

Roles of CD4+CD25+ T cells in the development of experimental murine allergic conjunctivitis

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Abstract

Background CD25+ regulatory T (T reg) cells play a suppressive role in experimental autoimmune uveoretinitis as well as experimental airway inflammation but their involvement in the development of allergic conjunctivitis (AC) remains unclear. We therefore investigated whether T reg cells play a role in the development of experimental AC (EC).

Methods BALB/c and C57BL/6 mice were actively immunized with ragweed (RW). The mice were treated with an anti-CD25 Ab (PC61) or control normal rat IgG (nrIgG) either 2 days prior to active immunization or during the induction phase (days 0, 2, 4, 6 and 8). Ten days after active immunization, the mice were challenged with RW-containing drops. Twenty-four hours after the challenge, the conjunctivas were harvested for histological analysis of eosinophil infiltration, and the spleens were harvested for cell culture for splenocyte transfer. Cultured splenocytes

were transferred into syngeneic mice, and 4 days after the transfer, the recipient mice were challenged with RW. Twenty-four hours after the challenge, conjunctivas were collected for histological analysis.

Results Pretreatment with PC61 did not affect EC in either strain of mice; however, treatment with PC61 during the induction phase significantly suppressed EC in C57BL/6 mice. In contrast, transfer of RW-primed splenocytes from mice treated with PC61 induced EC that was significantly more severe regardless of strain and treatment protocol.

Conclusions The finding that T reg cells play a suppressive role in the development of EC in splenocyte transfer experiments suggests that modulation of T reg cells may be a possible therapy for AC.

Keywords Allergic conjunctivitis · Effector T cell · Mouse · Regulatory T cell

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Introduction

Patients with allergic conjunctivitis (AC), which affects 30% of the population in the Western world, suffer from itching, tearing, and ocular hyperemia [3]. The most severe type of AC is vernal keratoconjunctivitis (VKC), which is characterized by the formation of giant papillae and can lead to corneal damage and vision loss [4]. Eosinophils are thought to be a predominant cause of tissue damage in VKC [4]. Although AC is an Ag-specific disease, eosinophils do not have Ag-specific receptors. Therefore, our group has focused on T cells to elucidate the mechanism by which VKC develops by using rat and mouse models of AC, which we call experimental immune-mediated blepharoconjunctivitis (EC) [32]. In Brown Norway rats, adoptive transfer of Ag-specific T cells induces EC

following a challenge with eye drops containing the Ag [6, 7]. In Balb/c mice, adoptive transfer of Ag-specific Th2 cells, but not Th1 cells, is able to induce the infiltration of eosinophils into the conjunctiva [23]. In addition, transfer of Ag-specific IgE is less potent at inducing eosinophil infiltration into the conjunctiva than transfer of Ag-primed splenocytes [8]. These data indicate that EC is a Th2-mediated disease.

We have also attempted to investigate how EC is regulated. One approach is the modulation of Th1 and Th2 cytokines by injection of recombinant cytokines or by using cytokine-deficient mice. In mice deficient in IL-12, a Th1-polarizing cytokine, significantly less conjunctival inflammation was observed when EC was induced [21]. In addition, endogenous IFN- γ , a typical Th1 cytokine, is positively involved in the development of EC in Balb/c mice [8]. These data suggest that EC is not regulated by a single cytokine. Another approach is to modulate costimulatory signals by treating mice with Abs specific to costimulatory molecules. Agonistic stimulation with 4-1BB (CD137) significantly reduces Th2 cytokine production by splenocytes and attenuated eosinophil infiltration into the conjunctiva [9]. In addition, agonistic stimulation with OX40 (CD134) significantly upregulates Th2 cytokine production by splenocytes and augments eosinophil infiltration into the conjunctiva [11]. These data suggest that modulation of costimulatory signals might be a possible therapy for AC.

Recently, a population of T cells, the so-called regulatory T (T reg) cells, has been shown to inhibit T-cell proliferation and effector function [5, 24]. T reg cells can be divided into two subsets: CD4+CD25+ thymus-selected naturally occurring T reg cells and inducible T reg cells, which produce immunosuppressive cytokines such as IL-10 and TGF- β [18]. There have been many reports demonstrating that CD4+CD25+ T reg cells play a regulatory role in the development of experimental autoimmune diseases [27]. However, only a small number of publications have examined their role in experimental allergic diseases. Previous studies using DO11.10 T-cell-receptor transgenic mice showed that CD4+CD25+ T reg cells can prevent allergic airway inflammation [1, 13, 15, 16, 29]. More recently, CD4+CD25+ T reg cells were demonstrated to suppress airway inflammation in wild-type immunocompetent mice by modulating pulmonary dendritic cell phenotype and function [19]. However, we have found mechanistic differences between experimental airway inflammation and EC. For instance, the interaction between inducible co-stimulator (ICOS) and its ligand B7-related protein-1 (B7RP-1) is not essential for the development of EC [10], while this interaction is important in the development of airway inflammation during the effector phase [31]. In addition, although airway inflammation involves mutual Th1 and Th2 cytokine responses [17],

Th2 cells play a more prominent role in the development of EC [23]. Thus, there is the possibility that CD4+CD25+ T reg cells have different roles in the development of EC and airway inflammation. To clarify this issue, we examined the roles of CD4+CD25+ T reg cells in the development of EC.

Materials and methods

Mice

Inbred BALB/c and C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). The mice were kept in pathogen-free conditions at the animal facility of Kochi Medical School and used when they were 6 to 12 weeks old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The number of mice used in each experimental group is described in the figure legends.

EC induction by active immunization and treatment with Abs

Short ragweed pollen (RW) from Polysciences, Inc. (Warrington, PA) adsorbed on alum (Sigma, St. Louis, MO) was injected into the left hind-footpad and the tail base. Fifty microliters of the emulsion (50 μ g of RW and 2 mg of alum) was injected into each site. The mice ($n=10$ per group) were injected intraperitoneally with 300 μ g of rat mAbs against CD25 (PC61, rat IgG1) [20, 22] or control rat IgG (MP Biomedicals Inc., Aurora, OH) on days 0, 2, 4, 6, and 8 after immunization (induction-phase treatment). In a separate experiment the mice were injected intraperitoneally with 1.5 mg of each Ab ($n=10$ per group) 2 days before being actively immunized as above (pretreatment). Ten days after the active immunization, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes were harvested for histological analysis; sera were obtained for measurement of Ig levels; and spleens were harvested to culture splenocytes for transfer and cytokine or proliferation assays.

EC induction by adoptive transfer of splenocytes from actively immunized and Ab-treated mice

Splenocytes harvested from mice that received either induction-phase treatment or pretreatment were cultured with RW extract (LSL Co., Tokyo, Japan) 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 (FCS)

from ICN Biomedical Japan Co. (Tokyo, Japan), 2-mercaptoethanol (2-ME, 5×10^{-5} M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). After incubation for 72 hours at 37°C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were injected intraperitoneally into naïve syngeneic mice ($n=8$ per group). Four days after the transfer, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 µl per eye). Twenty-four hours later, the eyes were harvested for histological analysis.

EC induction by adoptive transfer of in vitro-stimulated RW-primed splenocytes that were or were not depleted of CD25+ cells

Naïve mice were immunized with RW in both their left hind footpad and tail base and were not treated with any Abs. Ten days later, splenocytes were prepared and cultured with RW as described in the previous section. After incubation for 72 hours at 37°C in a humidified atmosphere with 5% CO₂, splenocytes were collected and treated with an anti-CD25 Ab (7D4, rat IgM) followed by treatment with complement (Cedarlane, Ontario, Canada) to deplete CD25+ cells. As a control, the splenocytes were treated with complement alone. After the transferring splenocyte number was adjusted so that each group would receive the same number of CD4+ cells (equivalent to 2×10^7 splenocytes in complement-treated group), the splenocytes were injected intraperitoneally into syngeneic naïve BALB/c mice. On day 4, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 µl per 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-µm-thick sections were cut and stained with Giemsa and sections from the central portion of the eye, including pupil and optic nerve head, were examined. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by two masked observers. Data are presented as conjunctival eosinophils per slide $\pm$ SEM of all the mice examined.

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. Cells were stimulated with RW extract at a final concentration of 25 µg/ml or with concanavalin A (Con A) at 5 µg/ml. After a 72-hour incubation at 37°C in a humidified

atmosphere containing 5% CO₂, the cultures were pulsed for 16 hours with 0.5 µCi/well [³H] thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested and the incorporated radioactivity was measured using standard techniques. The data are expressed as counts per minute (cpm).

Measurement of total and RW-specific Igs in serum

Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. Total serum Ig levels were assessed using ELISA, as detailed in [10], and the data are presented as concentrations of Igs. To assess RW-specific Ig levels in the serum, EIA plates (Costar, Corning, NY) were coated with RW extract (1 µg/ml) at 4°C overnight. After blocking with 1% bovine serum albumin in PBS, serum samples were added and incubated for 2 hours at room temperature or overnight at 4°C. We used the quantified RW-specific IgE (kindly provided by Prof. Fu-Tong Liu) and pooled hyperimmune serum as controls. The plates were washed with PBS plus 0.05% Tween 20 (PBS/T) (Wako, Osaka, Japan) and incubated for 2 hours at room temperature with alkaline phosphatase (ALP)-conjugated goat Ab specific for IgG₁ or IgG_{2a} (Zymed, San Francisco, CA). To detect IgE, biotin-conjugated rat anti-mouse IgE (BD Biosciences, Franklin Lakes, NJ) was added to each well for 2 hours at room temperature. After washing, avidin-ALP (Sigma) was added to each well for 1 hour. 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 minutes. Absorbance was measured at 405 nm. IgG1 and IgG2a concentrations are expressed as units (U)/ml relative to the pooled hyperimmune serum, whereas IgE concentrations are expressed as ng/ml.

Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 hours with RW extract (25 µg/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. The levels of IL-2, IL-4, IL-5 and IFN-γ produced were measured using commercially available cytokine kits (Duoset, R&D Systems, Minneapolis, MN) or the Bioplex system (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations.

Flow cytometric analysis

Freshly isolated or in vitro-stimulated splenocytes were incubated for 30 minutes on ice with FITC-labeled anti-mouse CD4 Ab (GK1.5, eBioscience, San Diego, CA).

After washing with cold 2% FCS-PBS, the cells were incubated with biotin-labeled anti-CD25 Ab (PC61, eBioscience) followed by incubation with streptavidin-PE (BD Biosciences, Franklin Lakes, NJ). Then, the cells were washed with cold 2% FCS-PBS and were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Dead cells were excluded using the viability dye 7-amino-actinomycin D (7-AAD). Acquisition and analysis were performed using CellQuest software. In Figs. 2, 3, 4, one representative dot-plot is shown from each group. In addition, mean % $\pm$ SEM of CD25-bright and -dim CD4⁺ cells is presented.

Statistical analysis

Serum Ig levels, splenocyte proliferation, the concentrations of cytokines in the culture supernatants and infiltrating eosinophil numbers were compared using Student's t-test.

Results

Treatment with PC61 during the induction phase does not affect actively-induced EC in BALB/c mice

To investigate the roles of CD4⁺CD25⁺ T cells in the development of EC during the induction phase, actively immunized BALB/c mice were treated with PC61 or control nrIgG every other day from the day of immunization to day 8. On day 10, the mice were challenged with RW-containing eye drops and 24 hours after the challenge, the conjunctivas were harvested to evaluate eosinophil infiltration into the conjunctiva. Slightly fewer eosinophils infiltrated into the conjunctiva of PC61-treated mice (21.6 ± 4.2) than those of nrIgG-treated mice (31.6 ± 4.7), but the difference did not reach statistical significance.

Effects of PC61 treatment on systemic immune responses

To examine whether PC61 treatment during the induction phase affected systemic immune responses, we evaluated both cellular and humoral immune responses. In flow cytometric analyses of splenocytes, only the number of CD25⁺ cells differed significantly between the two groups ($2.3 \pm 0.31\%$ in the nrIgG-treated group and $0.95 \pm 0.17\%$ in the PC61-treated group, $p < 0.01$). The proliferation of splenocytes in response to stimulation with RW was slightly, but not significantly, higher in the PC61-treated group, while Con A-induced proliferation was significantly higher in the PC61-treated group (Fig. 1a). Splenocytes from PC61-treated mice produced similar amounts of IL-4

Fig. 1 Effects of PC61 treatment during the induction phase on systemic immune responses in BALB/c mice. EC was induced in BALB/c mice by active immunization and these mice were treated with Abs during the induction phase ($n=10$ per group), and spleens were harvested for proliferation (a) or cytokine (b) assays and sera were harvested for measurement of Ig levels (c). (a) RW-induced proliferation was not different between the two groups, but PC61-treated splenocytes proliferated significantly more when stimulated with Con A than nrIgG-treated splenocytes. Data are presented as cpm; background cpm were 751 ± 51 in the nrIgG-treated group and 772 ± 60 in the PC61-treated group. (b) Following RW stimulation, IL-4 and IL-5 production were not different between the two groups, whereas PC61-treated splenocytes produced more IL-2 and IFN- γ . (c) Total serum IgG1 levels were significantly lower in PC61-treated mice, whereas the levels of other Igs did not differ significantly. ** $p < 0.05$, * $p < 0.01$

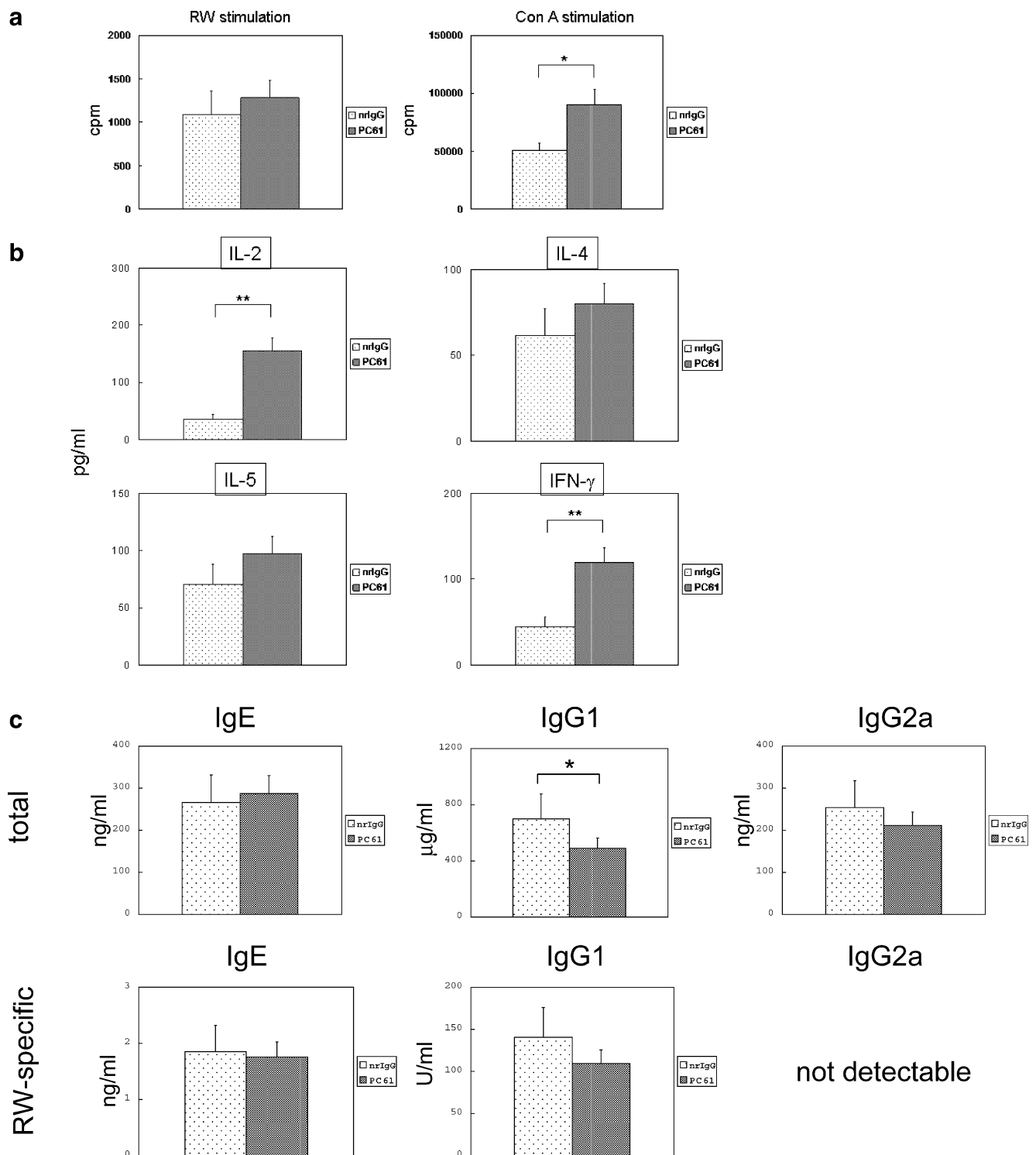
and IL-5 but significantly more IL-2 and IFN- γ following RW stimulation in vitro (Fig. 1b). Total IgG1 levels in the serum were significantly lower in the PC61-treated group, whereas total IgE and IgG2a and RW-specific IgE and IgG1 were not different between the two groups (Fig. 1c). Thus, PC61 treatment during the induction phase affected systemic immune responses, although not profoundly.

BALB/c mice receiving splenocytes from PC61-treated mice have EC that is significantly more severe than that in mice receiving splenocytes from nrIgG-treated mice

To further investigate whether PC61 treatment in vivo affects CD4⁺CD25⁺ T reg cells, we cultured splenocytes from PC61-treated mice with RW in vitro and then transferred them into syngeneic recipient mice. Flow cytometric analyses demonstrated that the percentage of CD25-bright CD4⁺ T cells, which could be considered to be T reg cells [2, 18], made up $4.86 \pm 0.76\%$ and $1.38 \pm 0.24\%$ of the transferring splenocytes in the nrIgG-treated and PC61-treated groups, respectively. CD25-dim CD4⁺ T cells, which could be considered to be effector T cells [2, 18], made up $2.26 \pm 0.39\%$ and $1.51 \pm 0.36\%$ in the transferring splenocytes from the nrIgG-treated and PC61-treated groups, respectively (Fig. 2a). Transfer of these in vitro-stimulated splenocytes from PC61-treated mice induced EC that was significantly more severe than that induced by splenocytes from nrIgG-treated mice (Fig. 2b).

Pretreatment with PC61 in vivo does not affect EC in BALB/c mice

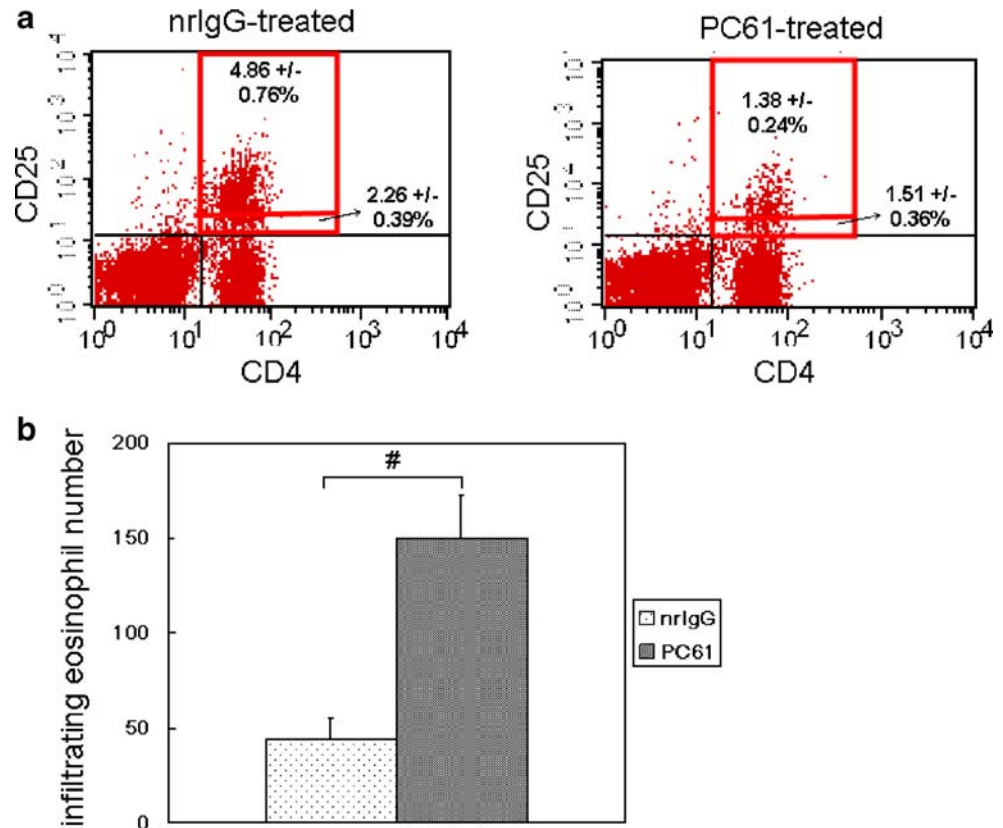
We showed above that PC61 treatment in vivo during the induction phase influences systemic immune responses but does not affect EC; however, the data in Fig. 2 suggest that T reg cells are likely to be affected by this in vivo treatment. This discrepancy may be because PC61 treatment affects not only T reg cells but also CD25-expressing



effector T cells [25]. Therefore, we next treated BALB/c mice with PC61 two days prior to active immunization; in a model of airway inflammation, this protocol efficiently depleted CD4⁺CD25⁺ T reg cells and upregulated airway inflammation [19]. Similar to the results we obtained when treatment was conducted during the induction phase, pretreatment with PC61 did not significantly affect EC

(Fig. 3a). The lack of effects on EC may be explained by the fact that the depleting Ab is not tracked longitudinally and worn off in the 48 hours prior to RW immunization. However, this possibility is less likely since depletion was noted in both the CD25-bright and -dim CD4⁺ compartments of splenocytes from PC61-treated mice (Fig. 3b). Transfer of in vitro-stimulated splenocytes from the mice

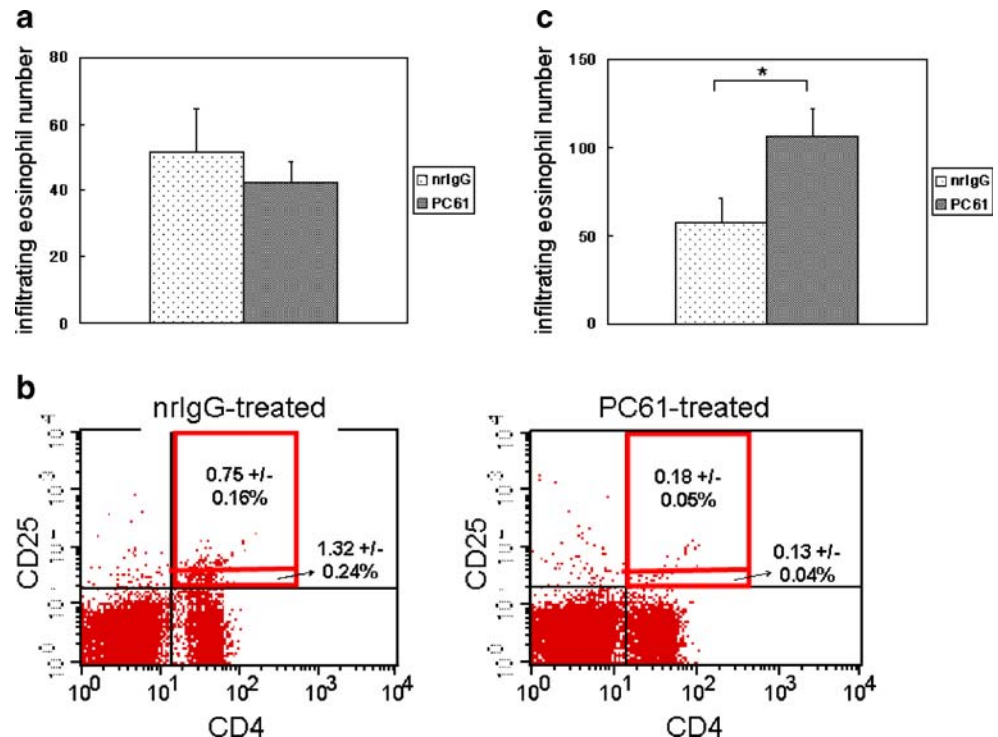
Fig. 2 Increased eosinophilic infiltration is observed in BALB/c mice upon adoptive transfer of splenocytes from mice treated with PC61 during the induction phase. EC was induced by transfer of RW-primed splenocytes from mice treated with Abs during the induction phase. (a) Flow cytometric analysis before splenocyte transfer. One representative dot-plot data together with mean $\pm$ SEM is shown. (b) Significantly more eosinophils infiltrated the conjunctivas in mice that received PC61-treated splenocytes than in mice that received nrIgG-treated splenocytes. Data are presented as the mean number of eosinophils per slide $\pm$ SEM. $p < 0.001$, $n = 8$ per group



pretreated with PC61 induced significantly more eosinophils than splenocytes pretreated with nrIgG (Fig. 3c). The mean percentages of CD25-bright and -dim CD4⁺ cells in

the transferred splenocytes were 5.92 ± 1.16 and 0.90 ± 0.21 in the nrIgG-treated group and 2.31 ± 0.34 and 0.36 ± 0.08 in the PC61-treated group.

Fig. 3 Effect of pretreatment with PC61 on EC in BALB/c mice. EC was induced in BALB/c mice that had been treated with Abs two days prior to active immunization. (a) Eosinophil infiltration was not significantly different between the groups of mice receiving PC61 and nrIgG ($n = 10$ per group). (b) Flow cytometric analysis. One representative dot-plot together with mean $\pm$ SEM is shown. (c) After splenocyte transfer, significantly more eosinophils infiltrated the conjunctivas in mice that received PC61-treated splenocytes than in mice that received nrIgG-treated splenocytes ($n = 8$ per group). Data are presented as the mean number of eosinophils per slide $\pm$ SEM. $*p < 0.05$



Treatment with PC61 in vivo does not affect EC in C57BL/6 mice

As shown above, PC61 treatment in vivo did not significantly affect the EC induced by active immunization in BALB/c mice. In a previous report, PC61 treatment in vivo upregulated airway inflammation more in a resistant strain than in a susceptible strain of mice [19]. We have previously shown that the BALB/c strain is more susceptible to EC than the C57BL/6 strain [12]. Therefore, we next examined whether treatment with PC61 in vivo affects EC in EC-resistant C57BL/6 mice. Treatment with PC61 during the induction phase significantly decreased eosinophil infiltration (Fig. 4a). However, similar to what we found in BALB/c mice, treatment with PC61 prior to active immunization did not significantly affect eosinophil infiltration into the conjunctiva (Fig. 4b). Figure 4c represents the typical flow cytometric data of CD25-bright and -dim CD4⁺ cells in the freshly isolated splenocytes from the mice treated with Abs prior to active immunization. Both the CD25-bright and -dim CD4⁺ compartments appear to be depleted (Fig. 4c).

C57BL/6 mice receiving splenocytes from PC61-treated mice develop EC that is significantly more severe than that in mice receiving splenocytes from nrIgG-treated mice

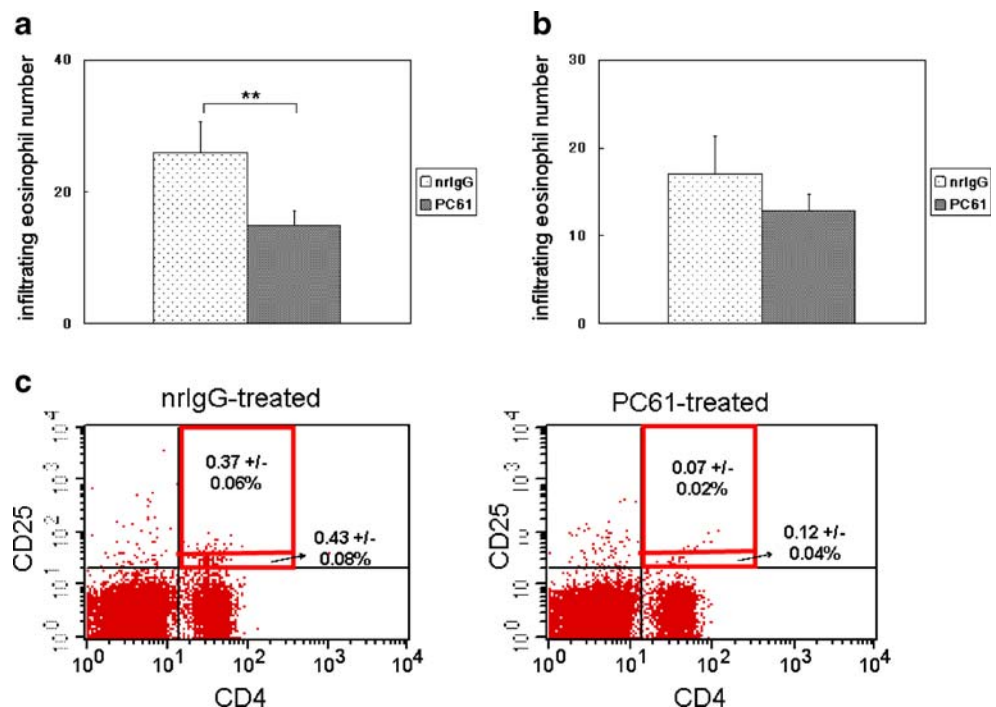
Next, to examine whether PC61 treatment in vivo affects CD4⁺CD25⁺ T reg cells in C57BL/6 mice, as it does in BALB/c mice, we cultured nrIgG- or PC61-treated splenocytes with RW in vitro and then transferred them into

syngeneic recipient mice. When the splenocytes were prepared from mice treated with Abs during the induction phase, CD25-bright and -dim CD4⁺ cells represented $2.80 \pm 0.52\%$ and $0.90 \pm 0.20\%$ of transferred nrIgG-treated splenocytes and $1.00 \pm 0.22\%$ and $0.62 \pm 0.13\%$ of transferred PC61-treated splenocytes. Higher numbers of eosinophils infiltrated the conjunctivas in recipients of splenocytes from mice treated with PC61 during the induction phase than from those treated with nrIgG, although the difference did not reach statistical significance (Fig. 5a). When the splenocytes were prepared from mice treated with Abs prior to active immunization, CD25-bright and -dim CD4⁺ cells represented $4.70 \pm 0.97\%$ and $0.94 \pm 0.19\%$ of transferred nrIgG-treated splenocytes and $1.83 \pm 0.41\%$ and $0.79 \pm 0.15\%$ of transferred PC61-treated splenocytes. Significantly more eosinophils infiltrated the conjunctivas in recipients of splenocytes from PC61-treated mice than in recipients of splenocytes from nrIgG-treated mice (Fig. 5b).

Depletion of CD25⁺ cells from transferred splenocytes abrogates EC in BALB/c mice

Finally, to examine whether the CD25⁺ cell population contains EC-inducible effector cells, we used an anti-CD25 Ab (7D4) and complement to deplete CD25⁺ cells from splenocytes obtained from RW-primed mice not treated with Abs. The CD25-depleted splenocytes or complement-treated control splenocytes were injected into the syngeneic recipient mice after adjusting the number of CD4⁺ cells to be equal in each group. The infiltration of eosinophils in the recipients of CD25-depleted splenocytes was the same as

Fig. 4 Effects of PC61 treatment either during the induction phase or prior to active immunization on EC induced by active immunization in C57BL/6 mice. EC was induced in C57BL/6 mice by active immunization with RW followed by RW challenge with RW-containing eye drops. The mice were treated with nrIgG or PC61 either during the induction phase (a, $n=10$ per group) or prior to active immunization (b, $n=10$ per group). (a) Significantly fewer eosinophils infiltrated the conjunctivas of mice treated with PC61 during the induction phase ($**p<0.01$). (b) Pretreatment with PC61 did not significantly affect eosinophil infiltration into the conjunctiva. Data are expressed as the mean $\pm$ SEM. (c) Flow cytometric analysis. One representative dot-plot together with mean $\pm$ SEM is shown



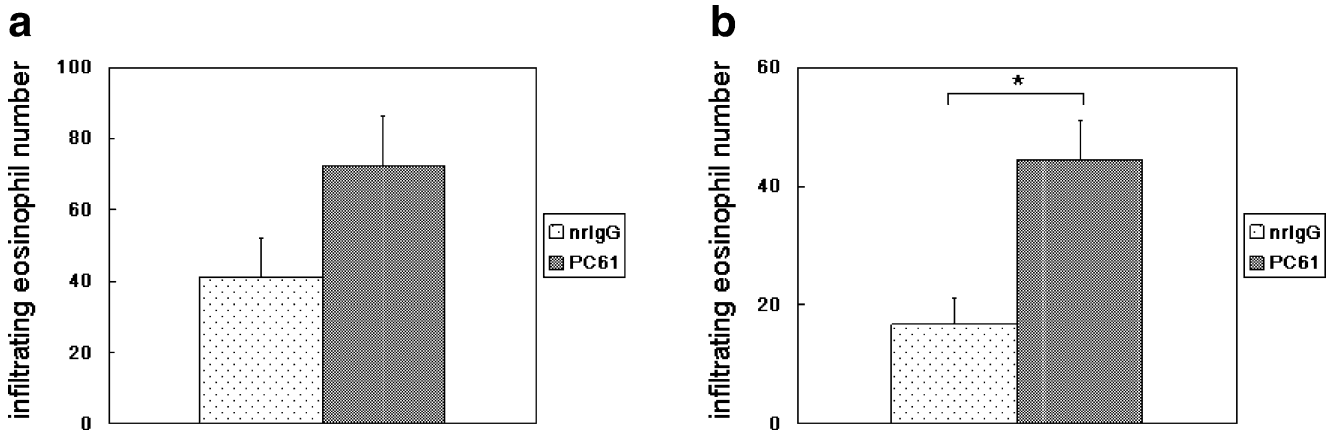


Fig. 5 Increased eosinophilic infiltration is observed in C57BL/6 mice upon adoptive transfer of splenocytes from mice treated with PC61. EC was induced by transfer of RW-primed splenocytes from mice treated with nrlgG or PC61 either during the induction phase (**a**, $n=8$) or prior to active immunization (**b**, $n=8$). More eosinophils infiltrated

the conjunctivas of mice that received PC61-treated splenocytes than nrlgG-treated splenocytes regardless of the experimental protocol, although the difference was only statistically significant when PC61 treatment was performed prior to active immunization (**b**, $*p<0.05$)

that in naïve mice (Fig. 6). In contrast, significantly more eosinophils infiltrated the conjunctivas of mice that received complement-treated splenocytes than that which received CD25-depleted splenocytes (Fig. 6).

Discussion

The initial hypothesis of this study was that depletion of CD25+ cells in vivo would potentiate EC, similar to the effect in mouse experimental airway inflammation, but contrary to our expectation, pretreatment with PC61, an anti-CD25 Ab, did not affect EC. Rather, we found that depletion of CD25+ cells in vivo either before immunization or during the induction phase suppressed EC only slightly in both BALB/c and C57BL/6 mice and the suppression was only significant in C57BL/6 mice treated with PC61 during the induction phase. More strikingly, transfer of splenocytes from mice treated with PC61 in vivo did induce EC that was significantly more severe than that induced by nrlgG-treated splenocytes in both strains of mice.

There are several possible explanations for the discrepancy between EC and experimental airway inflammation; one is that we did not deplete CD4+CD25+ T reg cells completely enough to abolish the suppressive effects of T reg cells and increase EC; a second is that CD25+ T reg cells play less a role in the development of EC than in airway inflammation; and a third is that PC61 treatment depletes not only T reg cells but also effector T cells.

Depletion of CD25+ T reg cells was not complete in this study, and CD4+CD25+ cells represented 0.61% of splenocytes after PC61 treatment in BALB/c mice and

0.19% in C57BL/6 mice, despite using a 1.5-fold higher dose of PC61 in our sensitization protocol than used in the previous report on airway inflammation [19], and the same treatment time point (2 days prior to active immunization). The depletion efficiency in the current study is similar to that seen in a previous report on experimental autoimmune uveoretinitis, in which 0.63% of splenocytes in the thymectomized mice were CD25+ T reg cells after

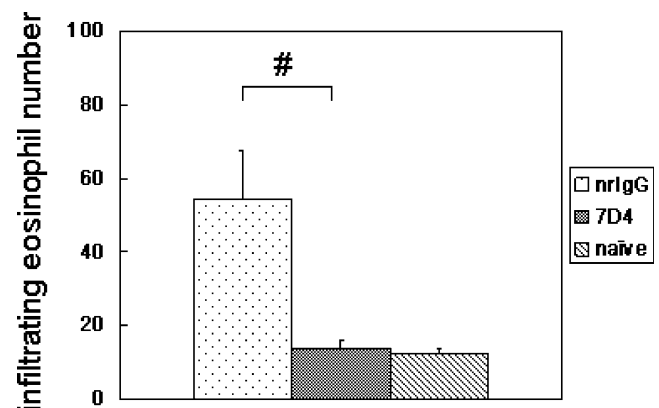


Fig. 6 Depletion of CD25+ cells from donor splenocytes abrogates the EC induced by splenocyte transfer in BALB/c mice. Splenocytes obtained from RW-primed mice without Ab treatment were cultured in vitro with RW for 3 days. CD25+ cells either were or were not depleted from these splenocytes, as detailed in the Section [Materials and Methods](#). After normalizing the number of CD4+ cells, the cells were transferred into syngeneic mice. Four days after the transfer, the recipient mice were challenged with RW. Twenty-four hours after the challenge, conjunctivas were harvested to evaluate eosinophil infiltration. As a control, the number of infiltrating eosinophils into the conjunctivas of naïve mice challenged with RW were counted. Significantly more eosinophils infiltrated the conjunctivas of mice that received complement-treated control splenocytes than of mice that received CD25-depleted splenocytes. $p<0.001$, $n=8$ per group

treatment with PC61; also in good agreement with our results, transfer of splenic CD4⁺ T cells from these mice increased the disease severity when compared with the control cells [30]. Furthermore, our data showing that transfer of splenocytes from mice treated with PC61 in vivo induced significantly more severe EC suggest that T reg cells do exist in vivo but were not able to exert their regulatory roles in vivo in the current experimental conditions. It is obviously necessary to titrate the Ab amounts for administration to find out the treatment protocol to completely deplete T reg cells.

In contrast to our data, PC61 treatment upregulated experimental airway inflammation, especially in airway inflammation-resistant C3H mice [19]. In flow cytometric analyses, PC61 treatment selectively depleted CD25-bright cells in the lung but minimally affected CD25-dim cells. CD25-bright and -dim cells are considered to be T reg cells and effector T cells, respectively, and these flow cytometric data correlate well with the severity of airway inflammation. In contrast, in our data, PC61 treatment prior to active immunization depleted both CD25-bright and -dim cells to the same extent. The reason why CD25-dim cells were not affected in experimental airway inflammation is unclear; however, depletion of CD25-dim cells as well as CD25-bright cells by PC61 treatment may, at least in part, play a role in reducing the severity of EC. The significant attenuation of eosinophil infiltration into the conjunctiva in C57BL/6 mice following induction-phase PC61 treatment supports this hypothesis. In addition, depletion of CD25⁺ cells in transferred splenocytes completely abolished EC in BALB/c mice, demonstrating that the CD25⁺ cell compartment contains effector T cells. Thus, it is most conceivable that PC61 treatment in vivo in our study affected both T reg cells and effector T cells, and therefore did not potentiate EC.

Adoptive transfer of splenocytes from actively immunized and PC61-treated mice induced more severe EC than did transfer of splenocytes from actively immunized and nrIgG-treated mice, regardless of mouse strains and treatment protocols. The two types of donor cells produced similar amounts of Th2 cytokines and Th1 cytokine production was significantly higher in PC61-treated splenocytes, indicating that the different severity of EC does not depend on the Th1/Th2 balance. Certain types of T reg cells produce immunosuppressive cytokines such as IL-10 and TGF- β [16, 27, 28]; however, in this study, IL-10 production was not affected by PC61 treatment in vivo (data not shown). The percentage of CD25⁺ splenocytes increased 3- to 10-fold after stimulation in vitro over the percentage in freshly isolated splenocytes. The CD25-bright CD4⁺ cell compartment in the transferring splenocytes from nrIgG-treated mice contained 2.5- to 3.5-fold more of these cells than the transferring splenocytes from PC61-

treated mice, but the CD25-dim CD4⁺ cell compartment differed only 1.5- to 2.5-fold. In vitro stimulation with RW preferentially activates effector T cells because regulatory T cells have less potential to proliferate [26, 28]. Therefore, stimulation in vitro may have affected the balance between the CD25-bright and the CD25-dim cell compartments in the nrIgG-treated and the PC61-treated groups. This response to stimulation may account for the augmented severity of EC following transfer of splenocytes from PC61-treated mice, and the results suggest that T reg cells are involved in the development of EC.

There is a lack of T reg cell-specific cell surface marker and it is often used to demonstrate the regulatory function of T reg cells by co-culture of T reg cells with effector T cells in proliferation assays. We have not functionally assessed whether PC61-treated splenocytes contain less T reg cells by this method. However, as a preliminary result, we found that CD4⁺CD25⁺ cells, especially CD25-bright cells, do express Foxp3 (unpublished result), which was identified to be an intracellular marker for naturally occurring thymus-selected CD4⁺CD25⁺ Treg cells [14]. Therefore, it could be regarded that PC61 treatment in this study depleted T reg cells although effector T cells were also depleted.

In conclusion, although PC61 treatment in vivo did not augment EC, T reg cells do play a role in the development of EC. Therefore, upregulation of the function of T reg cells could be a potential therapy for severe types of AC.

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