investigating the relationship between genetic background and Th1/Th2 balance (Kelso et al., 1991). Balb/c mice have been reported to predominantly exhibit Th2 responses to parasite infection or exogenous antigen challenge, whereas C57BL/6 mice exhibit Th1 dominant responses to the same stimuli (Mosmann and Coffman, 1989; Reiner and Locksley, 1995; Guler et al., 1996). This disparity has often been attributed to the genetic control of IL4 production by Balb/c mice, as the T cells in Balb/c mice are more likely to exhibit a Th2 phenotype and lose IL12 responsiveness than the T cells from other strains of mice (Hsieh et al., 1995; Abbas et al., 1996). However, Balb/c mice do not always exhibit Th2 immune responses and C57BL/6 mice are not always committed to Th1 immune responses. For example, Balb/c mice immunized with a low dose of *Leishmania major* show Th1 immune responses, including high IFN- $\gamma$  production, and successfully counter the infection (Menon and Bretscher, 1996).

To examine the involvement of genetic background in the development of EC in mice, we compared the susceptibility of Balb/c and C57BL/6 mice to EC induced by three different methods. Furthermore, to determine the degree to which endogenous IFN- $\gamma$  is involved in the development of EC in these two strains, we induced EC in IFN- $\gamma$  knockout (GKO) mice derived from these two different genetic backgrounds.

## 2. Materials and methods

### 2.1. Mice

Inbred Balb/c and C57BL/6 mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan. IFN- $\gamma$ -deficient C57BL/6 and Balb/c (IFN- $\gamma$ –/–, GKO) mice (Dalton et al., 1993) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in pathogen-free conditions at the animal facility of Kochi Medical School and used when they were 6- to 12-weeks-old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of

Animals in Ophthalmic and Vision Research. Each experimental group consisted of more than 10 mice.

### 2.2. EC induction by active immunization

Short ragweed pollen (RW, Polysciences, Inc., Warrington, PA) adsorbed on aluminum hydroxide (Sigma, St Louis, MO) was injected into the left hind-footpad and the tail base. Fifty microlitre of the emulsion (50  $\mu$ g of RW and 2 mg of aluminum hydroxide) was injected into each site. Ten days later, the immunized mice were challenged with RW in PBS (2 mg in 10  $\mu$ l) per eye. Twenty-four hours later, the eyes, sera and spleens were respectively harvested for histologic analysis, measurement of IgE, and T-cell culture for transfer, cytokine or proliferation assays.

### 2.3. EC induction by transfer of RW-specific IgE

Twenty-four hours after intravenous injection of 3.5  $\mu$ g of RW-specific IgE (Liu and Katz, 1984), which was prepared in Dr Liu's laboratory, the mice were challenged with RW in PBS (2 mg in 10  $\mu$ l) by eye drops. Twenty-four hours later, the eyes were harvested for histologic analysis.

### 2.4. EC induction by transfer of in vitro-stimulated RW-primed T cells

After sacrificing actively immunized mice, splenocytes were prepared and cultured with RW extract (LSL Co. Ltd, Tokyo, Japan) at final concentrations of 5  $\mu$ g ml<sup>–1</sup> in 75-cm<sup>2</sup> flasks at a concentration of 10<sup>7</sup> cells ml<sup>–1</sup> in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan Co., Tokyo, Japan), 2-mercaptoethanol (2-ME, 5  $\times$  10<sup>–5</sup> M), L-glutamine (2 mM), penicillin (100 U ml<sup>–1</sup>), and streptomycin (100  $\mu$ g ml<sup>–1</sup>). After incubation for 72 hr at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>, the cells were washed and CD4-positive cells were separated by commercially available columns (StemSep, Stemcell Technologies Inc., Vancouver, British Columbia, Canada). Syngeneic mice were then injected intravenously with 5  $\times$  10<sup>6</sup> separated CD4-positive T cells (confirmed by flow cytometric analysis to be >95% CD3-positive and >98% CD4-positive). Four days after the transfer, the recipient mice were challenged with RW in PBS (2 mg in 10  $\mu$ l). Twenty-four hours later, the eyes were harvested for histologic analysis.

### 2.5. Histologic analysis

The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2  $\mu$ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were

counted by two blinded observers. The sections counted were the central portion of the eye, which included the pupil and optic nerve head. Since the counting area varies depending on the severity of inflammation (when inflammation is severe, the thickness of the lamina propria mucosae increases), cell count data were expressed as infiltrating eosinophil number divided by area ( $\text{mm}^2$ ) measured by Scion Image (Scion Corporation, Frederick, MD). Data are presented as averages  $\pm$  SD of all the mice examined.

## 2.6. Cellular proliferation assay

Splenocytes from the individual mice in each group were combined and the following experiments were repeated three times. RBC-depleted splenocytes ( $3 \times 10^5$  cells/well) were cultured in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 5% FCS and 2-ME. Cells were stimulated with RW at final concentrations of 0.2, 1, 5, and  $25 \mu\text{g ml}^{-1}$  or with concanavalin A (Con A) at  $5 \mu\text{g ml}^{-1}$ . After an 80 hr incubation at  $37^\circ\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ , the cultures were pulsed for 16 hr with  $0.5 \mu\text{Ci/well}$  [ $^3\text{H}$ ]thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested and the incorporated radioactivity was measured by standard techniques. The data were expressed as delta counts per minute [cpm] (mean cpm of Ag-stimulated cultures – mean cpm of unstimulated control cultures).

## 2.7. Measurement of total IgE in serum

Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. Total IgE in sera was measured by ELISA. Briefly, affinity purified anti-mouse IgE ( $2 \mu\text{g ml}^{-1}$ , eBioscience, San Diego, CA) was coated in a 96-well EIA plate (Costar, Corning, NY) overnight at  $4^\circ\text{C}$ . The plates were then washed and incubated with blocking buffer (1% bovine serum albumin in PBS) for 3 hr at room temperature. The plates were washed again and the samples or IgE standards were applied to each well. After 2 hr incubation at room temperature, the plates were washed and biotin-conjugated rat anti-mouse IgE (BD biosciences, Franklin Lakes, NJ) was added to each well for 1 hr at room temperature. After washing, avidin-alkaline phosphatase (Sigma, St Louis, MO) was added to each well for 1 hr. After washing, the substrate *p*-nitrophenyl phosphate (*p*-nitrophenyl phosphate liquid substrate system, Sigma, St Louis, MO) was added to each well. Fifteen minutes later, absorbance was measured at 405 nm. The concentration of IgE was standardized by referring to the known concentrations of the IgE standards.

## 2.8. Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes ( $10^7$  cells  $\text{ml}^{-1}$ ) were cultured for 48 hr with Con A ( $5 \mu\text{g ml}^{-1}$ ) or RW ( $25 \mu\text{g ml}^{-1}$ ) in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. The levels of IL-4, IL-5, IL-13 and IFN- $\gamma$  produced were measured by using commercially available cytokine kits (Duoset, R & D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

## 2.9. Statistical analysis

Serum IgE levels, the concentrations of cytokines in the culture supernatants and infiltrating eosinophil numbers were compared by Student's *t*-test.

# 3. Results

## 3.1. Conjunctival eosinophil infiltration is more severe in Balb/c mice

To determine whether Balb/c and C57BL/6 mice differ in their susceptibility to EC, we induced EC in WT mice by three different methods, namely active immunization (Fig. 1(C) and (D)), passive immunization by transfer of RW-primed T cells (Fig. 1(E) and (F)), and passive immunization by transfer of RW-specific IgE (Fig. 1(G) and (H)). All three methods induced significant infiltration of eosinophils into the conjunctiva of the Balb/c mice; in contrast, in C57BL/6 mice, significant infiltration was induced only by active immunization (Figs. 1 and 2). When the two strains were compared, significantly more infiltration of inflammatory cells into the conjunctiva was noted in the Balb/c mice for all three methods of induction used (Fig. 2).

## 3.2. Th2 immune responses are more prominent in Balb/c mice

To study the relationship between susceptibility to EC and the type of systemic immune responses that are generated by the mice, sera and spleens were collected from actively immunized mice to examine their humoral and cellular immune responses, respectively. Both strains showed significantly increased total serum IgE levels after active immunization (Fig. 3(A)) and the magnitude of the increases was almost the same between the two strains (Balb/c: 10.3-fold, C57BL/6: 8.6-fold). When comparing between the two strains, the Balb/c titers were 129 times higher in naïve animals and 170 times higher after active immunization compared to the C57BL/6 titers (Fig. 3(A)). The splenocytes of both strains proliferated in response to RW (Fig. 3(B)). At the higher concentrations of RW in vitro such as 5 and  $25 \mu\text{g ml}^{-1}$ , the proliferative response of Balb/c

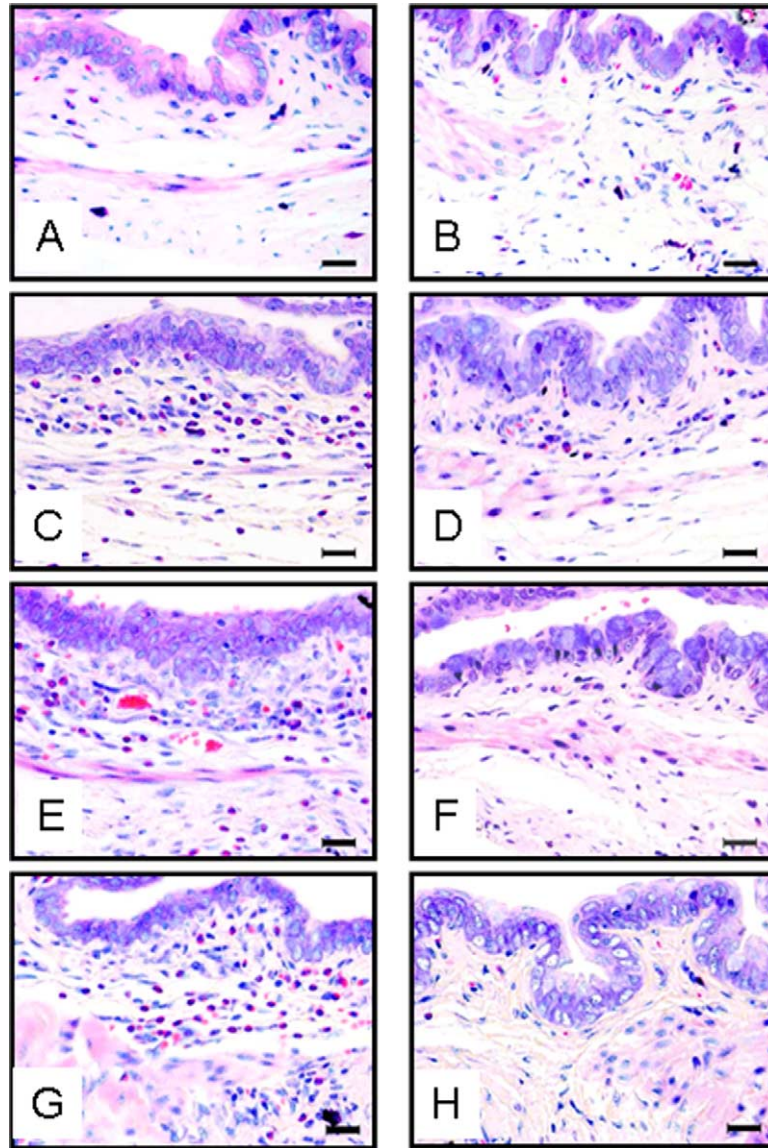


Fig. 1. Microphotographs of conjunctiva. EC was induced by active immunization (C, D), passive immunization by transfer of RW-primed T cells (E, F), or passive immunization by transfer of RW-specific IgE (G, H), followed by RW challenge in eye drops. Twenty-four hours later, the conjunctivas were harvested for histopathologic analysis. Naïve conjunctivas are shown in (A) and (B). The induction of EC is revealed by the infiltration in the conjunctiva of inflammatory cells. Representative photos of at least 10 mice per group are shown. (A), (C), (E), (G): Balb/c mice. (B), (D), (F), (H): C57BL/6 mice. Giemsa staining. Bar = 20  $\mu$ m.

splenocytes was higher than that of C57BL/6 splenocytes (Fig. 3(B)). The RW-stimulated Balb/c, but not C57BL/6, splenocytes produced IL-4 (Fig. 3(D)). In contrast, the RW-stimulated C57BL/6, but not Balb/c, splenocytes generated IFN- $\gamma$  (Fig. 3(C)). RW-stimulation induced similar levels of IL-5 (Fig. 3(E)) and IL-13 (Fig. 3(F)) in Balb/c and C57BL/6 mice. Moreover, Con A-stimulation induced Balb/c splenocytes to produce significantly more IL-4 and IL-13 than the C57BL/6 cells (Fig. 3(D) and (F)). In contrast, the C57BL/6 cells produced slightly more IL-5, although this was not statistically significant (Fig. 3(E)).

### 3.3. Endogenous IFN- $\gamma$ supports the development of EC in Balb/c mice

To investigate and compare the roles endogenous IFN- $\gamma$  plays in the development of EC in the two strains of mice, we induced EC in GKO mice on both backgrounds by active immunization and compared it to the EC in WT mice. EC could be induced in the GKO mice of both Balb/c (Fig. 4(A), (C) and (E)) and C57BL/6 mice (Fig. 4(B), (D) and (E)). However, compared to the EC induced in WT Balb/c mice, the GKO Balb/c mice showed significantly lower infiltration of eosinophils into the conjunctiva

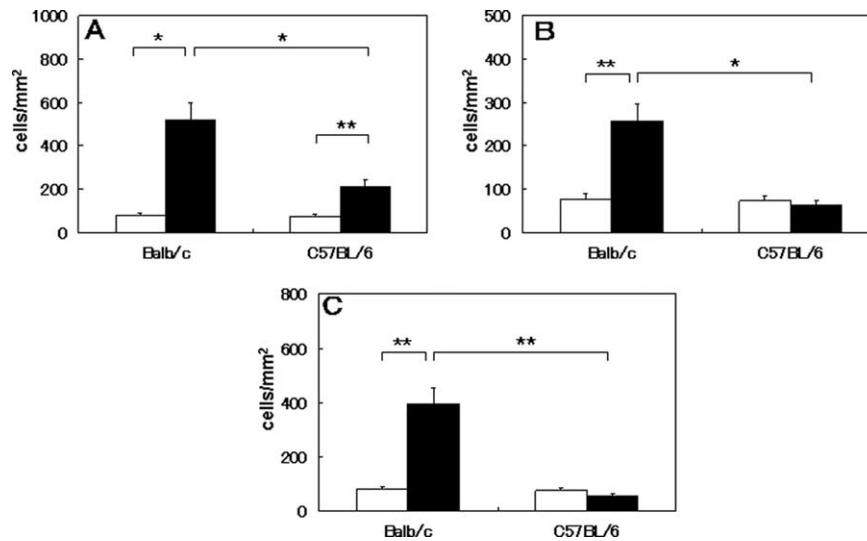


Fig. 2. Infiltrating eosinophil number. Infiltrating eosinophils into the conjunctiva were counted and the average number  $\pm$  SEM per mm<sup>2</sup> of at least 10 mice per group is shown. (A) Active immunization. (B) Passive immunization by transfer of RW-primed T cells. (C) Passive immunization by transfer of RW-specific IgE. Regardless of the method of EC induction used, more infiltrating eosinophils were detected in the conjunctiva of Balb/c mice than in the conjunctiva of C57BL/6 mice. \* $P < 0.05$ , \*\* $P < 0.01$ .

(Fig. 4(F)). In contrast, equivalent EC was induced in the WT and GKO mice of the C57BL/6 strain (Fig. 4(F)).

### 3.4. Th2 immune responses, except for total serum IgE and IL-5 production, are higher in the Balb/c GKO mice than in the C57BL/6 GKO mice

To investigate the differential involvement of endogenous IFN- $\gamma$  in the development of EC between the two strains, we examined the humoral and cellular immune responses produced by the GKO mice from the two strains. Like the WT mice of the two strains, total serum IgE was increased by active immunization in both GKO strains (Fig. 5(A)). However, unlike the total serum IgE pattern in the WT mice, the Balb/c mice had equivalent IgE levels to the C57BL/6 GKO mice (Fig. 5(A)). Splenocyte proliferation against RW was higher in C57BL/6 GKO mice at lower concentrations of RW such as 0.2 and 1  $\mu\text{g ml}^{-1}$ , but it was not different between the two GKO strains at higher concentrations such as 5 and 25  $\mu\text{g ml}^{-1}$  (Fig. 5(B)). Compared to their WT counterparts (Fig. 3(D) and (E)), both GKO strains showed upregulation of IL-4 and IL-5 production in response to in vitro antigenic challenge (Fig. 5(C) and (D)). However, like the WT mice, the Balb/c GKO mice produced more IL-4 than the C57BL/6 GKO mice (Fig. 5(C)). Similar to the WT mice (Fig. 3(D) and (F)), Con A-stimulation induced significantly more IL-4 and IL-13 production in Balb/c splenocytes than C57BL/6 splenocytes (Fig. 5(C) and (E)). Moreover, more IL-5 was generated in response to Con A-stimulation by the C57BL/6 GKO mice compared to the Balb/c GKO mice, although unlike the WT case, this difference was statistically significant (Fig. 5(D)).

## 4. Discussion

Two important conclusions evolve from this report. First, Balb/c mice are more prone to EC than C57BL/6 mice. Second, the role endogenous IFN- $\gamma$  plays in the development of EC differs between these two mouse strains.

To dissect the involvement of cellular and humoral immunity in the development of EC, we induced EC by transferring of RW-primed T cells or RW-specific IgE in both strains of mice. Since RW-primed splenocytes from Balb/c mice produced more IL-4, we expected that the EC induced by the transfer of RW-primed T cells would be more severe in Balb/c mice than in C57BL/6 mice. This was indeed the case. However, we found that even when EC was induced by transfer of RW-specific IgE, significantly more eosinophils infiltrated into the conjunctiva of Balb/c mice compared to C57BL/6 mice. This suggests that the mast cell-mediated inflammatory cascade plays a more significant role in the EC in Balb/c mice than in C57BL/6 mouse EC. To test this notion, we compared the mast cell numbers in the conjunctiva of naïve mice from both strains. However, a significant difference was not observed (data not shown). Thus, it remains unclear why the EC induced by the transfer of RW-specific IgE is more severe in Balb/c mice than in C57BL/6 mice. There is a report demonstrating that mast cells from Balb/c mice express more IL-5 than those from C57BL/6 mice after *Nippostrongylus brasiliensis* infection (Zhou et al., 1996). This difference may also be present in the conjunctiva of Balb/c and C57BL/6 mice. Future studies will focus on characterizing the differences between the conjunctival mast cells of the two strains.

Interestingly in Balb/c mice, high levels of total IgE in serum were noted even in naïve mice. In the induction of

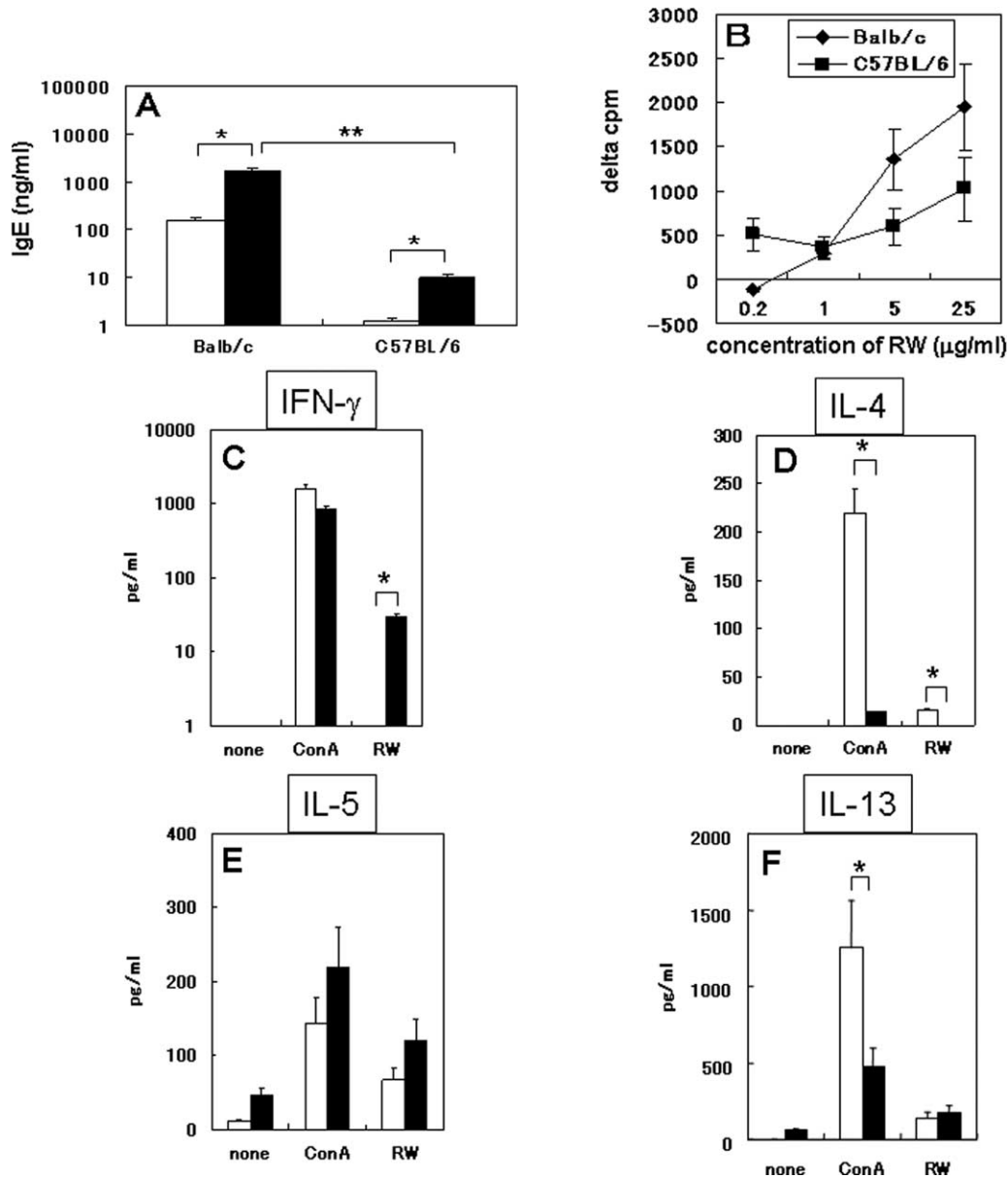


Fig. 3. Immune responses of actively immunized WT mice. Sera and spleens were harvested from mice developing EC due to active immunization for the measurement of serum IgE (A) and proliferation (B) or cytokine assays (C)–(F), respectively. As controls, sera were collected from naïve WT mice of both strains. (A) Total serum IgE was increased when EC was induced in both strains. Comparison of the two strains revealed that significantly higher levels of total IgE were present in Balb/c mice; however, the fold increase due to active immunization was similar between the two strains. White bars, naïve; black bars, EC-induced mice. (B) The splenocytes of both strains proliferated in response to RW. At the higher concentrations of RW in vitro such as 5 and 25  $\mu\text{g ml}^{-1}$ , the proliferative response of Balb/c mice was higher than that of C57BL/6 mice. Background cpm  $\pm$  SEM was  $1904 \pm 87$  in Balb/c mice and  $368 \pm 31$  in C57BL/6 mice. Error bars indicate SEM. (C)–(F) Cytokine assays. IFN- $\gamma$  (C), IL-4 (D), IL-5 (E) and IL-13 (F) levels in the culture supernatants of RW-primed splenocytes resulting from stimulation with RW or Con A were measured. As controls, culture supernatants without stimulants (none) were used. White bars, Balb/c mice; black bars, C57BL/6. \* $P < 0.05$ , \*\* $P < 0.01$ .

allergic responses, not only Ag-specific but also Ag non-specific responses are thought to be important. In fact, in experimental contact dermatitis, transfer of Ag-irrelevant IgE was able to induce inflammation in IgE knockout mice (Bryce et al., 2004). Therefore, the higher baseline levels of total IgE may participate in the more severe infiltration of eosinophils induced by active immunization in Balb/c WT mice compared with that in C57BL/6 WT mice.

Similar to a previous report (Kelso et al., 1991), Balb/c mice were more likely than C57BL/6 mice to produce Th2 immune responses upon active immunization with RW, as determined by serum IgE levels and IL-4/IL-13 production by their cultured splenocytes. It is possible that the higher Th2 immune responses in Balb/c mice are responsible for the greater infiltration of eosinophils into their conjunctiva compared to C57BL/6 mice. This is

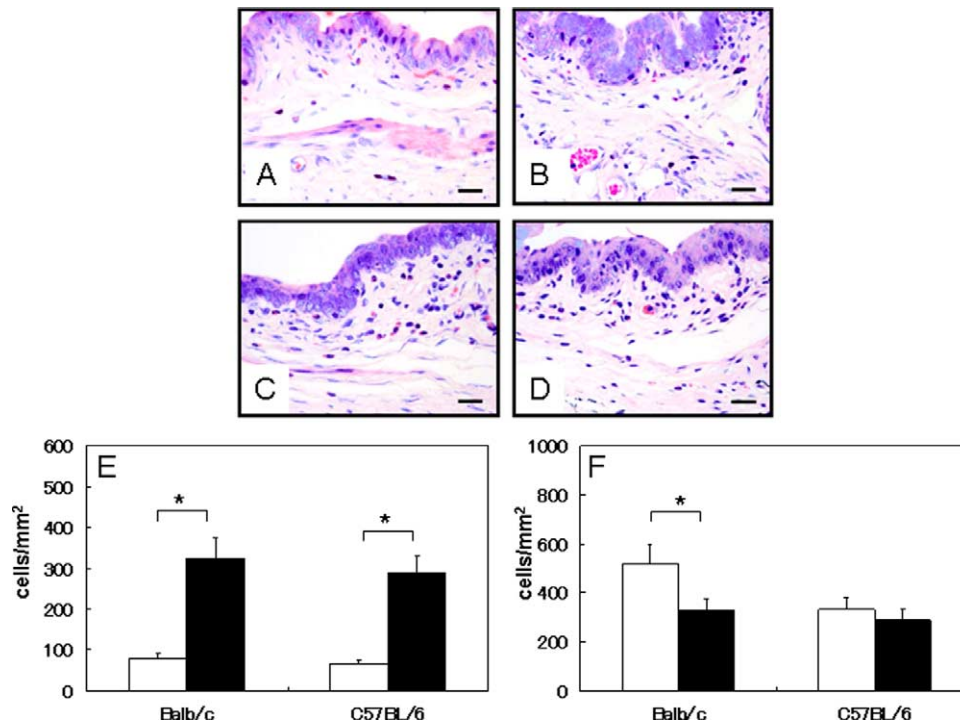


Fig. 4. Infiltration of eosinophils in GKO mice. EC was induced in GKO mice of either background by active immunization followed by RW challenge in eye drops. Twenty-four hours later, conjunctivas were harvested for histopathologic analysis. (A) Naïve GKO mice on the Balb/c background. (B) Naïve GKO mice on the C57BL/6 background. (C) GKO mice on the Balb/c background with EC. (D) GKO mice on the C57BL/6 background with EC. Both immunized GKO strains show infiltration of eosinophils in the conjunctiva, indicating the induction of EC. Giemsa staining. Bar = 20  $\mu$ m. (E) Eosinophil counts per mm<sup>2</sup> in the conjunctiva of GKO mice. White bar, naïve; black bar, EC-induced. \* $P$  < 0.05. (F) Comparison of eosinophil counts per mm<sup>2</sup> in the conjunctiva of EC-induced WT (white bar) and EC-induced GKO (black bar) mice. Fewer eosinophils were detected in EC-induced Balb/c GKO mice than in EC-induced Balb/c WT mice. \* $P$  < 0.05.

consistent with a previous report showing that infiltration of inflammatory cells into the conjunctiva was absent in IL-4 knockout mice (Magone et al., 2000). Alternatively, or in addition, the lower production of IFN- $\gamma$  upon RW stimulation by Balb/c mice may be involved in their higher susceptibility to EC. Therefore, we sought to compare the involvement of endogenous IFN- $\gamma$  in the development of EC between the two strains of mice. To this aim, we used GKO mice derived from the Balb/c and C57BL/6 backgrounds. A previous report using GKO mice (Magone et al., 2000) revealed that endogenous IFN- $\gamma$  inhibits conjunctival inflammation. However, we found here that endogenous IFN- $\gamma$  does not seem to inhibit the development of EC. This difference may be due to the different immunization protocol used in the two studies (Fukushima et al., in press). More importantly, we found the role of endogenous IFN- $\gamma$  played in the development of EC induced by our induction protocol differed between the two strains: endogenous IFN- $\gamma$  enhances the EC generated in Balb/c mice whereas its involvement in C57BL/6 mouse EC is negligible. There is an observation that may explain this phenomenon, namely, the lower levels of total serum IgE in Balb/c GKO mice (Fig. 5(A)) than in WT mice (Fig. 3(A)). Since the transfer of RW-specific IgE is

capable of inducing EC, the low IgE levels in Balb/c GKO mice may be related to the lower infiltration of eosinophils into their conjunctiva. In contrast, in C57BL/6 background mice, the total serum IgE levels after EC induction were slightly higher in GKO mice than in WT mice. Thus, IFN- $\gamma$  appears to contribute to the production of IgE in Balb/c but not in C57BL/6 mice, thereby enhancing the intensity of EC in Balb/c mice. Supporting this is that the culture of B cells in vitro with IFN- $\gamma$  has shown IFN- $\gamma$  participates differently in the IgE production of Balb/c and C57BL/6 mice (Matsushita and Katz, 1992).

Previous studies comparing experimental lung inflammation between the two strains of mice revealed that their susceptibility to lung inflammation depends on the experimental protocol (Zhang et al., 1997; Herz et al., 1998; Morokata et al., 1999). These reports suggest that the dose of Ag used for sensitization is an important factor that dictates the severity of lung inflammation. Although the antigen used in these reports was ovalbumin and thus different from RW, it was found that a high immunization dose of Ag (which was close to the immunization dose of RW we used) induced more severe lung inflammation in Balb/c mice (Zhang et al., 1997; Herz et al., 1998) whereas a lower Ag dose induced more severe lung inflammation in

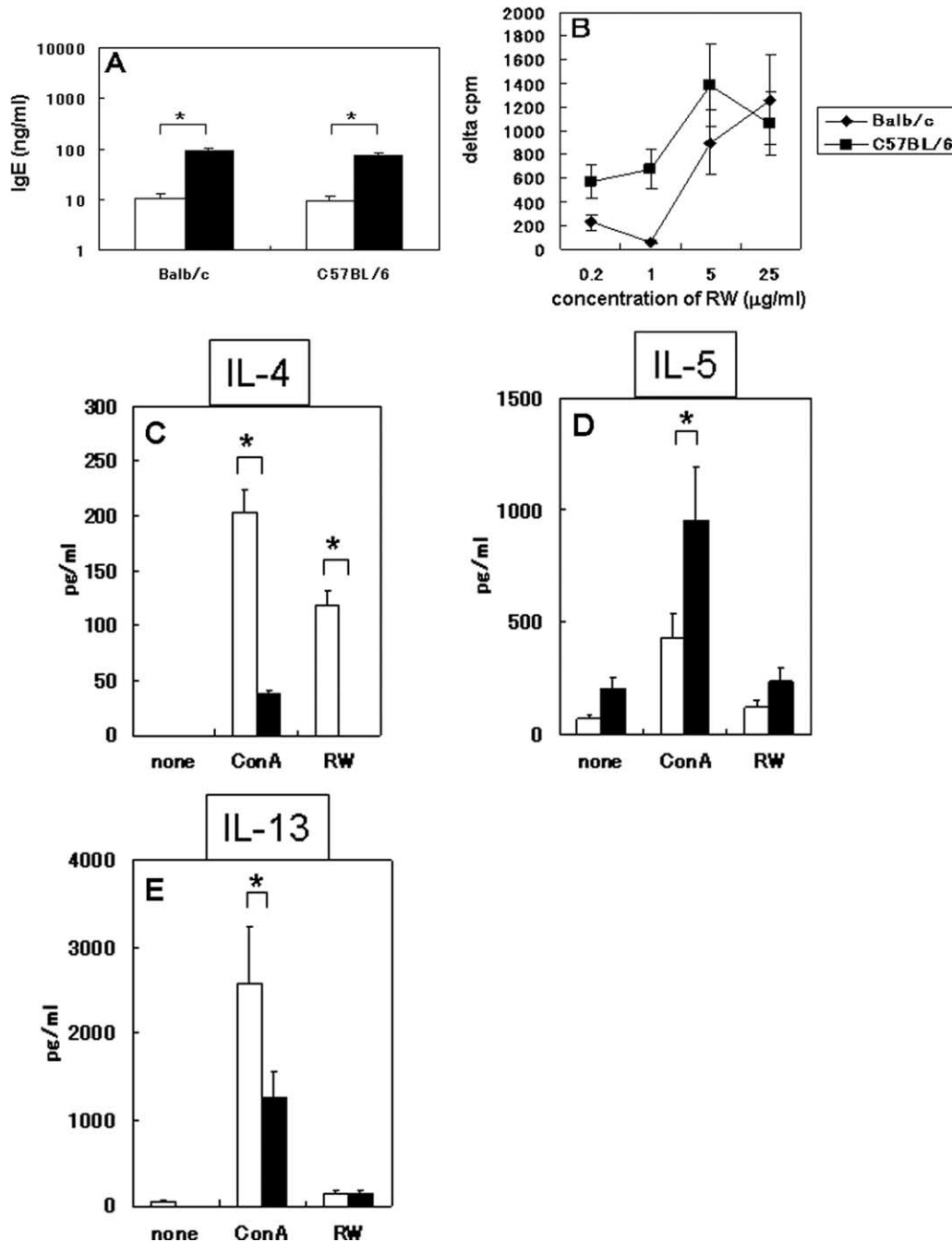


Fig. 5. Immune responses of actively immunized GKO mice. At the time of harvesting the conjunctivas mentioned in the legend to Fig. 4, sera and spleens were also harvested for the measurement of serum IgE (A) and perform proliferation (B) or cytokine assays (C)–(E), respectively. As controls, sera were collected from naïve GKO mice of both strains. (A) Total serum IgE was increased when EC was induced in both strains. White bars, naïve; black bars, EC-induced mice. (B) The RW-specific proliferation of splenocytes from the two immunized strains did not differ at the higher concentrations of RW such as 5 and 25  $\mu\text{g ml}^{-1}$ ; however, at lower concentrations of RW such as 0.2 and 1  $\mu\text{g ml}^{-1}$ , proliferation was higher in C57BL/6 mice. Background cpm  $\pm$  SEM was  $2631 \pm 149$  in Balb/c mice and  $331 \pm 56$  in C57BL/6 mice. Error bars indicate SEM. (C)–(F) Cytokine assays. IL-4 (C), IL-5 (D), and IL-13 (E) levels in the culture supernatants of RW-primed splenocytes due to stimulation with RW or Con A were measured. As controls, culture supernatants without stimulants (none) were used. White bars: Balb/c mice, black bars: C57BL/6. \* $P < 0.05$ .

C57BL/6 mice (Morokata et al., 1999). Thus, a relatively higher Ag dose for sensitization in our study may have led to more severe EC in Balb/c mice.

Accumulating evidence suggests that Th1 cells do not counteract Th2 cells in mouse experimental airway

inflammation by the transfer of T cells but rather augment the Th2-mediated eosinophilic infiltration (Randolph et al., 1999; Hansen et al., 1999). The mechanism by which Th1 cells assist the recruitment of Th2 cells has not been fully elucidated but it is believed that Th1 cell-generated

cytokines such as tumor necrosis factor- $\alpha$  may upregulate the expression of adhesion molecules in the inflammatory site (i.e. the lung) (Randolph et al., 1999). Thus, although it is well accepted that Th1 cells inhibit the function of Th2 cells both in vitro and in vivo (Fitch et al., 1993), Th1 cells also appear to assist Th2-mediated eosinophilic infiltration in certain circumstances. Taking these reports into consideration along with our observations, it may be that Th1 cells assist Th2-mediated eosinophilic infiltration in Balb/c mouse EC, whereas Th1 cells are involved to a lesser degree in the development of C57BL/6 mouse EC.

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