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CD8+ T Cells Play Disparate Roles in the Induction and the Effector Phases of Murine Experimental Allergic Conjunctivitis

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Abstract: Although CD4+ Th2 cells clearly play an essential role in the development of experimental allergic diseases, the functions CD8+ T cells may have in these diseases have been investigated less extensively and remain controversial. Here, we investigated the roles of CD8+ T cells in the development of experimental allergic conjunctivitis (EC). EC was induced in CD8 α -deficient (CD8KO) mice and wild-type (WT) mice by active immunization with short ragweed pollen (RW) followed by challenge with RW-containing eye drops. Alternatively, EC was induced by transferring RW-primed splenocytes followed by RW challenge. With regard to actively immunized mice, CD8KO mice showed significantly less severe eosinophil infiltration of the conjunctiva and lower total IgE levels, although the levels of the other Igs were equivalent between the two strains. Cytokine production by cultured splenocytes also did not differ, but the WT conjunctivas showed upregulated IL-5 and IL-6 expression and greater upregulation of IL-4 expression than the conjunctivas of CD8KO mice. Thus, CD8+ T cells may play a significant role during the induction phase by aiding IgE production and the generation of Th2 cytokines in the conjunctiva, thus promoting the development of EC. In contrast, splenocytes from CD8KO mice induced significantly more severe EC in WT mice than cells from WT mice. In addition, transfer of RW-primed splenocytes induced significantly more severe eosinophil infiltration in CD8KO recipient mice. Thus, CD8+ T cells promote the development of EC during the induction phase, but suppress it during the effector phase.

Key words: Allergic conjunctivitis, CD8, Eosinophil, T cell, Th2

Massive infiltration of eosinophils into the conjunctiva is the hallmark of severe types of allergic conjunctivitis (AC) such as vernal keratoconjunctivitis (VKC) (2). However, while AC is an Ag-specific disease, eosinophils do not have Ag-specific receptors. Therefore, certain types of cells that do have Ag-specific receptors are likely to be involved in the development of AC. To clarify this issue, our group has investigated the roles Ag-specific CD4+ T cells play in the development of experimental AC (experimental immune-mediated blepharoconjunctivitis, EC) (4, 6–8, 10, 12, 24). Our studies have shown that adoptively transferred Ag-

specific CD4+ T cells are able to induce EC, and that Ag-specific Th2 cells but not Th1 cells are capable of inducing eosinophil infiltration into the conjunctiva (23). These findings confirm that EC is a Th2-mediated disease.

While we were investigating the involvement of co-stimulatory molecules in the development of EC, we found that agonistic stimulation of 4-1BB, a member of TNF-receptor superfamily, suppressed eosinophil infiltration (9). Notably, the mice treated with agonistic anti-4-1BB Ab had a higher proportion of CD8+ T cells, especially CD8+/CD11+ cells (9). These findings suggested that CD8+ T cells may negatively regu-

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Abbreviations: AC, allergic conjunctivitis; EC, experimental immune-mediated blepharoconjunctivitis; CD8KO, CD8-deficient; RW, short ragweed pollen.

late the development of EC. Similarly, it has been reported that CD8⁺ T cells are involved in Th2-mediated experimental allergic airway disease. For example, in Brown Norway rats, a series of experiments suggest that CD8⁺ T cells negatively regulate the development of experimental airway inflammation (30). On the contrary, in Balb/c mice, treatment with an ablating anti-CD8 monoclonal Ab induced significantly less airway inflammation relative to the untreated controls (14). In addition, CD8 α -deficient (CD8KO) C57BL/6 mice exhibited significantly lower experimental lung inflammation than WT mice (22). These data suggest that, depending on the experimental settings, CD8⁺ T cells participate in different ways in airway inflammation. Consequently, it remains unclear how CD8⁺ T cells contribute to allergic inflammation. Furthermore, we have previously reported that Th2 cells play more prominent role in the development of AC (24), compared with allergic asthma and atopic dermatitis that are complex disorders involving mutual Th1 and Th2 cytokine responses (17, 32). Thus, it could be possible that involvement of CD8⁺ T cells in the development of EC differs from that of allergic airway inflammation.

Here, to investigate the roles CD8⁺ T cells play in the development of allergic inflammation in more detail, we analyzed the effect of knocking out CD8 on the induction and effector phases of EC in mice, which were respectively generated by actively immunizing the mice or by transferring of Ag-primed splenocytes into naïve animals.

Materials and Methods

Mice. Inbred wild-type (WT) C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan) and homozygous CD8KO mice on the C57BL/6 background (13) were obtained from the Jackson Laboratory (Bar Harbor, Me., U.S.A.). The mice were kept in specific pathogen-free conditions at the animal facility of Kochi Medical School and age- and gender-matched mice were 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.

Reagents. Short ragweed pollen (RW) was purchased from Polysciences, Inc. (Warrington, Pa., U.S.A.). RW extract was obtained from LSL Co., Ltd. (Tokyo). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, Mo., U.S.A.). FITC-labeled anti-CD3 (145-2C11) and anti-CD4 (GK1.5) and biotin-labeled anti-CD8 (53-6.7) were purchased from eBioscience, San Diego, Calif., U.S.A. FITC-labeled

anti-CD45R/B220 (RA3-6B2) and streptavidin-PE were obtained from BD Biosciences, Franklin Lakes, N.J., U.S.A.

Induction of EC by active immunization. RW adsorbed on alum was injected into the left hind-footpad and at the base of the tail of WT (number of mice (n)=20) and CD8KO mice (n =20). Fifty microliters of the emulsion (50 μ g of RW and 2 mg of alum) was injected into each site. From days 7 to 10, the eyes of the immunized mice were challenged daily with RW in PBS (2 mg in 10 μ l per eye). As a control, naïve WT mice were similarly challenged with RW (n =5). Twenty-four hours after the last challenge, the eyes, sera, and spleens were harvested for histological analysis or cytokine assays, measurement of Ig levels, and flow cytometric analysis, cytokine production or proliferation assays, respectively.

Induction of EC by adoptively transferring in vitro-stimulated RW-primed splenocytes. WT and CD8KO mice were actively immunized and challenged as described above and their splenocytes harvested on day 11 were cultured with RW extract at final concentrations of 5 μ g/ml in 75-cm² flasks at a concentration of 10^7 cells per ml in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan Co., Tokyo), 2-mercaptoethanol (2-ME, 5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (100 μ g/ml), penicillin (100 U/ml), and streptomycin (100 μ g/ml). After incubation for 72 hr at 37 C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes/mouse were intraperitoneally injected into WT or CD8KO mice. For this experiment, 20 WT recipients received WT donor cells, 10 CD8KO recipients received WT donor cells, and 10 WT recipient received CD8KO donor cells. In a separate experiment, the number of CD4⁺ T cells in the CD8KO donor cell population was adjusted before transfer to match the number in the WT donor cell population (per 2×10^7 cells). In this experiment, WT mice (n =8 per group) received the WT or CD8KO splenocytes. In all experiments, the eyes of the recipient mice were challenged daily from days 2 to 4 with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours after the last challenge, the eyes were harvested for histological analysis.

Histological analysis. The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2 μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas in the entire section were counted by two observers given blind samples. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. Since the

counts vary depending on the counting area, the cell count data are expressed as infiltrating eosinophil numbers divided by area (mm²) as measured by Scion Image (Scion Corporation, Frederick, Md., U.S.A.). The data are presented as averages $\pm$ SEM of all the mice examined.

Flow cytometric analysis. Splenocytes were incubated for 30 min on ice with FITC- or biotin-labeled Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% FCS-PBS. In the case of biotin-labeled Abs, the cells were then incubated with streptavidin-PE for 10 min and then washed again. These cells were analyzed on a FACScan (Becton Dickinson, Mountain View, Calif., U.S.A.) and acquisition and analysis were performed using CellQuest software. The data are presented as an average of the percentage of Ab-stained cells $\pm$ SEM per spleen.

Cellular proliferation assay. RBC-depleted splenocytes (3×10^5 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. The cells were stimulated with RW extract at final concentrations of 25 μ g/ml or with concanavalin A (Con A) at 5 μ g/ml. After a 72 hr incubation at 37 C in a humidified atmosphere containing 5% CO₂, the cultures were pulsed for 16 hr with 0.5 μ Ci/well [³H]-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 the change in counts per minute [cpm] (delta cpm: mean cpm of stimulated cultures – mean cpm of unstimulated control cultures) or stimulation index (SI: mean cpm of stimulated cultures/mean cpm of unstimulated control cultures).

Measurement of cytokines in the culture supernatants. RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 hr with Con A (5 μ g/ml) or RW extract (25 μ g/ml) in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. As a control, splenocytes were cultured in the same manner without any stimulants (expressed as “none” in the figure). The levels of IL-2, IL-4, IL-5, IL-10, IL-13 and IFN- γ that were produced were measured by using commercially available ELISA kits (Duoset; R&D Systems, Minneapolis, Minn., U.S.A.) according to the manufacturer’s recommendations.

Measurement of cytokines in the conjunctiva. Multiple cytokine levels including 17 cytokines (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , TNF- α) and 6 chemokines (eotaxin, KC, MCP-1, MIP-1 α , MIP-1 β , RANTES) in conjunctiva using Bio-

plex system (Bio-Rad, Hercules, Calif., U.S.A.) according to manufacturer’s recommendation. The excised conjunctivas from EC-developing WT and CD8 KO mice by active immunization ($n=4$ per group) were weighed and cut into small pieces. Conjunctivas homogenized in 100 μ l of lysis buffer were centrifuged at 6,000 rpm for 5 min. In total, 23 different cytokines and chemokines were measured in 50 μ l of conjunctiva supernatants. As the control, conjunctivas from naïve WT and CD8KO mice that had been challenged with RW were used ($n=4$ per group). Data are expressed as pg (cytokine)/mg (conjunctiva).

Measurement of Igs in serum. Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. Serum total Ig levels were assessed by ELISA, as detailed in elsewhere (11), and the data are presented as concentrations of Igs. To assess RW-specific Ig levels in the serum, EIA plates (Costar, Corning, N.Y., U.S.A.) were coated with RW extract (1 μ g/ml) at 4 C overnight. After blocking with 1% bovine serum albumin in PBS, serum samples were added and incubated for 2 hr at room temperature or overnight at 4 C. As controls, the quantified RW-specific IgE (kindly provided by Prof. Fu-Tong Liu) and pooled hyperimmune serum were used. The plates were washed with PBS plus 0.05% Tween 20 (PBS/T) (Wako, Osaka, Japan) and incubated for 2 hr at room temperature with alkaline phosphatase (ALP)-conjugated goat Ab specific for IgG₁ or IgG_{2a} (Zymed, San Francisco, Calif., U.S.A.). In the case of IgE, biotin-conjugated rat anti-mouse IgE (BD Biosciences) was added to each well for 2 hr at room temperature. After washing, avidin-ALP (Sigma) was added to each well for 1 hr. After washing with PBS/T, *p*-nitrophenyl phosphate (*p*-nitrophenyl phosphate liquid substrate system, Sigma) was added to each well and allowed to develop for 15 min. Absorbance was measured at 405 nm. IgG₁ and IgG_{2a} concentrations are expressed as units (U)/ml relative to the pooled hyperimmune serum, while IgE concentrations are shown as ng/ml.

Statistical analysis. Significant differences in infiltrating eosinophil numbers, serum Ig levels, splenocyte proliferation, splenocyte cytokine production and cytokine expression in the conjunctiva between the two groups were determined by Student’s *t*-test. *P* values less than 0.05 were considered significant.

Results

The Absence of CD8+ Cells Attenuates Eosinophil Infiltration in Actively Induced EC

To examine whether CD8+ T cells are involved in the initiation of EC, we induced EC in CD8KO and WT

mice by actively immunizing them with RW followed by RW challenge. Significantly less infiltration of eosinophils were observed in the conjunctivas of CD8KO mice (Fig. 1A). This was confirmed by the counting the cells in the conjunctivas of the two strains of mice (Fig. 1B). Flow cytometric analysis revealed that the spleens from the two groups of mice contained equivalent numbers of CD3+ cells; moreover, the sum of CD4+ and CD8+ splenic cells was equal to the total number of CD3+ cells in both the WT and CD8KO mice (Fig. 1C). Therefore, it appears that CD8+ T cells promote the infiltration of eosinophils into the conjunctiva when they are present at the induction phase of EC.

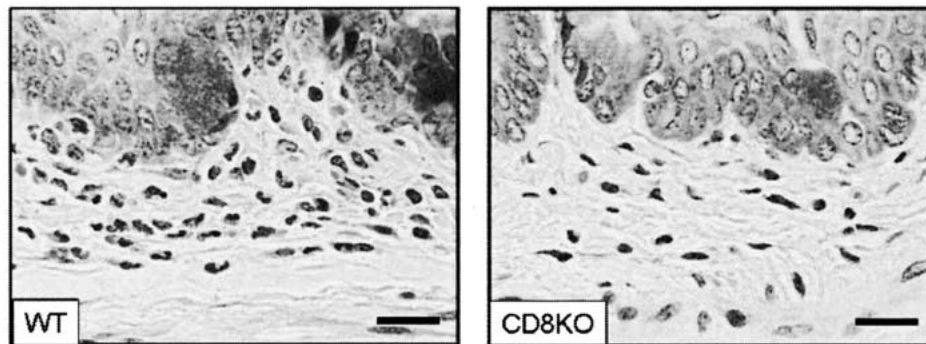
Comparison of the Immune Responses of Actively Immunized WT and CD8KO Mice

EC is mediated by both Ag-specific IgE and Ag-specific T cells (9, 12). Therefore, it could be possible that the lower eosinophil infiltration into the conjunctiva in

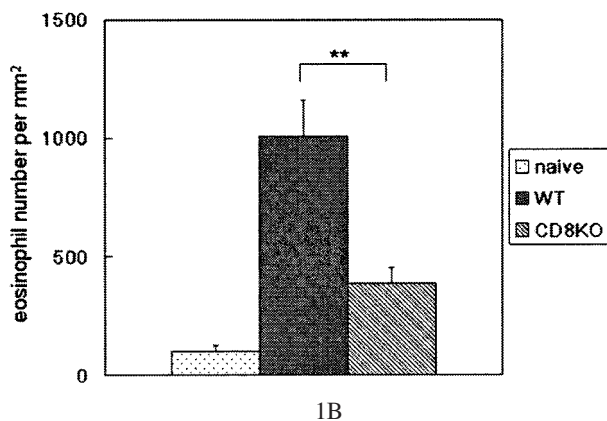
CD8KO mice is related to altered immune responses. To test this, we compared both the cellular and humoral immune responses of actively immunized WT and CD8KO mice. These mice did not differ markedly in the proliferation (Fig. 2A) or cytokine production (Fig. 2B) of their splenocytes against RW or Con A, although CD8KO mice showed more Con A-induced IL-2 production. However, the CD8KO mice had significantly lower total serum IgE levels (Fig. 2C), although the RW-specific IgE levels in the serum did not differ; moreover, the two groups had similar total and RW-specific IgG₁ and total IgG_{2a} levels (Fig. 2C). RW-specific IgG_{2a} was undetectable in both strains of mice.

Comparison of Cytokine and Chemokine Expressions in the Conjunctivas of Actively Immunized WT and CD8KO Mice

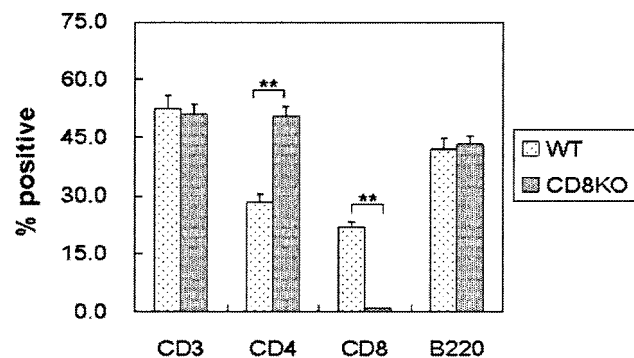
To compare the cytokine and chemokine expression in the conjunctivas of actively immunized and challenged WT and CD8KO mice, we harvested the con-



1A



1B



1C

Fig. 1. The infiltration of eosinophils into the conjunctiva in actively induced EC is attenuated in CD8KO mice. WT and CD8KO mice were immunized with RW in alum. From days 7 to 10, the mice were challenged daily with RW in eye drops and their conjunctivas and spleens were harvested 24 hr after the last challenge for histologic analysis and flow cytometric analysis, respectively. (A) Microphotograph. Bar=20 μ m. (B) The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm². ** P <0.01. (C) Flow cytometric analysis of splenocytes. ** P <0.01.

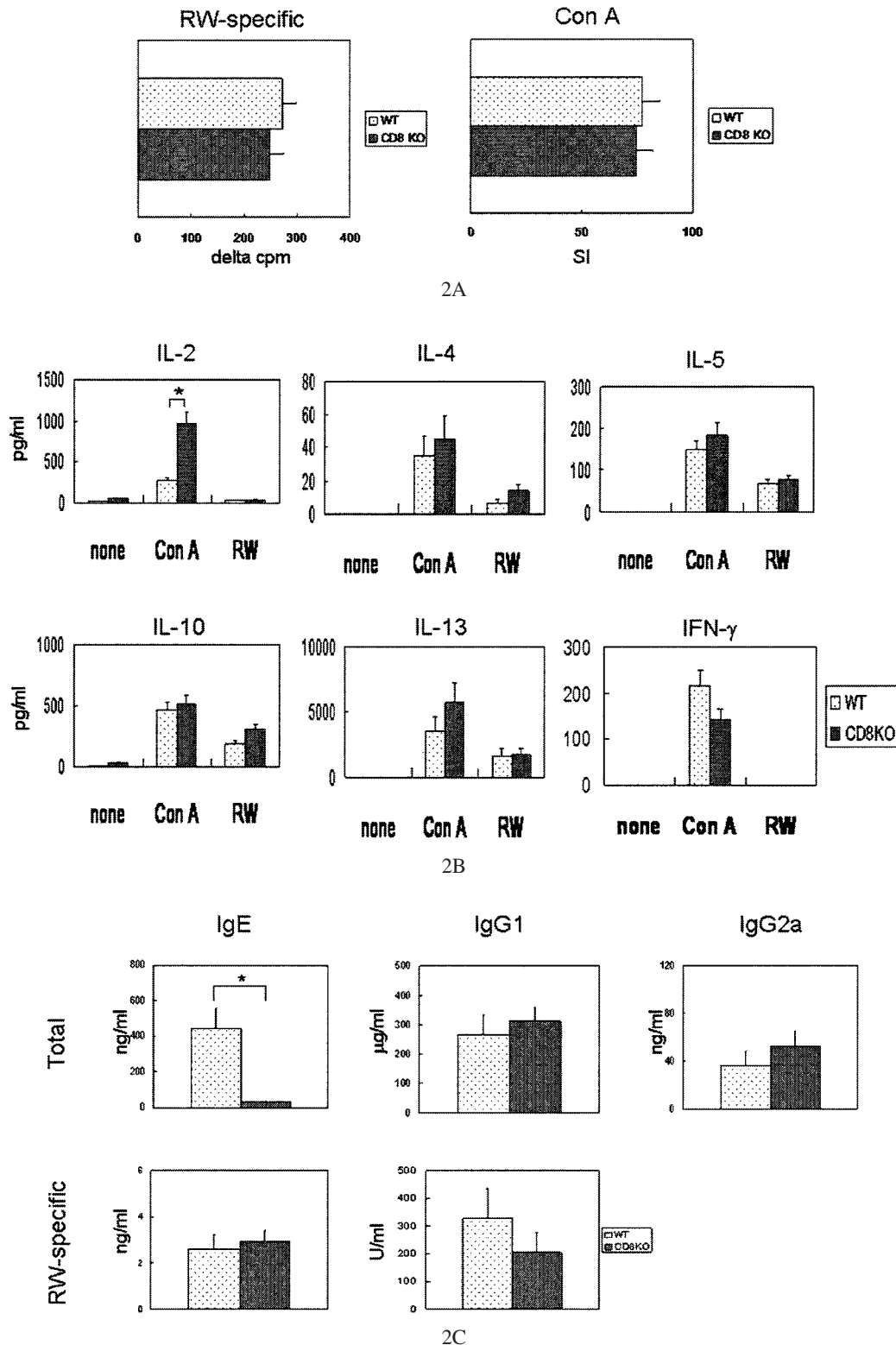


Fig. 2. Comparison of the systemic immune responses of WT and CD8KO mice that are developing actively induced EC. Spleens and sera were harvested from the mice described in the legend to Fig. 1 at the same time the conjunctivas were removed. (A, B) The splenocytes were cultured *in vitro* with RW or Con A or without any stimulants. (A) Splenocyte proliferation was evaluated by measuring [^{3} H]-thymidine incorporation. Background cpm $\pm$ SEM was 224 ± 6 and 407 ± 17 for WT and CD8KO mice, respectively. (B) The concentrations of IL-2, IL-4, IL-5, IL-10, IL-13 and IFN- γ in the supernatants of the cultured splenocytes were measured by ELISA (* $P < 0.05$). (C) Total and RW-specific IgE, IgG $_1$ and IgG $_{2a}$ levels in the serum were measured by ELISA (* $P < 0.05$).

junctivas and measured the levels of 17 cytokines and six chemokines. Conjunctivas from naïve mice challenged with RW served as negative controls. All cytokines/chemokines were detectable in the conjunctivas of actively immunized animals except for IL-13, IFN- γ and MIP-1 α . As shown in Fig. 3, IL-4, IL-5 and IL-6 expression was upregulated in WT mice conjunctivas after EC induction but only IL-4 showed a similar upregulation in CD8KO mice. However, the IL-4 levels in the WT conjunctivas were significantly higher than in the CD8KO conjunctivas. The conjunctival levels of the other detectable cytokines and chemokines did not differ significantly between the two groups.

Transfer of RW-Primed CD8KO Splenocytes Induces Significantly More Severe Eosinophil Infiltration into the Conjunctiva than That of WT Splenocytes

Although the proliferation and cytokine production of actively immunized WT and CD8KO splenocytes did not differ, it remains possible that the CD4+ T cells in CD8KO mice are not as capable of inducing EC as those in WT mice. To examine this, we transferred *in vitro*-stimulated RW-primed splenocytes from WT and CD8KO mice into naïve WT mice. The mice were then challenged with RW in eye drops and the eosinophils infiltrating into the conjunctiva were counted as described in "Materials and Methods." In the WT and CD8KO cell populations that were transferred, CD4+ T cells constituted 29% and 57% of the cells, respectively. When 2×10^7 of these cell populations were transferred into naïve animals, the CD8KO splenocytes induced three times the number of eosinophils infiltrating the conjunctiva that were induced by WT splenocytes; this difference was significant (Fig. 4A). Even when the

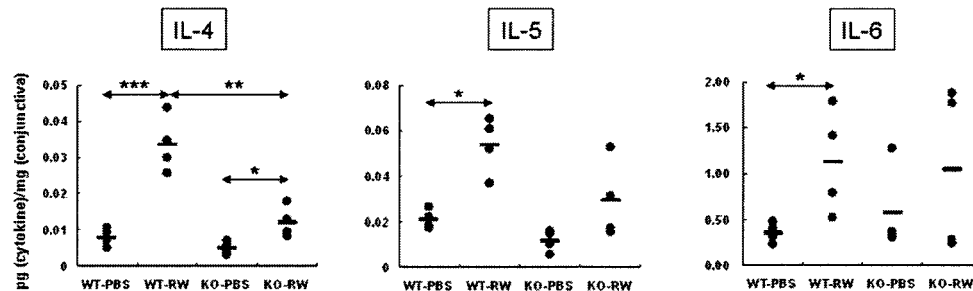


Fig. 3. Conjunctival IL-4 expression is reduced in CD8KO mice that are developing actively induced EC. Conjunctivas were harvested from actively immunized WT and CD8KO mice challenged with either PBS (WT-PBS, KO-PBS) or RW (WT-RW, KO-RW) and the levels of 17 cytokines and six chemokines were measured as described in "Materials and Methods." Data are expressed as pg (cytokine)/mg (conjunctiva). Each dot indicates individual mouse ($n=4$ per group), whereas horizontal bar indicates the average in each group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

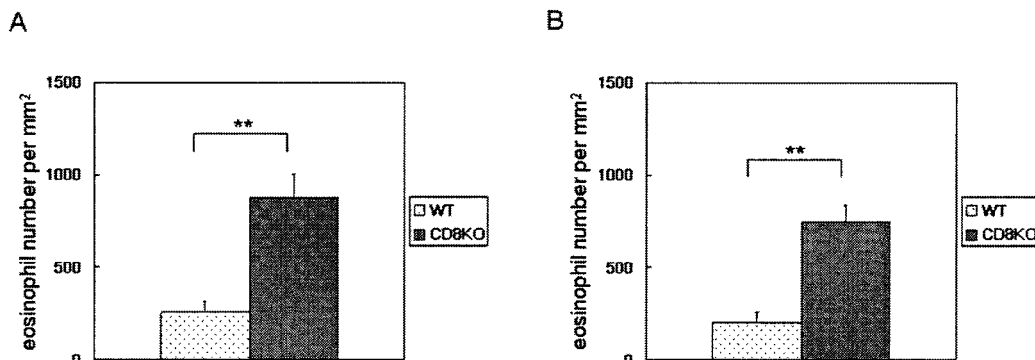


Fig. 4. Transferring RW-primed splenocytes from CD8KO mice into WT mice induces more severe eosinophil infiltration. Splenocytes from WT and CD8KO mice that are developing actively-induced EC were prepared and cultured *in vitro* with RW and then transferred into naïve WT mice. The eyes of the recipient mice were challenged daily from days 2 to 4 with RW in eye drops and 24 hr after the last challenge, their conjunctivas were subjected to histologic analysis. The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm². (A) When 2×10^7 splenocytes were transferred, the CD8KO-derived donor cells induced significantly more eosinophil infiltration. (B) Even when equal numbers of CD4+ T cells were transferred, CD8KO-derived donor cells induced significantly more severe eosinophil infiltration. ** $P<0.01$.

animals received equivalent numbers of CD4+ T cells, the number of eosinophils that infiltrated the WT conjunctiva was significantly higher by transfer of CD8KO-derived splenocytes (Fig. 4B). Thus, the CD4+ cells of actively immunized CD8KO mice are at least as capable of inducing EC as those from WT mice.

The Eosinophil Infiltration Induced by Transferring RW-Primed WT Splenocytes Is More Severe in CD8KO Recipients than in WT Recipients

To examine whether the resident CD8+ T cells in the recipient mice may affect the infiltration of eosinophils into the conjunctiva that is induced by transferring RW-primed splenocytes, we transferred *in vitro*-stimulated RW-primed WT splenocytes into naïve WT and CD8KO mice and then challenged the animals with RW in eye drops. Significantly more eosinophils infiltrated the conjunctivas of CD8KO recipient mice compared to WT recipient mice (Fig. 5). Thus, the resident CD8+ cells in the recipient mice suppress the eosinophilic infiltration into the conjunctivas that is induced by transferring RW-primed WT splenocytes.

Discussion

The role of CD4+ T cells play in the development of AC has been investigated in numerous experimental settings and accumulating evidence confirms that Th2-type CD4+ T cells play a crucial role in the development of severe AC (9, 24, 28). However, the role of

CD8+ T cells in experimental AC has not been investigated. Furthermore, there are a few reports that have investigated the role of CD8+ T cells are available in even experimental asthma (14–16, 22, 27, 29, 30), which is the most extensively analyzed model of experimental allergic diseases. Here, we sought to further elucidate the roles of CD8+ T cells play in experimental allergic diseases by inducing EC in CD8KO mice. To investigate the roles of CD8+ T cells in both the induction and effector phases of EC, the mice were either actively immunized or Ag-primed splenocytes were transferred to induce EC, respectively.

We found that CD8KO mice showed less severe eosinophilic infiltration of the conjunctiva upon active immunization than WT mice. CD8 α is expressed not only in the T-cell compartment but also by dendritic cells and $\gamma\delta$ T cells (1, 18). Therefore, one could argue that the lower infiltration of eosinophils in actively immunized CD8KO mice may be due to impaired function of dendritic cells. However, this possibility seems unlikely given the following observations: 1) flow cytometric analysis revealed equivalent frequencies of CD3+ cells in the spleens of actively immunized CD8KO and WT mice; moreover, the sum of CD4+ and CD8+ cells was equal to the total number of CD3+ cells in both WT and CD8KO mice; 2) the eosinophil infiltration induced by transferring primed CD8KO splenocytes was significantly more severe than when the splenocytes originated from WT mice; and 3) when the primed splenocytes from WT mice were transferred into naïve CD8KO mice, significantly more severe eosinophil infiltration into the conjunctiva was observed compared to when the splenocytes were transferred into WT mice. In addition, the actively immunized WT and CD8KO mice show generally equivalent splenocyte proliferation and cytokine release upon *in vitro* culture with antigen. Thus, it is unlikely that the APC function in CD8KO mice is impaired.

Why then do actively immunized CD8KO mice show less severe eosinophil infiltration into the conjunctiva? With regard to the systemic immune responses of the actively immunized WT and CD8KO mice, the only clear difference observed was that the latter mice has significantly lower serum total IgE levels, although the RW-specific IgE levels in the serum was the same. With regard to this, it should be noted that in the induction of allergic responses, not only Ag-specific but also Ag non-specific responses are thought to be important. In fact, in experimental contact dermatitis, transferring Ag-irrelevant IgE induced inflammation in IgE knockout mice (3). Therefore, the higher levels of total IgE in the WT mice may contribute to their more severe eosinophilic infiltration upon active immunization as

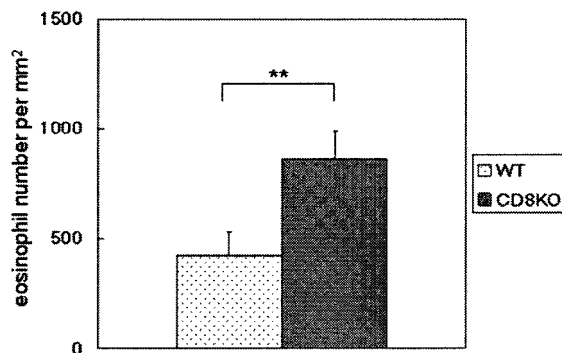


Fig. 5. Eosinophil infiltration is more severe in CD8KO than WT recipients when RW-primed WT splenocytes are transferred. Splenocytes from WT mice developing actively-induced EC were prepared, cultured *in vitro* with RW, and then transferred into naïve WT and CD8KO recipient mice. The eyes of the recipient mice were challenged daily from days 2 to 4 with RW in eye drops and 24 hr after the last challenge, their conjunctivas were subjected to histologic analysis. The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm². ** $P < 0.01$.

compared to the response by CD8KO mice. Notably, most previous reports suggest that CD8⁺ T cells suppress IgE production (19, 20, 25). Why the total serum IgE levels in CD8KO mice are lower in our system remains to be elucidated.

Our previous report demonstrating that injecting IL-4 alone into the subconjunctival space induced eosinophil infiltration into the conjunctiva (5) suggests that IL-4 in the conjunctiva is important for conjunctival infiltration by eosinophils. In addition, we have reported that the severity of eosinophil infiltration upon splenocyte transfer correlates positively with the ability of transferred population to produce IL-4 (24). Thus, both local and systemic Th2 cytokines are apparently important for eosinophil infiltration into the conjunctiva. When we analyzed the expression of various cytokines and chemokines in the conjunctivas of actively immunized WT and CD8KO mice, we found that Th2 cytokines, in particular IL-4, were upregulated more markedly by active EC induction in WT mice. Nevertheless, the cultured CD8KO splenocytes produced similar levels of Th2 cytokines as WT splenocytes. Thus, in our experimental system, the IL-4 expression in the conjunctiva and spleen that is induced by active immunization differs. One explanation for this discrepancy could be that IL-4-producing type 2 CD8⁺ effector memory T cells (21, 26, 31), which preferentially traffic to nonlymphoid tissues and acquire effector function more rapidly than central memory CD8⁺ T cells, may have selectively infiltrated into the conjunctiva. However, it remains unclear how CD8⁺ T cells augment the IL-4 levels in the conjunctiva of mice developing actively-induced EC.

Our data here clearly show that CD8⁺ T cells are important for the development of EC induced by active immunization. However, the nature of this experiment means that it was unclear whether the CD8⁺ T cells exert their effects either during the induction and/or the effector phase. Therefore, we next investigated the role of CD8⁺ T cells specifically play during the effector phase of EC by cell transfer experiments. Transferring RW-primed WT splenocytes into CD8KO mice induced more severe eosinophil infiltration than when the same cell population was transferred into WT mice. This suggests that high numbers of Ag non-specific CD8⁺ T cells inhibit the development of EC during the effector phase. The data that transferring RW-primed CD8KO splenocytes induced significantly more severe eosinophil infiltration compared to WT splenocytes suggests that Ag-primed CD8⁺ T cells inhibit the development of EC during the effector phase. In addition, transferring RW-primed WT splenocytes depleted of CD8⁺ T cells into naïve WT mice also induced sig-

nificantly more severe eosinophil infiltration than the undepleted population (data not shown). Taken together, our data indicate that CD8⁺ T cells play suppressive roles during the effector phase of EC.

With regard to experimental asthma, Ag-primed CD8⁺ T cells contribute to the development of experimental airway hyperresponsiveness in mice via a mechanism that may be mediated by IL-5 (14) and IL-13 (22). Martin et al. demonstrated that transferring Ag-primed CD8⁺ T cells suppressed late airway responses in Brown Norway rats (30). They also recently reported that the transfer of Ag-primed CD8⁺ $\alpha\beta$, but not $\gamma\delta$, T cells induced significant late airway responses (15). These reports support the notion that Ag-primed CD8⁺ T cells promote the development of airway inflammation. This is contrary to our own observation here that indicate that Ag-primed CD8⁺ T cells play a negative role in the development of EC during the effector phase. Although the reason for this discrepancy is unclear at the present, it may be related to differences in the immunization protocols, Ags and species that were used. Consistent with a previous report that suggested resident CD8⁺ T cells suppress CD4⁺ T cell-dependent late allergic airway responses in Brown Norway rats (16) is our observation that transferring *in vitro*-stimulated RW-primed WT splenocytes into CD8KO recipient mice induced more severe eosinophil infiltration than when WT recipients were used.

In conclusion, our data clearly demonstrate that CD8⁺ T cells promote the development of EC during the induction phase but play a suppressive role during the effector phase.

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