

The Control of Allergic Conjunctivitis by Suppressor of Cytokine Signaling (SOCS)3 and SOCS5 in a Murine Model¹

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Allergic conjunctivitis (AC) is a common allergic eye disease characterized by clinical symptoms such as itchiness, conjunctival congestion, elevated Ag-specific IgE, mast cell activation, and local eosinophil infiltration. In this study we established a murine model for Ag-induced AC to understand the pathogenesis of the disease. Cell transfer experiments indicated that AC can be divided into early and late phase responses (EPR and LPR). EPR was associated with IgE responses, leading to itchiness, whereas LPR was characterized by local eosinophil infiltration. Both EPR and LPR were significantly inhibited in STAT6-deficient mice, and adoptive transfer of Th2 cells reconstituted LPR. Furthermore, SOCS3 was highly expressed at the disease site, and T cell-specific expression of SOCS3 deteriorated clinical and pathological features of AC, indicating that Th2-mediated SOCS3 expression controls the development and persistence of AC. Reduction of the expression level in SOCS3 heterozygous mice or inhibition of function in dominant-negative SOCS3 transgenic mice clearly reduced the severity of AC. In contrast, constitutive expression of SOCS5, a specific inhibitor of IL-4 signaling, resulted in reduced eosinophil infiltration. These results suggest that negative regulation of the Th2-mediated response by dominant-negative SOCS3 and SOCS5 could be a target for therapeutic intervention in allergic disease. *The Journal of Immunology*, 2005, 175: 5489–5497.

Allergic conjunctivitis (AC)³ is a spectrum of disorders ranging from acute forms of seasonal AC to chronic and severe sight-threatening forms, such as vernal keratoconjunctivitis. Seasonal AC is by far the most common form, and its prevalence and incidence have been increasing. The development of AC is characterized by topical and constitutive Ag sensitization, and patients suffer from several inflammatory symptoms, including itching, redness, lid swelling, and chemosis. Recent studies have expanded the basic knowledge of the pathophysiological mechanisms in AC, which is now divided into early and late phases. The presence of Ag-specific IgE is indispensable for the early phase response (EPR), which is characterized by itching, but not for the late phase response (LPR). The association of IgE with granule-associated preformed and newly formed mediators in mast cells has been well documented in the type 1 allergic response (1), and conjunctival biopsy samples from chronic AC patients contain a significantly higher proportion of mast cells and a higher level of mediators (2), providing clinical evidence that mast cell activation is found in the tear fluid and serum of AC patients.

The mechanisms that regulate the LPR, however, are not fully understood. The attraction of eosinophils to sensitized areas is a

pathological feature of the LPR in AC. Eosinophil attraction is reconstituted upon cell transfer from Ag-sensitized animals in the absence of Ag-specific IgE (3–6). Th2 cytokine responses are known to be associated with many cases of allergic disorders (7, 8), such as asthma and atopic dermatitis (AD), but treatment with Abs against Th2 cytokines is insufficient in human asthmatic patients (9, 10), suggesting that such allergic diseases are a complex disorder, in which the significance of Th2 cells remains elusive. AC provides another example of this situation. Treatment with rIL-12 enhances the inflammatory response of AC in the conjunctiva (11); thus, the relevance of Th1 and Th2 cells to the development of AC remains unclear.

Suppressor of cytokine signaling (SOCS) molecules act as negative regulators of cytokine signaling and are known to be involved in the pathogenesis of many inflammatory diseases (12–14). The discovery of SOCS proteins has provided new insight into the cytokine regulation of Th1 and Th2 immune responses. Members of the SOCS family have been identified due to their marked conserved Src homology 2 (SH2) domain and a unique conserved motif, referred to as the SOCS box. Eight members of the SOCS protein family have been identified: cytokine-inducible SH2 domain-containing protein and SOCS1 to -7. SOCS3 has been characterized as a protein related to cytokine-inducible SH2 domain-containing protein 1 and SOCS1, and the expression of SOCS3 is transiently induced in a variety of immune and inflammatory situations by a wide variety of cytokines, including IFN- γ , IL-2, IL-3, IL-4, IL-6, and IL-10, as well as by pathogen-associated molecular patterns. At an inflammatory site, SOCS3 works as a proinflammatory mediator by suppressing IL-6 signaling via gp130 (15). SOCS3 is induced by IL-6 as well as by IL-10 in macrophages through STAT3 activation and preferentially inhibits the IL-6-mediated signaling pathway (16). However, SOCS3 has distinct targets and functions in T cells. We have demonstrated a correlation between SOCS3 expression and Th2-mediated allergic disease in both mice and humans (17). SOCS3 is highly expressed in peripheral T cells from human patients with Th2 type diseases such as atopic asthma and dermatitis, and its expression tightly correlates with the severity of disease. Serum IgE concentrations

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³ Abbreviations used in this paper: AC, allergic conjunctivitis; AD, atopic dermatitis; dn, dominant negative; EPR, early phase response; LPR, late phase responses; RW, ragweed pollen; SH2, Src homology 2; SOCS, suppressor of cytokine signaling; Tg, transgenic.

are also increased in patients with high SOCS3 expression. In T cells, SOCS3 is selectively induced under Th2 culture conditions in the presence of IL-4. The induced SOCS3 specifically binds to the cytoplasmic region of IL-12R β and inhibits IL-12-mediated STAT4 activation. Such SOCS3-mediated Th1 inhibition subsequently enhances Th2 development and increases the incidence of allergic airway hyper-responsiveness.

SOCS5 is highly expressed in lymphoid organs such as spleen, lymph nodes, thymus, and bone marrow in human tissue. We found that SOCS5 is a potential regulator of IL-4 signaling through an SH2 domain-independent interaction between the IL-4R α -chain and SOCS5, and forced expression of SOCS5 in T cells clearly blocks Th2 differentiation (18), indicating that SOCS5 is a negative regulator for Th2-mediated allergic responses. However, SOCS5-deficient CD4⁺ T cells show normal Th1/Th2 differentiation, indicating that endogenous expression levels of SOCS5 may be dispensable for regulation of Th cell differentiation (19).

In the present paper we report a murine model for ragweed pollen (RW)-induced allergic conjunctivitis that allows separate assessment of EPR and LPR in AC. In many allergic inflammatory diseases, elevation of the plasma level of IgE and attraction of eosinophils have been reported to correlate with appearance of Th2 cells producing IL-4 and IL-5 (20, 21). The appearance of Th2 cells is tightly regulated through the IL-4-mediated STAT6 activation pathway; thus, STAT6 is a critical transcriptional factor that regulates Th2-mediated immune responses (22, 23). To address the functional relevance of the IL-4/STAT6 pathway in our AC mouse model, we examined the significance of STAT6 in EPR and LPR. STAT6 deficiency clearly reduced both responses, demonstrating that the presence of Th2 cells may be crucial for generation of both EPR and LPR in AC. We also attempted to investigate the role of the SOCS proteins as negative regulators of cytokines. SOCS3 and SOCS5 are expected to be the key modulators of allergic immune diseases; therefore, we studied the effect of T cell-specific expression of SOCS3, dominant-negative SOCS3 (dnSOCS3), and SOCS5 in the RW-induced mouse AC model. Our results provide new insights into cytokine signal-based therapeutic strategies that may be widely applicable to allergic diseases.

Materials and Methods

Animals and reagents

BALB/c and C57BL/6 mice were purchased from Clea Japan. The wild-type SOCS3, dnSOCS3 (F25A), and SOCS5 transgenic (Tg) mice under the control of the *Ick* proximal promoter and the intronic enhancer of the Ig H chain were generated and backcrossed into C57BL/6 mice over 10 generations (SOCS3, F25A Tg, and SOCS5 Tg mice, respectively). SOCS3^{+/−} mice were provided by J. N. Ihle (St. Jude's Research Hospital, Memphis, TN), and were backcrossed into C57BL/6 mice over 10 generations. STAT6 KO mice were a gift from Dr. S. Akira (Osaka University, Osaka, Japan) and were also backcrossed into BALB/c mice over 10 generations. OVA-specific TCR, DO11.10 Tg mice with a BALB/c background were provided by Dr. K. Murphy (Washington University, St. Louis, MO). All animal procedures conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Anti-CD3 (CT3) mAb was purchased from Caltag Laboratories. Anti-CD4 mAb (L3T4) was obtained from BD Pharmingen. Anti-major basic protein Ab was provided by Dr. J. Lee (Mayo Clinic, Scottsdale, AZ). Anti-SOCS3 polyclonal Ab (sc-9023) was purchased from Santa Cruz Biotechnology.

Induction of experimental AC by active immunization

BALB/c or C57BL/6 mice (6–9 wk old) were systemically s.c. sensitized with RW (100 μ g/200 μ l/mouse; Polyscience) emulsified in aluminum hydroxide hydrate gel (Sigma-Aldrich) on day 0. On days 7 and 14, mice were immunized i.p. with RW (100 μ g/mouse) in PBS. A week after the second immunization, mice were challenged with RW (1 mg/5 μ l PBS/eye) via eye drops. Clinical symptoms in the conjunctiva within 15 and 30 min after administration of the Ag eye drop were evaluated as chemosis, red-

ness, tearing, discharge, and scratching behavior, based on the criteria described in Table I. Clinical appearance and photographs were evaluated by two blinded observers, one of whom was an experienced ophthalmologist. Scratching behavior was monitored for 30 s, and the frequency of scratching was counted and evaluated as follows: one to three times, mild; four to six times, moderate; and more than seven times, severe. The final score was calculated as the sum of both eyes in each mouse. After 24 h, eyes were collected for histological analysis, and infiltrating cell number was counted in the conjunctiva.

Experimental AC by adoptive transfer

Spleens were harvested from RW-immunized donor animals. These RW-primed splenocytes were stimulated in vitro with soluble RW extract (25 μ g/ml; LSL). After 3 days of stimulation, CD4⁺ T cells were enriched using a MACS separation column (Miltenyi Biotec). Five million cells were i.v. transferred into naive syngeneic recipient mice. Four days after the transfer, RW (1 mg/5 μ l PBS/eye) was introduced onto the ocular surface of the recipient mice. Eyes were collected for histological examination 24 h after the administration of RW eye drops. Whole blood was collected 24 h after RW challenge, and total and RW-specific IgE levels were measured by ELISA.

Histological examination

The methods used for conventional histological analysis have been described previously (3, 5, 24). Briefly, vertical plane sections, including the optic nerve, were subjected to Giemsa and H&E staining. Sections for immunohistochemistry were prepared following Kawamoto's method (25). The eyes were immediately frozen in 3% carboxymethylcellulose gel in liquid nitrogen. Four-micrometer-thick sections were cut and fixed in ice-cold methanol, and thereafter washed with PBS. Slides were blocked with 1% normal mouse serum, 1% FCS, and 1% BSA in PBS; washed; and then further blocked with anti-FcR Ab (2.4G2). After washing, slides were exposed to the first Abs overnight, followed by an additional 1-h exposure to HRP secondary Ab. All slides were developed with 3,3'-diaminobenzidine.

Lymphocyte culture and cytokine production analysis

Splenocytes from RW-primed mice were collected 8 days after the last immunization. The splenocytes (5×10^6 cells/ml) were stimulated with RW (25 μ g/ml) in RPMI 1640 medium supplemented with 10% FCS, 0.1 M HEPES, MEM, 10 mM sodium pyruvate, and 5×10^{-5} 2ME. Supernatants were collected on day 3, and IFN- γ , IL-4, IL-5, and IL-13 levels were measured by ELISA with Abs obtained from BD Pharmingen and R&D Systems.

Statistical analysis

All results are presented as the mean and SE. Comparisons between groups were performed using a *t* test, and *p* values are given where appropriate.

Results

Establishment of a RW-specific AC mouse model

To generate an allergen (Ag)-induced experimental AC murine model, BALB/c and C57BL/6 mice were immunized with RW in the presence of aluminum and challenged via eye drops. The severity of clinical symptoms in EPR was scored immediately after

Table I. Grading table for clinical symptoms^a

	Absent	Mild	Moderate	Severe
Lid swelling	0	1	2	3
Chemosis	0	1	2	3
Redness	0	1	2	3
Discharge and tear	0	1	2	3
Scratching	0	1	2	3

^a Animals were examined clinically for signs of EPR 15 min after topical application of RW. Lid swelling, conjunctival redness, chemosis, discharge and tearing, and scratching behavior were graded based on the grading table. Clinical appearance and photographs were evaluated by two blinded observers. Scratching behavior was monitored for 30 s and the frequency of scratching was counted and evaluated as follows: one to three times, mild; four to six times, moderate; and more than seven times, severe. A score was given for each eye, and the final results show the sum of scores across both eyes of each mouse. Scores in the figures are the average of the total points for each mouse.

the administration of eye drops. The RW challenge induced clear lid swelling, tearing, severe discharge, and extensive scratching (Fig. 1A), and RW-immunized mice clearly showed higher levels of total and RW-specific IgE concentrations (Fig. 1B). Moreover, the mice challenged with RW, but not those challenged with PBS, showed a strong LPR with massive eosinophil infiltration into the conjunctiva 24 h after the RW challenge (Fig. 1, C and D) and increased IL-4, IL-5, and IL-13 in regional lymph nodes (Fig. 1E). RW-specific EPR and LPR were similarly observed in BALB/c and C57BL/6 mice maintained in specific pathogen-free condition (Figs. 1 and 2E, and 3). Therefore, this experimental AC model must be considered consistent with the criteria for the pathological symptoms of AC in human allergic conjunctivitis.

Experimental AC is mediated by CD4⁺ T cells

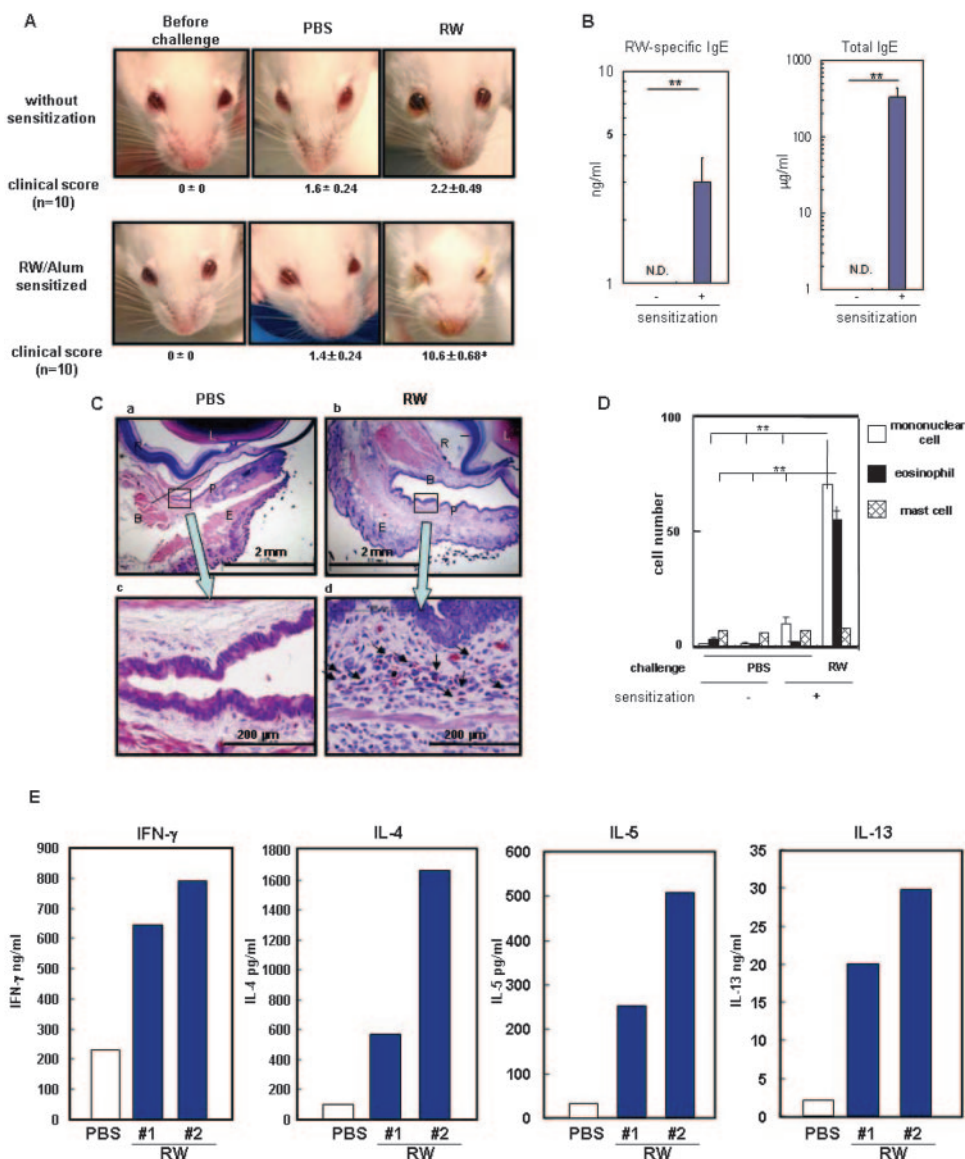
To identify which cell populations are responsible for EPR and LPR in the RW-specific experimental AC model, whole splenic cells from RW-immunized BALB/c mice were adoptively transferred into normal BALB/c mice. IgE production and the characteristic clinical symptoms of EPR were not found in any recipient mice after RW challenge (data not shown). In contrast, eosinophils migrated into the Ag-challenged eye 24 h after the challenge (Fig.

2A), demonstrating that LPR was mediated by RW-immunized splenocytes. We also transferred a T cell-depleted fraction, but these cells did not reconstitute LPR (data not shown). We next transferred purified CD4⁺ and CD8⁺ T cells from RW-immunized BALB/c mice. BALB/c mice adoptively immunized with CD4⁺ cells, but not CD8⁺ cells, exhibited clear eosinophil infiltration in the recipient BALB/c mice (Fig. 2, B and C), indicating that LPR in AC is regulated by CD4⁺ T cells.

Eosinophil infiltration in AC is regulated by Th2 cell-mediated immune response

We next studied whether eosinophil attraction observed in LPR is also initiated by Th2-mediated responses. To prepare an Ag-primed Th1 and Th2 population, OVA-specific TCR DO11.10 Tg mice were used. Th1 and Th2 cells were induced by in vitro stimulation with a specific peptide. These cells were transferred into syngeneic BALB/c mice, which were then challenged with OVA peptide 323–339 via administration of eye drops. Significant eosinophil infiltration was observed after transfer of Th2 cells, whereas little eosinophilic infiltration was found in the conjunctiva of OVA-specific Th1 cell recipients (Fig. 2D).

FIGURE 1. Establishment of RW-specific experimental AC. BALB/c mice were immunized with RW as described in *Materials and Methods*. Mice with or without RW sensitization were challenged with RW or vehicle only via application of eye drops. **A**, Clinical appearances and scores (average $\pm$ SE) of the EPR, 15–30 min after challenge, are shown. Mice with RW sensitization and RW challenge exhibited symptoms such as lid swelling, tearing, and discharge accompanied by frequent scratching. **B**, Total IgE and RW-specific IgE production in the serum was measured. **C**, Eyes were collected 24 h after RW challenge. Pathological features of conjunctivas in plane vertical sections, including the optic nerve, are shown. LPR characterized by local eosinophilic infiltration was confirmed in the RW-sensitized and RW-challenged mice (**b** and **d**), but not in the PBS-challenged mice (**a** and **c**). Black arrows indicate eosinophils. Giemsa stain (L, lens; R, retina; E, lower eye lid; P, palpebral conjunctiva; B, bulbar conjunctiva). **D**, Mononuclear cells ($\square$), eosinophils ($\blacksquare$), and mast cells ($\boxtimes$) in the palpebral conjunctiva and bulbar conjunctiva were counted. **, $p < 0.01$. Representative pictures of three independent experiments are shown ($n = 10$). **E**, Cervical and brachial lymph node cells were obtained from an RW-sensitized/PBS-treated mouse (PBS) and two independent RW-sensitized/RW-challenged mice (nos. 1 and 2). T cells were enriched and stimulated with anti-TCR mAb for 24 h. IFN- γ , IL-4, IL-5, and IL-13 concentrations in culture supernatant were measured by ELISA.



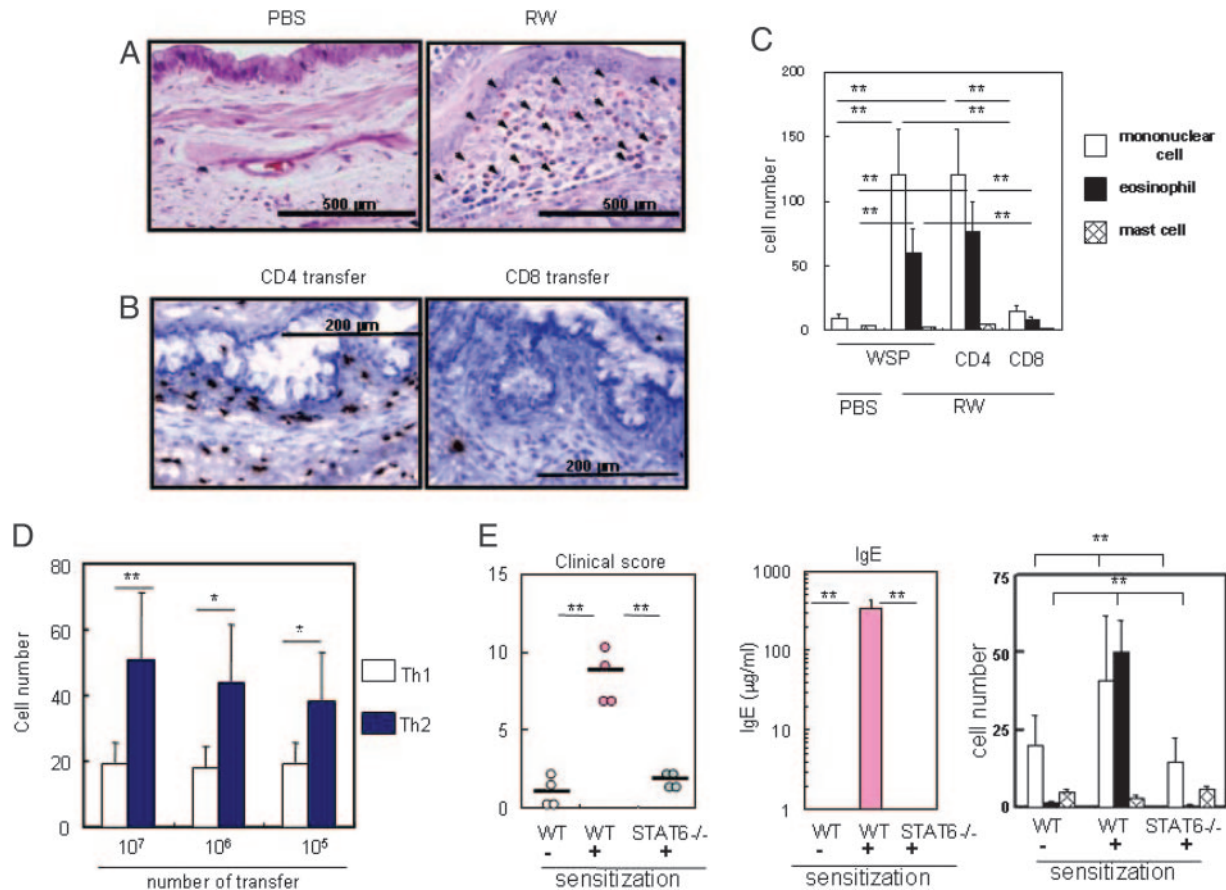


FIGURE 2. Th2 cells were responsible for EPR and LPR in AC. Experimental AC was induced by adoptive transfer system in BALB/c mice. **A**, RW-immunized BALB/c splenocytes (10×10^6) were adoptively transferred into normal BALB/c mice. Subsequent application of RW eye drops was performed 4 days later. Eyes were collected 24 h after RW challenge. The numbers of infiltrating cells were counted in sections with Giemsa stain. Arrows indicate eosinophils. **B**, $CD4^+$ T cells and $CD8^+$ T cells were purified from spleen cells of RW-immunized BALB/c mice and transferred into normal syngeneic mice (5×10^6 cells/mouse). Recipient mice received subsequent RW challenge on day 4 after transfer. Eyes were collected 24 h after RW challenge. Immunohistochemistry was performed using anti-major basic protein Ab, which specifically binds to eosinophilic granules. Brown staining indicates eosinophils. Representative data from two independent experiments are shown. **C**, Mononuclear cells (□), eosinophils (■), and mast cells (▨) were counted in each conjunctiva; averages are shown ($n = 8$ /group). WSP, Whole spleen cells. **D**, OVA-specific Th1 and Th2 cells were generated from splenocytes of DO11.10 TCR Tg mice with a BALB/c background. Syngeneic mice received i.v. injection of the indicated number of Th1 or Th2 cells. Eye drops of OVA peptide 323–339 ($10 \mu\text{g}$) were administered 4 days after the transfer, and eyes were collected 24 h later. Eosinophils were counted in each conjunctiva ($n = 4$ /group). **E**, STAT6 knockout (STAT6^{-/-}) and wild-type (WT) control BALB/c mice were immunized with RW. Mice with and without sensitization were exposed to RW by eye drops. The clinical score in EPR ($n = 4$ /group), the IgE titer in sera, and local cellular infiltration in conjunctiva were evaluated ($n = 8$ /group). Mononuclear cells (□), eosinophils (■), and mast cells (▨) were counted. *, $p < 0.05$; **, $p < 0.01$.

We then examined the involvement of Th2 cells in AC in the absence of a Th2-mediated response. Therefore, EPR and LPR were induced in STAT6-deficient BALB/c mice, in which Th2 cell development was abrogated. RW-induced IgE production was completely abolished in STAT6-deficient mice, and the number of eosinophils in the conjunctiva was drastically reduced (Fig. 2E). Taken together, the data clearly demonstrated that both EPR and LPR in the Ag-induced AC model are regulated by Th2 cells.

Accumulation of SOCS3-expressing cells in the conjunctivas with AC

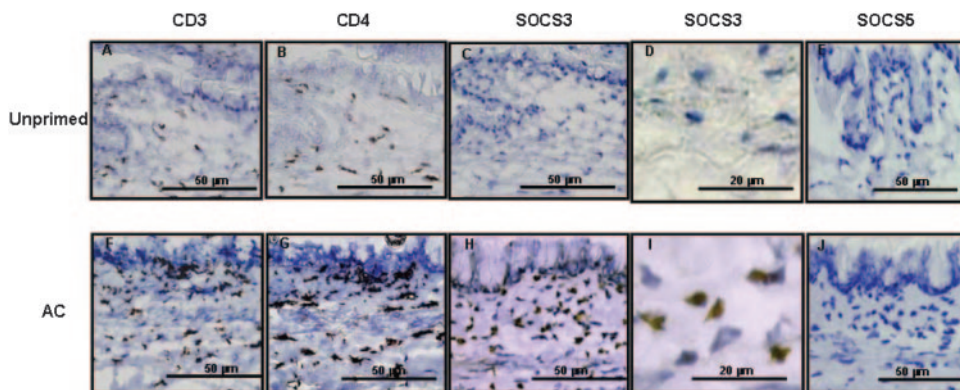
SOCS3 was profoundly expressed in Th2 cells derived from mouse and human T cells (17). Therefore, we next examined SOCS3 and SOCS5 protein expression at the inflammatory site by immunohistochemical analysis. When mice were challenged with eye drops of RW, massive accumulation of $CD3^+$ and $CD4^+$ cells was found in the conjunctivas with AC (Fig. 3). In these challenged mice, SOCS3-expressing, but not SOCS5-expressing, cells

migrated in high numbers into the inflammatory site, whereas unprimed C57BL/6 conjunctiva showed no SOCS3-expressing cells (Fig. 3). The accumulation of SOCS3-expressing cells at the inflammatory site may be coincident with the accumulation of $CD4^+$ T cells. These results suggest that migration of the SOCS3-expressing cells may be implicated in the development and exacerbation of AC.

T cell-specific SOCS3 overexpression causes exacerbation of AC symptoms

Negative regulation of Th1 differentiation by SOCS3 results in enhanced Th2 differentiation, suggesting that constitutive SOCS3 expression in T cells would enhance EPR and LPR in RW-induced AC, which is mainly regulated by the Th2 response. If this were true, down-regulation of the expression or function of SOCS3 protein may block the development and persistence of symptoms in AC. To test these possibilities, we first examined whether constitutive expression of SOCS3 in T cells exacerbates RW-induced AC; thus, EPR and LPR were analyzed in SOCS3 Tg mice with a

FIGURE 3. SOCS3 and SOCS5 expression in the conjunctiva with AC. Experimental AC was induced in C57BL/6 WT mice as described in Fig. 1. Eyes were enucleated 24 h after Ag challenge. Immunohistochemical analysis was performed as described in *Materials and Methods*. Sections were stained with anti-CD3 Ab (A and F), anti-CD4 Ab (B and G), anti-SOCS3 Ab (C, D, H, and I), and anti-SOCS5 Ab (E and J).



C57BL/6 background. Normal C57BL/6 mice showed EPR and LPR similar to those of BALB/c mice (Figs. 1 and 4), whereas SOCS3 Tg mice exhibited 2-fold increases in RW-specific IgE production and clinical score in EPR (Fig. 4A) and 6-fold higher eosinophil infiltration in LPR compared with normal wild-type littermate C57BL/6 mice (Figs. 1 and 4B). Splenocytes from RW-

immunized SOCS3 Tg mice produced significantly higher levels of IL-5 than those from C57BL/6 mice, although IL-4 was not detectable (Fig. 4C). Transfer of T cells from immunized SOCS3 Tg into normal C57BL/6 mice initiated a marked increase in eosinophil infiltration (Fig. 4D), indicating that enhanced SOCS3 expression exacerbated Th2-mediated AC.

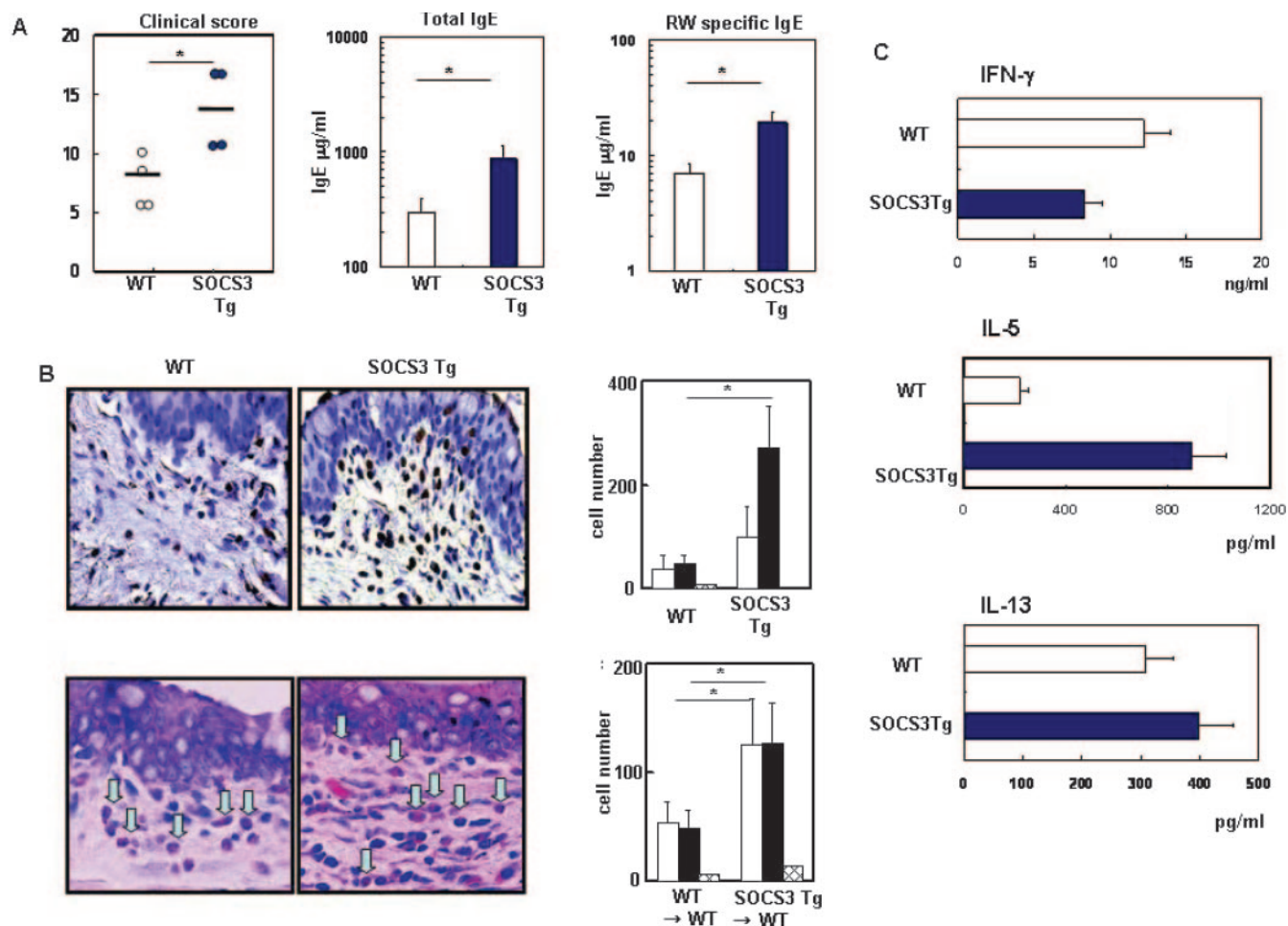


FIGURE 4. Forced expression of SOCS3 on T cell-enhanced incidents in AC. A, SOCS3 Tg and wild-type (WT) mice with a C57BL/6 background were immunized, and experimental AC was induced as described in Fig. 1. EPR was monitored by clinical score, total IgE in sera, and RW-specific IgE. B, Pathological features of conjunctiva 24 h after RW challenge are shown. Immunohistochemistry was performed as described in Fig. 2. Brown staining indicates eosinophils. Mononuclear cells ($\square$), eosinophils ($\blacksquare$), and mast cells ($\boxtimes$) were counted in each conjunctiva ($n = 4/\text{group}$). C, Spleens were harvested from RW-immunized SOCS3 Tg mice and WT mice. These splenocytes were stimulated with RW for 3 days. The amounts of IFN- γ , IL-5, and IL-13 in the supernatants were measured by ELISA. D, CD4 $^{+}$ T cells were purified from RW-immunized splenocytes from SOCS3 Tg and WT mice and transferred into naive WT mice i.v. Recipient mice were challenged with RW on day 4 after transfer. Mononuclear cells ($\square$), eosinophils ($\blacksquare$), and mast cells ($\boxtimes$) were counted in the conjunctiva 24 h after challenge. *, $p < 0.05$; **, $p < 0.01$.

Down-regulation of *SOCS3* expression inhibits development of AC

Next, we explored whether the *SOCS3* expression level in T cells affects IgE production and eosinophil attraction in RW-induced AC using heterozygous *SOCS3*-deficient mice, in which *SOCS3* expression was half that in control mice. The reduction in *SOCS3* expression clearly improved the EPR clinical score in *SOCS3*^{+/-} mice, although IgE production and eosinophil infiltration showed only minor reductions (Fig. 5A).

These results suggest that *SOCS3* expression levels in primed CD4⁺ T cells may affect the severity of LPR in AC. To examine this possibility, RW-immunized CD4⁺ T cells from either *SOCS3*^{+/+} or *SOCS3*^{+/-} mice were adoptively transferred into normal C57BL/6 mice. The RW-immunized *SOCS3*^{+/-} CD4⁺ T cells exhibited increased IFN- γ production, whereas IL-4 and IL-13 production was markedly attenuated (Fig. 6B). Although a slight reduction in eosinophil infiltration was observed in *SOCS3*^{+/-} mice, the CD4⁺ T cells transferred from *SOCS3*^{+/-} mice showed prominent reduction of conjunctival eosinophils (Fig. 5C). These data clearly demonstrate that the severity of both EPR and LPR could be controlled by manipulation of *SOCS3* expression levels in CD4⁺ T cells.

Impairment of *SOCS3* function in T cells inhibits development of AC

We have previously reported that the point mutation of *SOCS3* protein (F25A) in the kinase inhibitory region acted as dominant negative, and that T cell-specific expression of dn*SOCS3* F25A alters the Th cell polarization profile into a Th1-dominant profile (17). Thus, the RW-induced AC model system was examined in F25A Tg mice to determine whether impairment of *SOCS3* function would affect the development of AC. F25A Tg mice with a C57BL/6 background showed significantly attenu-

ated clinical symptoms and IgE production in EPR as well as reduced eosinophil attraction in LPR (Fig. 6A). Splenocytes from RW-immunized F25A Tg mice produced significantly higher levels of IFN- γ and lower levels of IL-5 than those from WT C57BL/6 mice (Fig. 6B). The transfer of immunized F25A T cells into normal C57BL/6 mice resulted in reduced eosinophil infiltration (Fig. 6C). These results clearly suggest that the severity of AC is drastically reduced by inhibition of *SOCS3* function, and that symptoms of Th2-mediated AC could be regulated by *SOCS3* expression in CD4 T cells.

SOCS5 in T cells attenuates eosinophilic infiltration

SOCS5 is a negative regulator of IL-4 signaling through an SH2 domain-independent interaction, and T cells from *SOCS5* Tg mice have impaired Th2 differentiation (18). Because the development of AC is attributed to a Th2-mediated immune response, we postulated that *SOCS5* overexpression in T cells may be effective in reducing AC. Therefore, we examined the symptoms and cytokine profiles in RW-immunized Lck-*SOCS5* T cells. RW-immunized splenocytes from Lck-*SOCS5* Tg mice produced a 7-fold higher amount of IFN- γ compared with normal littermate mice, whereas of the Th2 cytokines, only IL-13 showed a clear reduction (Fig. 7A). No detectable amount of IL-4 was found in either group (data not shown), and there was no difference in IL-5 production (Fig. 7A). Adoptive transfer of RW-immunized *SOCS5* Tg T cells into normal C57BL/6 mice resulted in much less conjunctival eosinophilic infiltration compared with that induced by immunized littermate T cells (Fig. 7B). These results suggest that the development of LPR in AC can be improved with *SOCS5* expression in T cells, most likely via inhibition of the Th2-mediated AC response. Thus, enhancement of the expression of *SOCS5* in CD4⁺ T cells may be a viable therapeutic approach for AC.

Discussion

This murine model for RW-specific AC provides an appropriate system to investigate EPR and LPR in AC, and the adoptive transfer system clearly segregates these two distinct reactions. The present study indicates that allergic ocular disease and the scratching behavior that occurred within 30 min after eye drop administration are regulated by Th2 cytokine-mediated Ag-specific IgE production. This is supported by our previous use of a rat model to demonstrate that transfer of Ag-specific IgE into normal rats induces the characteristic clinical symptoms of EPR, but not eosinophilic infiltration into the inflammatory ocular site, which is apparently increased in LPR. In this study we have also demonstrated that transfer of Ag-primed Th2 cells reconstitutes eosinophilic infiltration into the inflammatory ocular site in LPR, and that the Th2-mediated EPR appears to be indispensable for eosinophil attraction in the LPR of AC.

The Th2-type cytokines, IL-4, IL-5, and IL-13, are known to be involved in many aspects of allergic pathophysiology: IL-4 is essential for IgE production (26, 27), IL-5 is required for eosinophilopoiesis (28), and IL-13 regulates the airway response in asthma (8, 29). The significance of these cytokines and the credibility of the Th2 hypothesis for allergic diseases have been evaluated by antagonistic Abs or other inhibitors in allergic patients and murine model systems. There are a limited number of preliminary reports that support the utility of soluble IL-4R (30, 31) and anti-IL-5 Abs (10, 32) in the treatment of human allergic asthma. The soluble IL-4R shows modest efficacy in the treatment of moderate asthma, whereas treatment with an anti-IL-5 Ab only partially inhibits eosinophilopoiesis, without improving asthma pathogenesis. Treatment with Abs against other Th2 cytokines, including IL-9 (33,

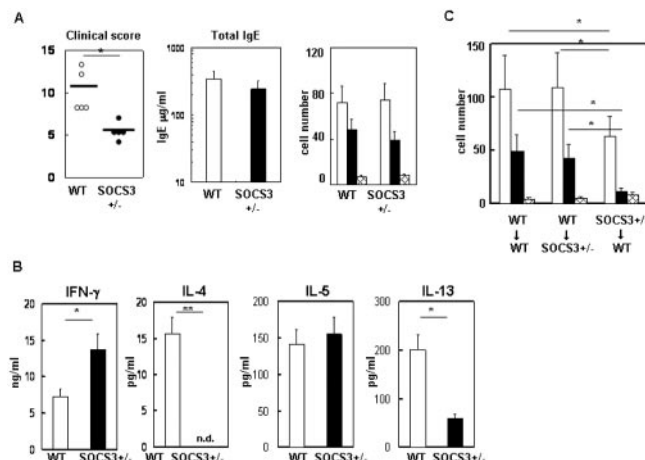
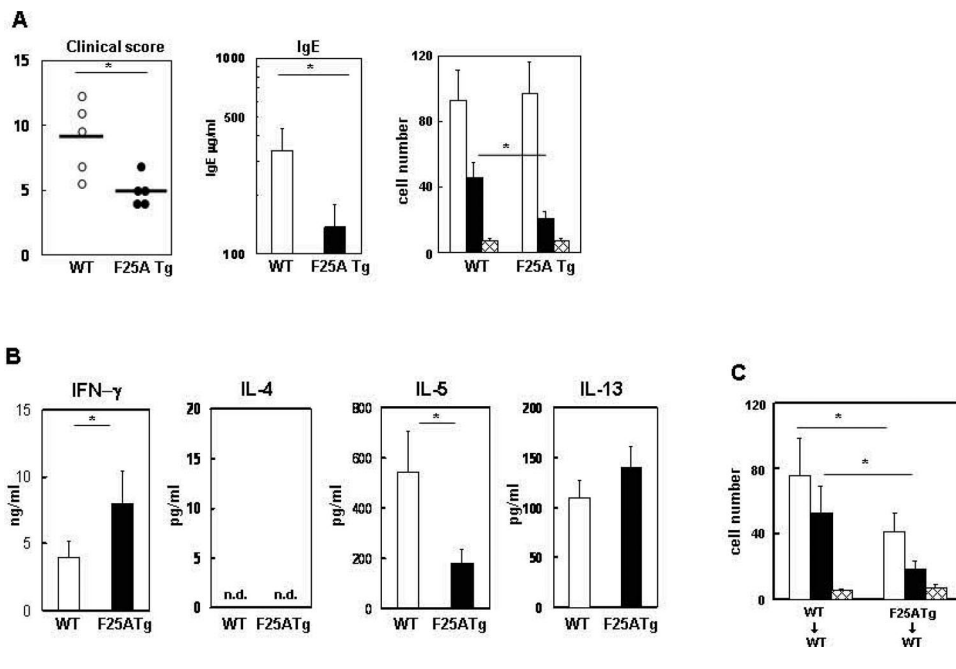


FIGURE 5. Reduction of incidents in AC in heterozygous *Soxs3* mice with a C56BL/6 background. **A**, Experimental AC was induced in *SOCS3*^{+/-} and wild-type (WT) mice as described in Fig. 1. The clinical score in EPR and IgE titer in sera were evaluated. Mononuclear cells (□), eosinophils (■), and mast cells (▨) were counted in the conjunctiva 24 h after the RW challenge ($n = 5$ /group). **B**, RW-immunized splenocytes were stimulated with RW for 3 days, and production of IFN- γ , IL-4, IL-5, and IL-13 against RW was measured by ELISA. **C**, CD4⁺ T cells were purified from spleen cells from RW-immunized *SOCS3*^{+/-} mice. Immunized CD4⁺ T cells (5×10^6) were transferred from WT to WT, WT to *SOCS3*^{+/-}, or *SOCS3*^{+/-} to WT recipients, and LPR was evaluated by cellular infiltration in the conjunctiva 24 h after the local RW exposure. Mononuclear cells (□), eosinophils (■), and mast cells (▨) were counted in each conjunctiva ($n = 5$ /group). *, $p < 0.05$; **, $p < 0.01$.

FIGURE 6. Overexpression of dnSOCS3 and F25A and decreased EPR and LPR in AC. **A**, Experimental AC was induced in F25A Tg and wild-type (WT) mice with a C57BL/6 background as described in Fig. 1. Clinical score in EPR and IgE in the sera were evaluated. Mononuclear cells (■), eosinophils (□), and mast cells (▨) were counted in the conjunctiva 24 h after the RW challenge. **B**, RW-immunized splenocytes were stimulated with RW, and the productions of IFN- γ , IL-4, IL-5, and IL-13 in supernatants were compared between WT and F25A Tg mice. **C**, CD4⁺ T cells were purified from RW-immunized splenocytes and transferred (5×10^6) from WT to WT or from F25A Tg into WT. Recipient animals were treated with RW eye drops. LPR was evaluated by cellular infiltration in the conjunctiva 24 h after local RW exposure. Mononuclear cells (□), eosinophils (■), and mast cells (▨) were counted in each conjunctiva ($n = 5$ /group). *, $p < 0.05$.



34), IL-10 (35), and IL-13 (36), also appears to be insufficient for significant improvement of allergic asthma model in mice. In a murine model of asthma and AD, IgE production was abrogated in the absence of IL-4- and IL-13-mediated signaling, but eosinophil infiltration and characteristic clinical symptoms were not affected. Similarly, STAT6-deficient NC/Nga mice do not show the reduction of AD skin lesion development (37). Therefore, allergic asthma and AD are complex disorders that involve mutual Th1 and Th2 cytokine responses, and the significance of Th2 cytokines continues to remain inconclusive.

The present work addressed the more prominent role of Th2 cells in the development of EPR and LPR in AC. This role was clearly demonstrated by two independent experiments: one in which STAT6-deficient mice showed a marked reduction of IgE production, AC-specific clinical symptoms, and eosinophilic infiltration (Fig. 2E), and a second in which transfer of OVA-primed Th2 cells reconstituted eosinophila (Fig. 2D). This idea is consistent with previous reports that suggest that IL-4 and IL-13 expression levels are elevated in tears and cytology samples from AC patients (38). Similarly, results from a murine AC model suggest

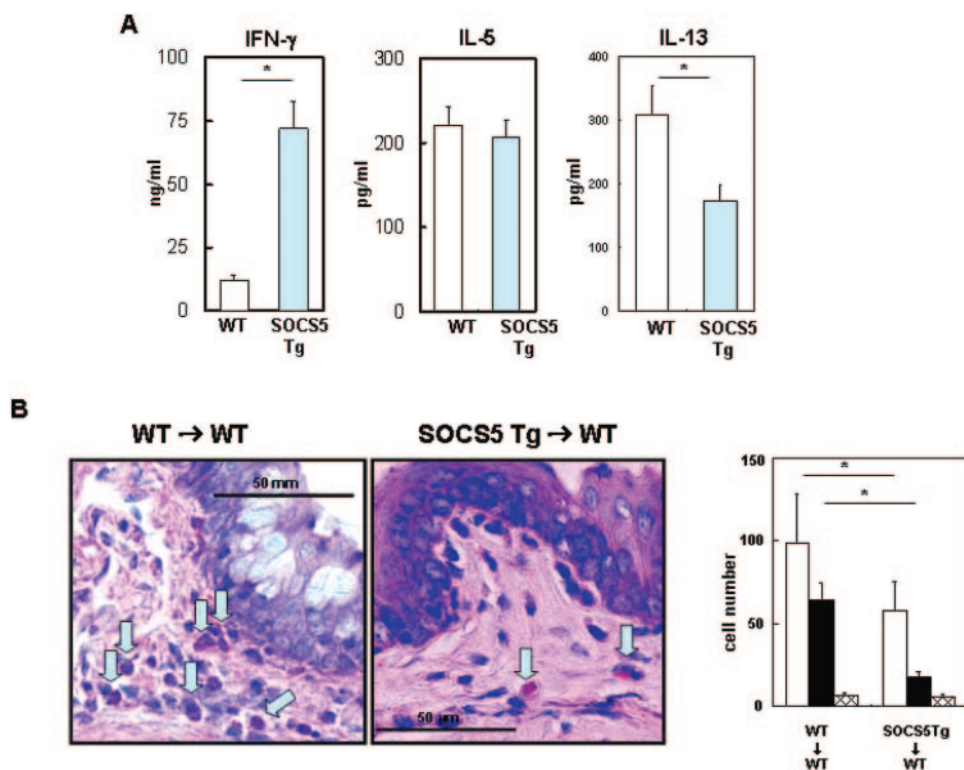


FIGURE 7. Forced expression of SOCS5 in CD4⁺ T cells attenuates AC. **A**, SOCS5 Tg and wild-type (WT) mice with a C57BL/6 background were immunized with RW. Spleens were harvested, and cytokine production of splenocytes against RW stimulation was tested. The amounts of IFN- γ , IL-5, and IL-13 in the supernatant were measured by ELISA. **B**, CD4⁺ T cells were purified from RW-immunized mice. Immunized CD4⁺ T cells (5×10^6) were transferred from WT to WT or from SOCS5 Tg to WT mice. **B**, Pathological features of conjunctiva 24 h after RW eye drop administration are shown. Cellular infiltration was evaluated with sections with Giemsa stain. The arrow indicates an eosinophil. Mononuclear cells (□), eosinophils (■), and mast cells (▨) were counted in each conjunctiva. *, $p < 0.05$. Representative data shown are the average of three experiments ($n = 8$).

that IL-4-deficient mice have reduced AC, whereas IFN- γ -deficient mice show massive eosinophil infiltration (11). However, this model system also provided controversial evidence that IL-12 deficiency and anti-IL-12 mAb treatment lead to reduction of eosinophil infiltration into conjunctiva. IL-12 is known to be a potent inducer of the classic Th1 cytokine, IFN- γ , and also to act as a strong inhibitor of Th2 polarization (39). Our previous results using a rat AC model have shown that treatment with rIL-12 induces striking infiltration of mononuclear cells at the challenged site (5), and similar results have been reported by another group in a mouse AC model (11), suggesting the possibility that IL-12 may have an uncharacterized and unexpected role in enhancing the inflammatory responses in ocular allergy.

The cytokine negative regulator, SOCS3, binds to a cytoplasmic region of the IL-12R β 2-chain and inhibits IL-12-mediated STAT4 activation (17). Therefore, we expected that constitutive expression of SOCS3 would inhibit infiltration of eosinophils in the LPR in AC. However, SOCS3 Tg mice showed clear enhancement of both EPR and LPR in AC; consistent with this, heterozygous deletion of the *Sox3* gene and overexpression of a dnSOCS3 were effective in prevention of EPR and LPR (Figs. 5 and 6). We have previously shown that excess expression of SOCS3 in CD4⁺ T cells leads to enhanced IL-4 and impaired IFN- γ production, indicative of a bias to Th2 differentiation (17). Because the expression of SOCS3 in CD4 T cells was much higher than that in allergic patients, SOCS3 expression is tightly associated with the severity of allergic asthma and dermatitis. The present data show that this is also the case in AC; SOCS3 expression levels were specifically increased at the inflammatory site in conjunctiva (Fig. 3). Therefore, the present results indicate that SOCS3-mediated inhibition of IL-12 signaling appears to regulate the balance of Th cell development, rather than inhibit the IL-12-mediated inflammatory response in the LPR. It is well documented that the expression and function of the IL-12R are clearly impaired in Th2 cells (40); thus, IL-12 is unlikely to be associated with Th2-mediated eosinophil attraction.

SOCS5 is known to be a potential negative regulator of IL-4 signaling through a nontyrosine-based interaction with the IL-4R. We have reported that T cell-specific forced expression of SOCS5 clearly blocks Th2 differentiation (18). However, the observation with SOCS5-deficient CD4⁺ T cells suggest that endogenous expression levels of SOCS5 may not be crucial for regulation of the balance of Th cell cytokine production (19). These controversial results might be elucidated by low expression of SOCS5 during priming with a particular Ag. Transfer of CD4⁺ T cells that constitutively expressed SOCS5 clearly enhanced IFN- γ production and decreased IL-13 production and eosinophilic infiltration in the conjunctiva (Fig. 7). Therefore, in terms of regulating the balance of Th cells, SOCS5 has a similar beneficial effect for dnSOCS3. These results seem to indicate that unlike other allergic diseases, the pathology of AC is strictly regulated by an IL-4-mediated Th2 immune response. The down-regulation of Th2 dominance in the EPR by T cell-specific expression of SOCS5 or dnSOCS3 may therefore be a possible strategy for LPR control in ocular allergic disease.

Corticosteroids and mast cell stabilizers are currently the main pharmacological strategy for treatment of AC patients, but neither of these approaches has sufficient efficacy without leading to unpleasant side effects. Thus, many kinds of immunomodulatory and antimediator therapies have been investigated, but none of them has proved successful for AC. The present results support the idea that modification of SOCS3 and SOCS5 function or expression, using pharmacological, antisense, or DNA vaccine approaches,

might be a novel therapeutic strategy for ocular allergic diseases such as AC.

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Disclosures

The authors have no financial conflict of interest.

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