

Endogenous IL-17 Does Not Play a Significant Role in the Development of Experimental Murine Allergic Conjunctivitis

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Key Words

Conjunctivitis, allergic • Eosinophil • IL-17 • T cell

Abstract

Background: Endogenous IL-17 is needed for the Ag sensitization that results in murine allergic asthma, a Th2-mediated disease. Here, we aimed to investigate the role IL-17 plays in the development of murine experimental allergic conjunctivitis (EC) which is also a Th2-mediated disease. **Methods:** To induce EC, wild-type (WT) and IL-17-deficient (IL-17 KO) mice on the BALB/c and C57BL/6 backgrounds were immunized with ragweed (RW) in alum and challenged with RW in eye drops. Alternatively, EC was induced by adoptively transferring RW-primed splenocytes followed by challenge with RW-containing eye drops. Twenty-four hours after the RW challenge, the conjunctivas and spleens were harvested for histological analyses and cytokine assays, respectively. **Results:** The WT and IL-17 KO mice on both backgrounds did not differ in terms of the severity of actively induced EC, as evaluated by the conjunctival eosinophil infiltration. They also did not differ with regard to the phenotypes of the inflammatory cells infiltrating the conjunctivas, although primed IL-17 KO splenocytes stimulated in vitro with RW extract did produce significantly higher amounts of IL-4, IL-13 and IFN- γ than WT splenocytes. Reciprocal adoptive transfer experiments also demonstrated that the IL-17 from both the donor splenocytes and the recipient mice is not

involved in the development of EC. **Conclusions:** Endogenous IL-17 does not appear to play a significant role in the development of EC.

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Introduction

Allergic conjunctivitis (AC) is a disease the severity of which ranges from mild seasonal AC to vernal keratoconjunctivitis [1]. In the conjunctivas of vernal keratoconjunctivitis patients, inflammatory cells that include eosinophils are abundant [2]. The correlation between eosinophil numbers in tear fluid and the severity of AC in atopic patients [3] suggests that eosinophils play a role in the development of severe AC.

Our group has used an experimental AC (EC) model in rats [4–7] and mice [8–10] to investigate how eosinophils infiltrate the conjunctiva during the development of AC. We found that T cells, not IgE, play a prominent role in conjunctival eosinophil infiltration [8]. Furthermore, it appears that Ag-specific Th2 cells, not Th1 cells, initiate the conjunctival eosinophil infiltration in EC [9].

Recently, IL-17-producing Th17 cells were shown to play a crucial role in the development of organ-specific autoimmune diseases that are believed to be mediated by Th1 cells, such as experimental autoimmune encephalo-

myelitis [11] and collagen-induced arthritis [12]. With regard to the role Th17 cells play in Th2-mediated allergic diseases, IL-17-deficient (IL-17 KO) mice have only insignificant differences compared to wild-type (WT) mice with regard to airway hyperresponsiveness, total cell number in the bronchoalveolar fluid, pulmonary pathology and antigen-specific Ig levels [13]. In contrast, a study examining IL-17 receptor-deficient mice has shown that endogenous IL-17 is needed for the Ag sensitization that results in allergic asthma [14]. Thus, it is still controversial whether Th17 cells participate in Th2-mediated diseases. To address this question, we induced EC in WT and IL-17 KO mice to determine whether endogenous IL-17 is required for the development of EC, a Th2-mediated disease.

Materials and Methods

Mice

Inbred WT mice on BALB/c and C57BL/6 backgrounds were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Homozygous IL-17 KO mice on BALB/c and C57BL/6 backgrounds were established as previously reported [13]. The mice were kept in specific pathogen-free conditions at the animal facility of the 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 (Warrington, Pa., USA). RW extract was obtained from LSL (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, Mo., USA).

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 and IL-17 KO mice ($n = 13$ /group on the BALB/c background and $n = 10$ /group on the C57BL/6 background). Fifty microliters of the emulsion (50 μ g of RW and 675 μ g of alum) was injected into each site. The eyes of the immunized mice were challenged on day 10 (BALB/c) or days 7–10 (C57BL/6) with RW in PBS (2 mg in 10 μ l/eye). Twenty-four hours after the (last) challenge, the eyes and spleens were harvested for histological analysis and cytokine production, respectively.

Induction of EC by Adoptively Transferring *in vitro*-Stimulated RW-Primed Splenocytes

WT and IL-17 KO mice on the BALB/c background were actively immunized and challenged as described above and their day 11-harvested splenocytes were cultured with RW extract at a final concentration of 5 μ g/ml in 75-cm² flasks at a concentration of 10^7 cells/ml in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (ICN Biomedical Japan,

Tokyo, Japan), 2-mercaptoethanol (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 h at 37°C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes/mouse were injected intraperitoneally into naïve WT or IL-17 KO mice. In one experiment, 6 WT recipients and 6 IL-17 KO recipients received the same WT donor cells. In a separate experiment, two groups of 7 WT recipients received either WT or IL-17 KO donor cells. In both experiments, the eyes of the recipient mice were challenged 4 days after the transfer with RW in PBS (2 mg in 10 μ l/eye). Twenty-four hours later, 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 blinded samples. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. The data are presented as averages $\pm$ SEM of all the mice examined.

Immunohistochemistry

Conjunctivas from actively immunized and primed BALB/c mice were harvested 24 h after RW challenge. The conjunctivas were embedded in OCT compound (VWR, Suwanee, Ga., USA), and flash frozen in liquid nitrogen. Vertical 4- μ m sections were prepared and fixed in methanol. Endogenous peroxidase activity was inhibited with 0.1% NaN₃ and 0.3% H₂O₂ in distilled water for 10 min at room temperature. The samples were exposed to primary antibodies for 30 min and then to appropriate biotinylated secondary antibodies for another 30 min. Antibody binding was revealed by using an avidin-biotin complex kit (Vector Laboratories, Burlingame, Calif., USA) followed by development with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The antibodies used were rat anti-mouse CD3 (CT-CD3, Caltag, Burlingame, Calif., USA), anti-mouse CD11b (M1/70.15, Invitrogen, Carlsbad, Calif., USA), and rabbit anti-mouse major basic protein (MBP, provided by Dr. Lee, Mayo Clinic, Scottsdale, Ark., USA).

Measurement of Cytokines in Culture Supernatants

RBC-depleted splenocytes (10^7 cells/ml) from naïve WT mice or actively immunized and challenged WT and IL-17 KO mice were cultured for 48 h with 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% fetal calf serum and 2-mercaptoethanol. The levels of IL-4, IL-12, IL-13, IL-17 and IFN- γ that were produced were measured by using Luminex100 (Luminex, Austin, Tex., USA) and the Bio-Plex Cytokine Assay Panel (Bio-Rad Laboratories, Hercules, Calif., USA) according to the manufacturer's instructions. The levels of IL-6 and TGF- β_1 were measured by cytokine ELISA kit (BioSource International, Camarillo, Calif., USA) according to the manufacturer's recommendation. The data of four individual experiments are shown as averages $\pm$ SEM (pg/ml).

Quantitative Real-Time RT-PCR

RNA from the conjunctivas from naïve and challenged BALB/c WT or actively immunized and challenged BALB/c WT

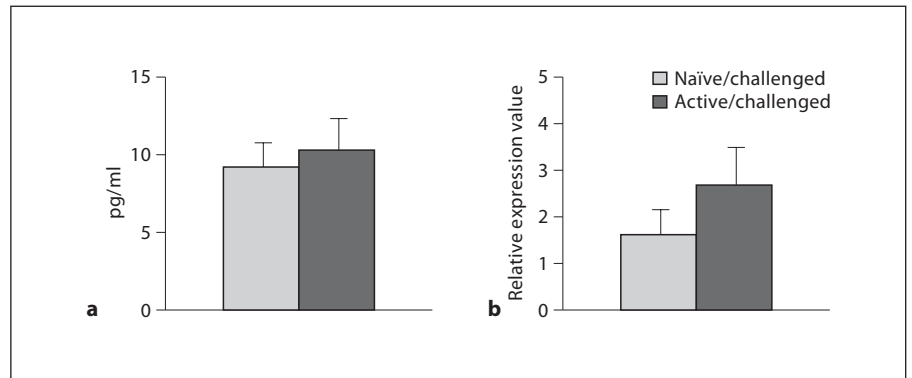


Fig. 1. Comparison of IL-17 production between naïve and EC-developing mice. **a** Splenocytes were prepared from naïve BALB/c WT or actively immunized and challenged BALB/c WT mice. These splenocytes were stimulated in vitro with RW extract and 48 h later, supernatants were collected to measure IL-17 levels. Data are expressed as mean $\pm$ SEM. $n = 3/\text{group}$. **b** Twenty-four hours after RW challenge, the conjunctivas were collected from

naïve and challenged BALB/c WT or actively immunized and challenged BALB/c WT mice. Expression levels of IL-17 mRNA were evaluated by the real-time RT-PCR method. Data are presented as relative expression values calculated by dividing the expression level of IL-17 by the expression level of HPRT. $n = 3/\text{group}$.

mice was purified using Trizol RNA isolation reagent (Invitrogen Life Technologies, Carlsbad, Calif., USA) according to the manufacturer's protocol. Total RNA from each tissue sample was incubated with M-MLV reverse transcriptase (RT; Invitrogen Life Technologies) according to the manufacturer's protocol using random primers. Quantitative real-time PCR was done using ABI Prism 7000 Sequence Detection Systems (Applied Biosystems, Foster City, Calif., USA). Probe and primer sequences for IL-17a and hypoxanthine phosphoribosyl transferase (HPRT) were obtained from Applied Biosystems. Each reaction was performed in 25 μl with 50% TaqMan 2 $\times$ PCR Master Mix (Applied Biosystems), 100 nM each of the forward and reverse primer, and 200 nM of probe. Conditions for the PCR were 2 min at 50°C, 10 min at 95°C, and then 40 cycles, each consisting of 15 s at 95°C, and 1 min at 60°C. Arbitrary cDNA values of individual samples were determined using standard curves obtained from a 4-fold serial dilution of a reference cDNA sample. Relative expression values were calculated by dividing the expression level of IL-17a by the expression level of HPRT.

Statistical Analysis

Significant differences between the tested groups with regard to infiltrating eosinophil numbers, splenocyte cytokine production and IL-17 RNA expression levels were determined by Student's *t* test. *p* values <0.05 were considered significant.

Results

Comparison of IL-17 Production between Naïve and EC-Developing Mice

To investigate whether IL-17 expression is augmented by the induction of EC, we measured the expression levels of IL-17 in the spleens and the conjunctivas by a bioassay

(the Bio-Plex Cytokine Assay Panel) and real-time RT-PCR, respectively. By stimulation with RW extract in vitro, splenocytes from actively immunized and challenged BALB/c WT mice produced slightly more IL-17 than those from naïve BALB/c WT mice, although the difference did not reach significance (fig. 1a). Similarly, IL-17 expression levels in the conjunctivas from actively immunized and challenged BALB/c WT mice were slightly but not significantly higher than those from BALB/c WT mice (fig. 1b).

IL-17 KO and WT Mice Develop Equally Severe EC after Active Immunization and Challenge

WT and IL-17 KO mice on the BALB/c and C57BL/6 backgrounds were actively immunized with RW in alum and then challenged once on day 10 (BALB/c) or 4 times consecutively on days 7–10 (C57BL/6) with RW in eye drops. Twenty-four hours after RW challenge on day 10, the conjunctivas were harvested for histological analyses. The BALB/c WT mice evinced conjunctival infiltration of inflammatory cells (fig. 2a) and knocking out IL-17 had no effect on the degree of eosinophil infiltration into the conjunctiva (fig. 2b). With regard to C57BL/6 mice, conjunctival infiltration of inflammatory cells was minimal in both WT and IL-17 KO (data not shown). Immunohistochemical analysis of the conjunctivas of the BALB/c mice revealed that for both the WT and IL-17 KO mice, the conjunctival infiltrating cells were composed of T cells, macrophages and eosinophils (fig. 2c), with very few polymorphonuclear leukocytes and B cells being de-

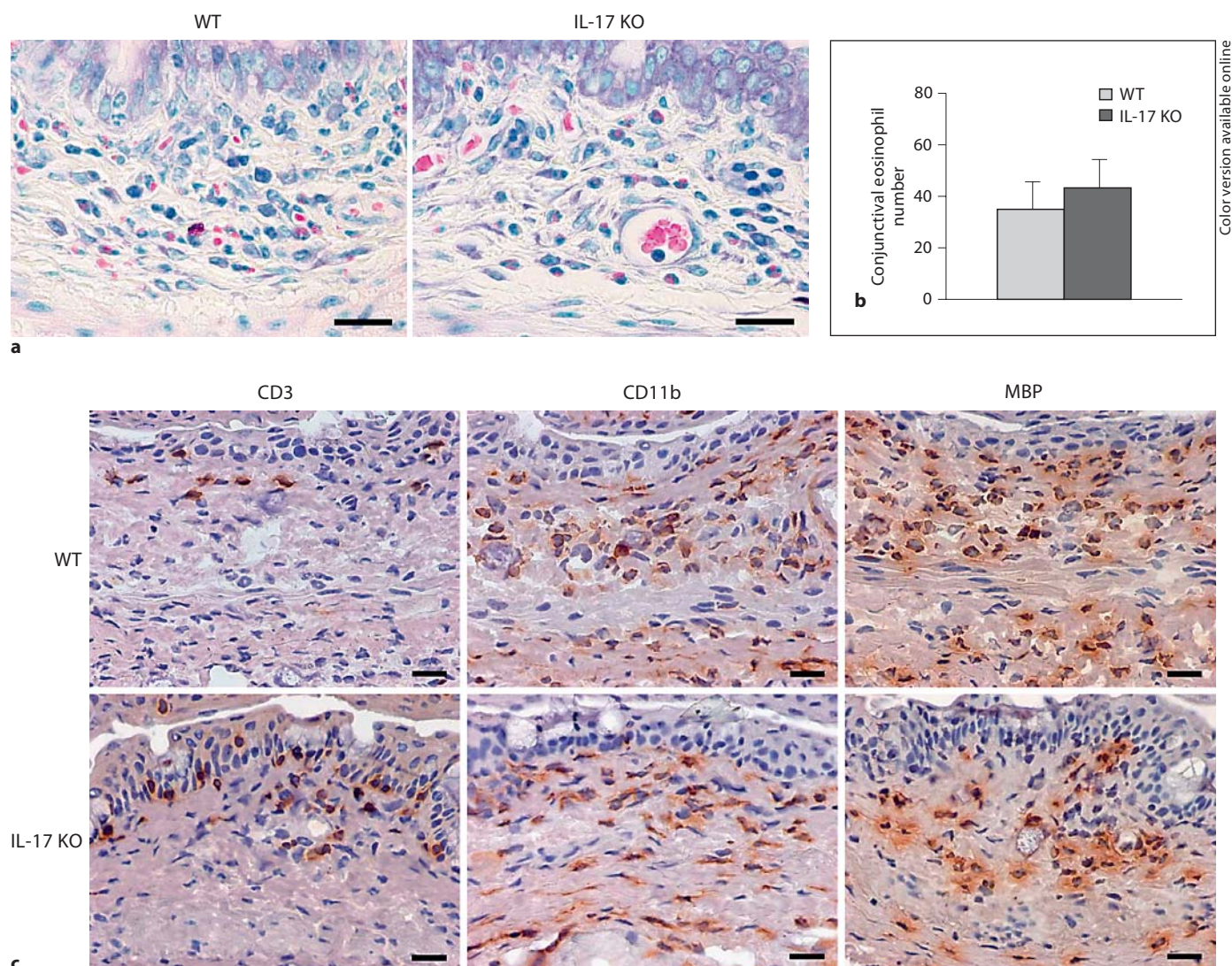


Fig. 2. Severity of EC in actively immunized WT and IL-17 KO mice. EC was induced in BALB/c WT and IL-17 KO mice by active immunization with RW in alum followed by RW challenge in eye drops. Twenty-four hours after the RW challenge, the conjunctivas were harvested for histological and immunohistochemical analyses. **a** Microphotographs of the Giemsa-stained conjuncti-

vas. Bar = 20 μ m. **b** Conjunctival eosinophil numbers. Conjunctival eosinophil numbers were counted in each slide and the data are presented as mean conjunctival eosinophil numbers $\pm$ SEM. $n = 13/\text{group}$. **c** Microphotographs of the immunohistochemically analyzed BALB/c conjunctivas. Bar = 20 μ m.

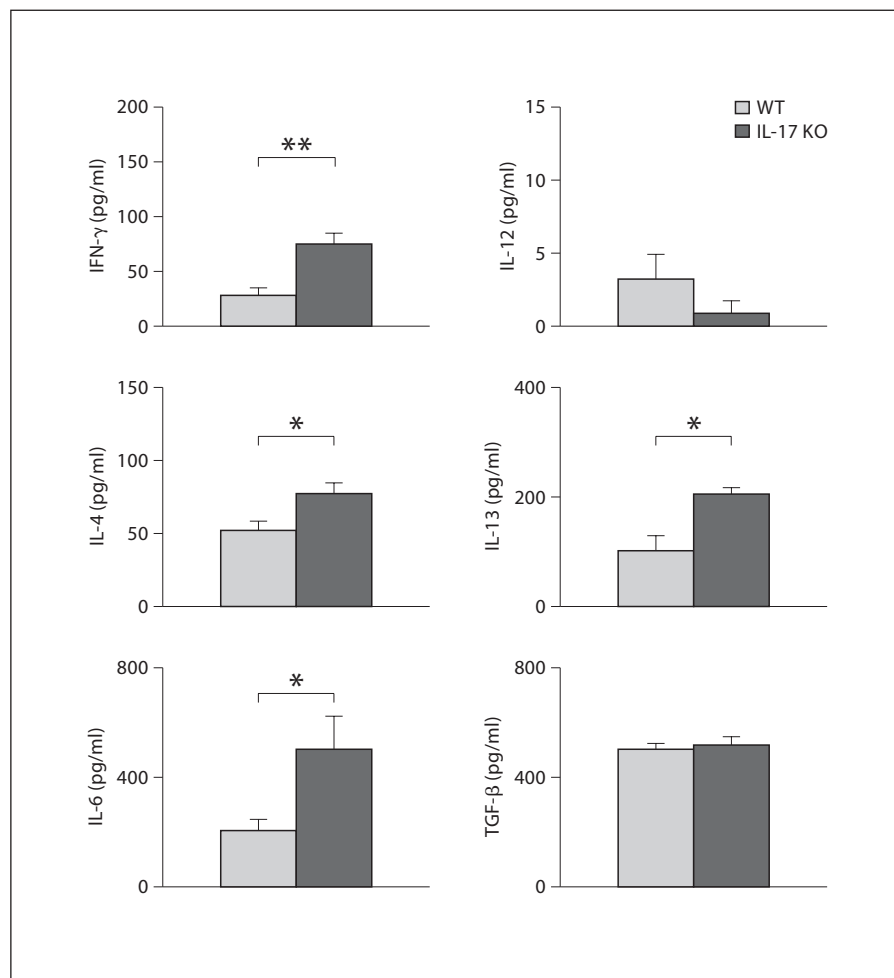
tected (data not shown). Slightly more, but not significantly different, T cells were detected in the conjunctiva of IL-17 KO mice (fig. 2c).

Immune Responses in IL-17 KO Mice

To investigate whether knocking out IL-17 affects the immune response induced by RW immunization, the spleens and blood were collected from the IL-17 KO and WT BALB/c mice at the time the conjunctivas were har-

vested. The splenocytes were stimulated in vitro with RW extract for 48 h and the culture supernatants were subjected to cytokine analysis. The splenocytes from the IL-17 KO mice produced significantly higher amounts of IL-4, IL-6, IL-13 and IFN- γ but did not differ significantly from WT splenocytes in terms of their IL-12 and TGF- β productions (fig. 3). The levels of total IgG1, IgG2a and IgE in the serum did not differ between the actively immunized WT and IL-17 KO mice (data not shown).

Fig. 3. Cytokine production by splenocytes from actively immunized BALB/c mice after in vitro stimulation with RW extract. The spleens of the actively immunized and primed BALB/c mice were collected at the time the conjunctivas were harvested and the splenocytes were stimulated in vitro with RW extract for 48 h. The culture supernatants were then tested to measure cytokine levels. The data are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.



WT and IL-17 KO Mice Develop Equally Severe EC after Splenocyte Transfer

To investigate whether IL-17 plays a role in the development of EC during the effector phase, EC was induced by transferring RW-primed splenocytes followed by RW challenge in eye drops. Since the severity of EC in C57BL/6 was extremely weak as mentioned above, experiments were performed by using BALB/c mice. The WT recipient mice developed equally severe EC regardless of whether they received RW-primed WT splenocytes or RW-primed IL-17 KO splenocytes (fig. 4a). Similarly, IL-17 KO recipient mice that received RW-primed WT splenocytes did not differ in disease severity from WT recipient mice that received the same cells (fig. 4b). Thus, it appears that IL-17 does not play a role in the development of EC during the effector phase.

Discussion

Consistent with a previous report that the severity of lung inflammation was not different between WT and IL-17 KO mice [13], we found that WT and IL-17 KO mice did not differ in terms of the severity of EC (as measured by the numbers of conjunctival eosinophils) or the phenotypes of the conjunctival infiltrating inflammatory cells. It is of note that IL-17 KO mice used in that study [13] were identical to the mice used in this study. In contrast, another study reported that allergic asthma was attenuated in IL-17 receptor-deficient mice [14]. In our study, the IL-17 deficiency did significantly affect the systemic immune responses as the splenocytes of the actively immunized and challenged IL-17 KO mice showed significantly upregulated IFN- γ and IL-4 production. This is in good agreement with the notion that IL-17 negatively regulates both Th1 and Th2 immune responses [15].

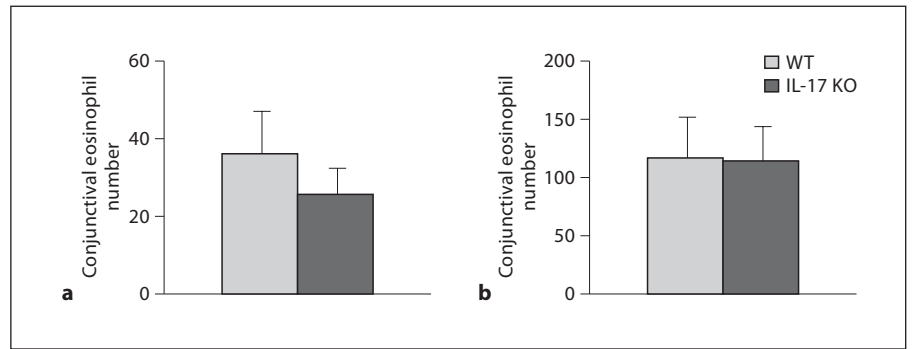


Fig. 4. Severity of EC in WT and IL-17 KO BALB/c mice after adoptive transfer of RW-primed splenocytes. The spleens of WT and IL-17 KO BALB/c mice were collected at the time the conjunctivas were harvested and the splenocytes were cultured in vitro with RW extract for 3 days before being transferred into WT or IL-17 KO mice. Four days later, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis to determine conjunctival eosino-

phil numbers. **a** Effect of receiving different donor cells on EC severity in WT mice. RW-primed splenocytes from WT or IL-17 KO mice were transferred into naïve WT mice. **b** Effect on EC severity of transferring WT cells into different recipient mice. RW-primed splenocytes from WT mice were transferred into naïve WT or IL-17 KO mice. The data are presented as mean conjunctival eosinophil numbers $\pm$ SEM.

EC is mediated by Th2 cells [9] and is negatively regulated by IFN- γ , a Th1 cytokine [16]. Consequently, it appears that the IL-17 KO mice develop EC as well as the WT mice because they still have the Th1/Th2 balance needed to develop it, probably because both Th1 and Th2 responses were equally upregulated. An interesting finding in the previous report was that lung inflammation was reduced in IL-17-deficient (IL-17 $^{-/-}$) DO11.10 transgenic mice compared with IL-17 $^{+/-}$ DO11.10 transgenic mice [13]. Thus, as explained in this report [13], the adjuvant alum may also have induced an excessive Th2 response in our study and, therefore, no difference of the severity of EC was observed between WT and IL-17 KO mice.

It has been reported that intranasal administration of IL-17 during the effector phase of allergic asthma reduced pulmonary eosinophil recruitment and bronchial hyper-reactivity [14]. In another report, intratracheal IL-17F

gene transfer during the effector phase of allergic asthma significantly augmented the infiltration into the lung of neutrophils but not eosinophils [17]. Thus, IL-17 appears to be involved in the effector phase of allergic asthma. To investigate whether IL-17 is involved in the development of EC during the effector phase, we performed adoptive transfer experiments with RW-primed splenocytes. Equivalent disease severity was elicited regardless of the donor/recipient combination examined. Thus, it appears that IL-17 does not play a role in the development of EC during the effector phase. Furthermore, subconjunctival injection of recombinant IL-17 prior to RW challenge did not affect conjunctival eosinophil infiltration and did not increase conjunctival neutrophil infiltration (unpubl. results). It is unclear why IL-17 participates in allergic asthma but not EC. Further studies are necessary to clarify why IL-17 plays a different role in the development of EC compared to allergic asthma.

References

- 1 Ehlers WH, Donshik PC: Allergic ocular disorders: a spectrum of diseases. *CLAO J* 1992; 18:117–124.
- 2 Bonini S, Magrini L, Rotiroti G, Lambiase A, Tomassini M, Rumi C, Bonini S: The eosinophil and the eye. *Allergy* 1997;52:44–47.
- 3 Fukagawa K, Nakajima T, Tsubota K, Shimura S, Saito H, Hirai K: Presence of eotaxin in tears of patients with atopic keratoconjunctivitis with severe corneal damage. *J Allergy Clin Immunol* 1999;103:1220–1221.
- 4 Fukushima A, Yoshida H, Iwamoto H, Yoshida O, Ueno H: The role of cellular immunity both in the induction and effector phases of experimental allergic blepharoconjunctivitis (EAC) in rats. *Exp Eye Res* 1997;65:631–637.

- 5 Yoshida O, Yoshida H, Iwamoto H, Nishino K, Fukushima A, Ueno H: Genetic background determines the nature of immune responses and experimental immune-mediated blepharoconjunctivitis (EC). *Curr Eye Res* 1999;18:117–124.
- 6 Iwamoto H, Nishino K, Magone TM, Whitcup SM, Yoshida O, Yoshida H, Ozaki A, Fukushima A, Ueno H: Experimental immune-mediated blepharoconjunctivitis in rats induced by immunization with ragweed pollen. *Graefes Arch Clin Exp Ophthalmol* 2000;238:346–351.
- 7 Fukushima A, Ozaki A, Fukata K, Ishida W, Ueno H: Ag-specific recognition, activation, and effector function of T cells in the conjunctiva with experimental immune-mediated blepharoconjunctivitis. *Invest Ophthalmol Vis Sci* 2003;44:4366–4374.
- 8 Fukushima A, Ozaki A, Jian Z, Ishida W, Fukata K, Ueno H, Liu FT: Dissection of antigen-specific humoral and cellular immune responses for the development of experimental immune-mediated blepharoconjunctivitis in C57BL/6 mice. *Curr Eye Res* 2005;30:241–248.
- 9 Ozaki A, Seki Y, Fukushima A, Kubo M: The control of allergic conjunctivitis by suppressor of cytokine signaling (SOCS)3 and SOCS5 in a murine model. *J Immunol* 2005;175:5489–5497.
- 10 Fukushima A, Yamaguchi T, Ishida W, Fukata K, Taniguchi T, Liu FT, Ueno H: Genetic background determines susceptibility to experimental immune-mediated blepharoconjunctivitis: comparison of Balb/c and C57BL/6 mice. *Exp Eye Res* 2006;82:210–218.
- 11 Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD: Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003;421:744–748.
- 12 Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ: Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 2003;198:1951–1957.
- 13 Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, Sekikawa K, Asano M, Iwakura Y: Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 2002;17:375–387.
- 14 Schnyder-Candrian S, Togbe D, Couillin I, Mercier I, Brombacher F, Quesniaux V, Fossiez F, Ryffel B, Schnyder B: Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 2006;203:2715–2725.
- 15 Harrington LE, Mangan PR, Weaver CT: Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr Opin Immunol* 2006;18:349–356.
- 16 Magone MT, Whitcup SM, Fukushima A, Chan CC, Silver PB, Rizzo LV: The role of IL-12 in the induction of late-phase cellular infiltration in a murine model of allergic conjunctivitis. *J Allergy Clin Immunol* 2000;105:299–308.
- 17 Oda N, Canelos PB, Essayan DM, Plunkett BA, Myers AC, Huang SK: Interleukin-17F induces pulmonary neutrophilia and amplifies antigen-induced allergic response. *Am J Respir Crit Care Med* 2005;171:12–18.