

Participation of CD11b and F4/80 Molecules in the Conjunctival Eosinophilia of Experimental Allergic Conjunctivitis

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Key Words

Allergic conjunctivitis • CD11b • Eosinophil • F4/80 • Mouse

Abstract

Background: CD11b and F4/80 are macrophage surface markers. How these molecules participate in allergic eosinophil infiltration remains unclear. We examined the roles CD11b and F4/80 play in the conjunctival eosinophil infiltration associated with experimental allergic conjunctivitis. **Methods:** Ragweed-immunized BALB/c mice were challenged with ragweed in eye drops to induce conjunctival eosinophil infiltration. The effect of challenge on conjunctival CD11b+ and F4/80+ cell numbers was determined by immunohistochemistry. In the same model, blocking anti-CD11b and anti-F4/80 Abs were injected intraperitoneally during the induction or the effector phase, or subconjunctivally 2 h before challenge, to determine their effect on challenge-induced conjunctival eosinophilia. To examine whether eosinophils express CD11b and F4/80 molecules, splenocytes from IL-5 gene-electroporated mice were subjected to flow cytometric analysis. To clarify the involvement of CD11b and F4/80 in conjunctival eosinophil infiltration, mice were intraperitoneally injected with anti-CD11b and anti-F4/80 Abs and then subconjunctivally injected with eotaxin to induce

conjunctival eosinophilia. **Results:** Ragweed challenge elevated conjunctival CD11b+ and F4/80+ cell numbers. Systemic anti-CD11b and anti-F4/80 Ab treatments during the effector phase, but not in either the induction phase or the local injection of Ab, suppressed conjunctival eosinophil infiltration in ragweed-induced conjunctivitis. Most splenic eosinophils from IL-5 gene-introduced mice expressed CD11b and F4/80. Systemic anti-CD11b and anti-F4/80 Ab treatment suppressed conjunctival eosinophilia induced by subconjunctival eotaxin injection. **Conclusions:** CD11b and F4/80 appear to participate in conjunctival eosinophil infiltration in allergic conjunctivitis. Their involvement in conjunctival eosinophilia appears to be due to their expression on eosinophils rather than on macrophages.

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Introduction

The hallmark of severe forms of allergic conjunctivitis (AC) is that eosinophil numbers in tear fluids of patients with AC increase in parallel with the severity of corneal tissue damage [1]. Moreover, major basic protein (MBP) and eosinophil cationic protein, which are secreted by activated eosinophils, are detected in the ulcerated cornea

of patients with atopic keratoconjunctivitis [2] and vernal keratoconjunctivitis, which are the severe forms of AC. Thus, it is clear that eosinophil infiltration into the conjunctiva causes the corneal tissue damage associated with severe AC.

We have investigated how eosinophils infiltrate the conjunctiva during the development of AC by using an experimental animal model of AC called experimental conjunctivitis (EC) [3]. These series of experiments revealed that Ag-specific T cells, rather than Ag-specific IgE, play a crucial role in the conjunctival eosinophil infiltration [4, 5]. We also found that Ag-specific T cells recognize the Ag in the conjunctiva, after which they are activated and secrete cytokines in the conjunctiva [6]. It is likely that these T cells recognize the Ag presented by conjunctiva-residential Ag-presenting cells, such as macrophages and dendritic cells [7]. These cells are known to express various cell surface markers. In particular, macrophages are known to express CD11b [8] and F4/80 [9].

Here, we sought to investigate how CD11b and F4/80 participate in the EC-associated conjunctival eosinophil infiltration by blocking CD11b and F4/80 molecules with specific Abs. We found that while these molecules do not play a role in the induction phase of EC, they clearly participate during the effector phase to promote conjunctival eosinophilia. These observations suggested that the blocking of CD11b and F4/80 molecules affected the eosinophils directly rather than by altering the Ag-presenting function of the conjunctiva-resident macrophages. Our additional experiments reveal that CD11b and F4/80 are expressed by eosinophils and that in this context these molecules participate in EC-associated conjunctival eosinophil infiltration.

Materials and Methods

Mice

Inbred wild-type BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were kept in specific pathogen-free conditions at the animal facility of Kochi Medical School, and age- and gender-matched mice were used when they were 6–12 weeks old. All research adhered to the Association for Research in Vision and Ophthalmology statement on '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). Hybridomas producing Ab to CD11b (5C3) and an Ab to F4/80 were obtained from ATCC (Manassas, Va., USA). For in vivo blocking treatments, these Abs were puri-

fied from ascites using a protein G column. Both preparations contained less than 100 pg/ml endotoxin. Normal rat IgG (nrIgG) was purchased from MP Biomedicals (Aurora, Ohio, USA). For immunohistochemistry, anti-mouse CD11b (Invitrogen, Carlsbad, Calif., USA) and anti-mouse macrophages (F4/80 antigen (BMA Biomedicals, Augst, Switzerland) were purchased, and rabbit anti-mouse MBP was kindly provided by Dr. Lee (Mayo Clinic). Biotinylated rabbit polyclonal anti-rat immunoglobulins (Dako Cytomation, Glostrup, Denmark) and biotinylated goat polyclonal anti-rabbit immunoglobulins (BD Biosciences Pharmingen, San Diego, Calif., USA) were used as secondary Abs. For flow cytometric analysis, FITC-labeled anti-mouse CD11b (BioLegend, San Diego, Calif., USA) and PE-labeled anti-mouse macrophage (F4/80; Cedarlane Laboratories, Burlington, Ont., Canada) were purchased. Recombinant mouse CCL11/eotaxin was obtained from R&D Systems (Minneapolis, Minn., USA).

EC Induction by Active Immunization and Treatment with Abs by Intraperitoneal Injection

RW adsorbed on alum was injected into the left hind footpad and the tail base. Fifty microliters of the emulsion (50 µg of RW and 675 µg of alum) were injected into each site. The mice were injected intraperitoneally with 200 µg of purified anti-CD11b, anti-F4/80 or nrIgG (n = 10 per group) on days 0, 2, 4, 6 and 8 after immunization (induction phase treatment). In a separate experiment, the Abs were injected intraperitoneally once 2 h prior to RW challenge (n = 10 per group, effector phase treatment). In both experiments, the eyes of the immunized mice were challenged on day 10 with RW in PBS (2 mg in 10 µl per eye). Twenty-four hours later, the eyes, sera and spleens were harvested for histological analysis, measurement of RW-specific Ig levels, and cytokine assays, respectively.

EC Induction by Active Immunization and Treatment with Abs by Subconjunctival Injection

Mice were actively immunized as described above and then, on day 10, injected once subconjunctivally with 10 µg/10 µl of purified anti-CD11b (n = 5), anti-F4/80 (n = 5), or nrIgG (n = 4) 2 h prior to RW challenge (local treatment). Twenty-four hours after challenge, the eyes of the immunized mice were harvested for histological analysis.

Subconjunctival Injection of Eotaxin and Treatment with Abs by Intraperitoneal Injection

Mice injected intraperitoneally with 200 µg nrIgG, anti-CD11b, or anti-F4/80 Abs (n = 3 per group) were injected 2 h later subconjunctivally with recombinant mouse eotaxin (0.1 µg in 10 µl). 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. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by 2 masked observers. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. The data are presented as means ± SEM of all the mice examined.

Immunohistochemistry

Conjunctivas from RW-immunized mice that had not been treated with any Abs were harvested 24 h after RW challenge ($n = 3$). In addition, conjunctivas from naïve mice were collected ($n = 3$). The conjunctivas were embedded in OCT compound (VWR, Suwanee, Ga., USA) and snap frozen in liquid nitrogen. Vertical 4- μ m sections were prepared and fixed in methanol. Endogenous peroxidase activity was inhibited by incubation with 0.1% NaN_3 and 0.3% H_2O_2 in distilled water for 10 min at room temperature. The samples were exposed to the anti-MBP, anti-CD11b, or anti-F4/80 Abs for 30 min and then to the appropriate biotinylated secondary Ab for another 30 min. As a control, the samples were exposed to biotinylated rabbit polyclonal anti-rat immunoglobulins but not to any first Abs. Ab binding was revealed by using an Avidin-Biotin-Complex kit (Vector Laboratories, Burlingame, Calif., USA) followed by development with 3,3'-diaminobenzidine tetrahydrochloride (Sigma).

Electroporation of IL-5 Gene and Flow Cytometric Analysis

To boost the eosinophil compartment, mice were electroporated with the IL-5 gene as previously described [10]. Briefly, the tibialis anterior muscles of mice ($n = 3$) were injected with a plasmid DNA expressing IL-5 as the vector, after which a pair of electrode needles was inserted into the DNA injection site to deliver electric pulses. Fourteen days later, the spleens were harvested and subjected to flow cytometric analysis. As previously reported [11, 12], cells with high levels of side scatter can be regarded as eosinophils. The splenocytes were incubated for 30 min on ice with FITC- or PE-labeled Abs at optimal concentrations and then washed with cold 2% FCS-PBS. These cells were analyzed on a FACScan (Becton Dickinson, Mountain View, Calif., USA) and data were analyzed using CellQuest software.

Measurement of RW-Specific IgG1, IgG2a and IgE in Serum

Twenty-four hours after RW challenge of actively immunized mice treated with Abs during the induction phase, the blood was collected and serum was prepared. RW-specific IgG1, IgG2a and IgE in the sera were measured by ELISA as previously described [13]. IgG1 and IgG2a concentrations are expressed as units (U)/ml relative to the pooled hyperimmune serum, while IgE concentrations are shown as ng/ml.

Measurement of Cytokines in Culture Supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with 25 μ g/ml RW extract in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 50 μ M 2-ME. The levels of IL-2, IL-4, IL-5, IL-10, TNF- α and IFN- γ in the culture supernatants were measured by using Luminex100 (Luminex Corporation, Austin, Tex., USA) and the Bio-Plex Cytokine Assay Panel (Bio-Rad Laboratories, Hercules, Calif., USA) according to the manufacturers' instructions.

Statistical Analysis

Differences between the Ab-treated and nrlgG-treated mice in terms of their serum Ig levels, cytokine production and infiltrating eosinophil numbers were tested for significance by Student's t test and Mann-Whitney U test, respectively. $p < 0.05$ was considered significant.

Results

Upregulation of CD11b and F4/80 Expression in Conjunctiva by Induction of EC

First, we examined whether the induction of EC causes CD11b- and F4/80-expressing cells to infiltrate the conjunctiva. EC was induced by active immunization with RW in alum and 10 days later the mice were challenged with RW in eye drops. Twenty-four hours later, the conjunctivas were collected and subjected to immunohistochemical analysis. Although it is ideal to evaluate the state of EC by using a clinical grading score, clinical findings such as hyperemia and discharge were hardly detectable. Therefore, we decided to evaluate the severity of EC by conjunctival eosinophilia. While CD11b+ and F4/80+ cells were present in the conjunctivas of naïve mice, the induction of EC elevated the numbers of conjunctival CD11b+ and F4/80+ cells (fig. 1). As a positive control, MBP expression was examined, which revealed that the induction of EC also increased conjunctival MBP+ eosinophil numbers (fig. 1).

Effect of Systemic Treatment with Anti-CD11b and Anti-F4/80 Abs during the Induction Phase of EC

To investigate whether CD11b+ and F4/80+ cells participate in the development of EC during the induction phase, Abs against CD11b and F4/80 were injected intraperitoneally 5 times every other day, starting on the day of immunization. As a control, nrlgG was injected. On day 10, the mice were challenged with RW in eye drops, and 24 h later the conjunctivas, blood and spleens were harvested for histological analysis, measurement of RW-specific Ig levels in the serum, and RW-recall cytokine production, respectively. Mice injected with anti-CD11b, anti-F4/80 and nrlgG exhibited similar levels of conjunctival eosinophilia and, indeed, the conjunctival eosinophil counts of the 3 groups did not differ significantly (fig. 2a). The 3 groups also did not differ significantly in terms of RW-specific IgG1, IgG2a and IgE levels (fig. 2b). However, cytokine assays revealed that compared to nrlgG treatment, anti-CD11b Ab treatment significantly increased RW-specific IL-4 and IL-10 production by splenocytes, while anti-F4/80 Ab treatment significantly increased IFN- γ and significantly decreased IL-10 levels (fig. 2c). Thus, anti-CD11b and anti-F4/80 Ab treatment during the induction phase significantly affected RW-specific systemic immune responses, but this did not result in changes in conjunctival eosinophil infiltration.

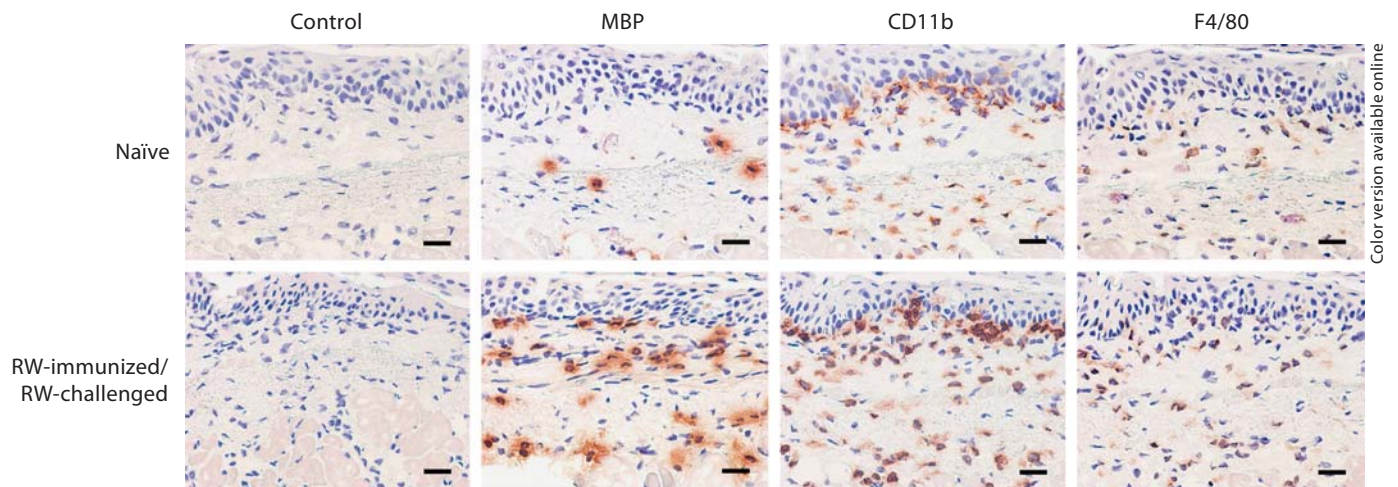


Fig. 1. Expression of CD11b and F4/80 in the conjunctiva. BALB/c mice were immunized with RW and challenged 10 days later with RW in eye drops (RW-immunized/RW-challenged; $n = 3$). Twenty-four hours later, the conjunctivas were harvested and subjected to immunohistochemistry. Conjunctivas from naïve

mice were also examined ($n = 3$). While CD11b+ and F4/80+ cells exist in the conjunctivas of naïve mice, their numbers are increased by the induction of EC. The control samples were exposed to a secondary Ab alone. Representative images are shown. Scale bar = 20 μ m.

Effect of Treatment with Anti-CD11b and Anti-F4/80 Abs during the Effector Phase of EC

To investigate whether CD11b+ and F4/80+ cells participate in the development of EC during the effector phase, Abs against CD11b and F4/80 were injected intraperitoneally once 2 h prior to RW challenge. As a control, nrIgG was injected. Intraperitoneal injection of anti-CD11b and anti-F4/80 Ab during the effector phase reduced conjunctival eosinophil infiltration (fig. 3a). This was confirmed by counting the conjunctival eosinophils, which revealed that the groups treated with anti-CD11b and anti-F4/80 had significantly fewer conjunctival eosinophils than the nrIgG-treated mice (fig. 3b). Next, to investigate whether CD11b+ and F4/80+ cells participate in the development of EC in the conjunctiva, Abs against CD11b and F4/80 were injected subconjunctivally once 2 h prior to RW challenge. As a control, nrIgG was injected. The local injection of anti-CD11b and anti-F4/80 Ab during the effector phase did not significantly affect the severity of conjunctival eosinophilia (fig. 3c).

Expression of CD11b and F4/80 on Eosinophils

As described above, the blocking of CD11b and F4/80 during the effector phase, but not during the induction phase, suppressed conjunctival eosinophil infiltration in EC. These data suggest that eosinophils themselves express CD11b and F4/80 and the blocking of these molecules directly affects the infiltrating capability of eosino-

phils. A good way to test whether conjunctiva-infiltrating eosinophils express CD11b and F4/80 would be to assess by immunohistochemical analysis whether these molecules colocalize with MBP in the conjunctiva. However, due to high background autofluorescence, we were unable to perform these experiments to our satisfaction. Thus, we instead evaluated the ability of splenic eosinophils to express CD11b and F4/80 by flow cytometric analysis. For this, we obtained the splenocytes from mice, in which the IL-5 gene was over-expressed due to electroporation of the IL-5 gene, and stained these cells with anti-CD11b and anti-F4/80 Abs. CD11b and F4/80 expression was detected in the whole splenocyte compartment (gated as R3 in the upper plot of fig. 4). As previously described [11, 12], the granulocytes that are gated on the basis of their high forward and side scatters (gated as R2 in the lower plot of fig. 4) mainly consist of eosinophils. The percentage of cells in the R2 gate relative to the whole splenocyte numbers in R3 was calculated by flow cytometric analysis to be $35.9 \pm 2.1\%$ (fig. 4). The vast majority of the cells in R2 expressed CD11b ($93.6 \pm 0.5\%$) and F4/80 ($90.9 \pm 1.1\%$; fig. 4).

CD11b and F4/80 Participate in Conjunctival Eosinophil Infiltration Induced by Eotaxin

Finally, to investigate whether the CD11b and F4/80 molecules expressed by eosinophils are directly involved in the conjunctival eosinophil infiltration in EC, naïve

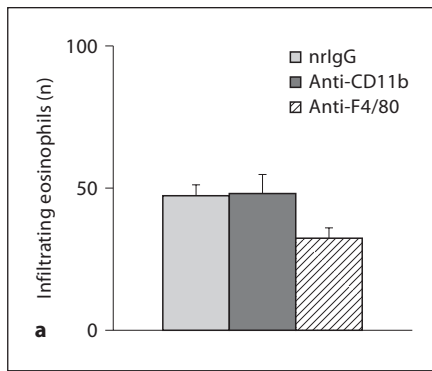
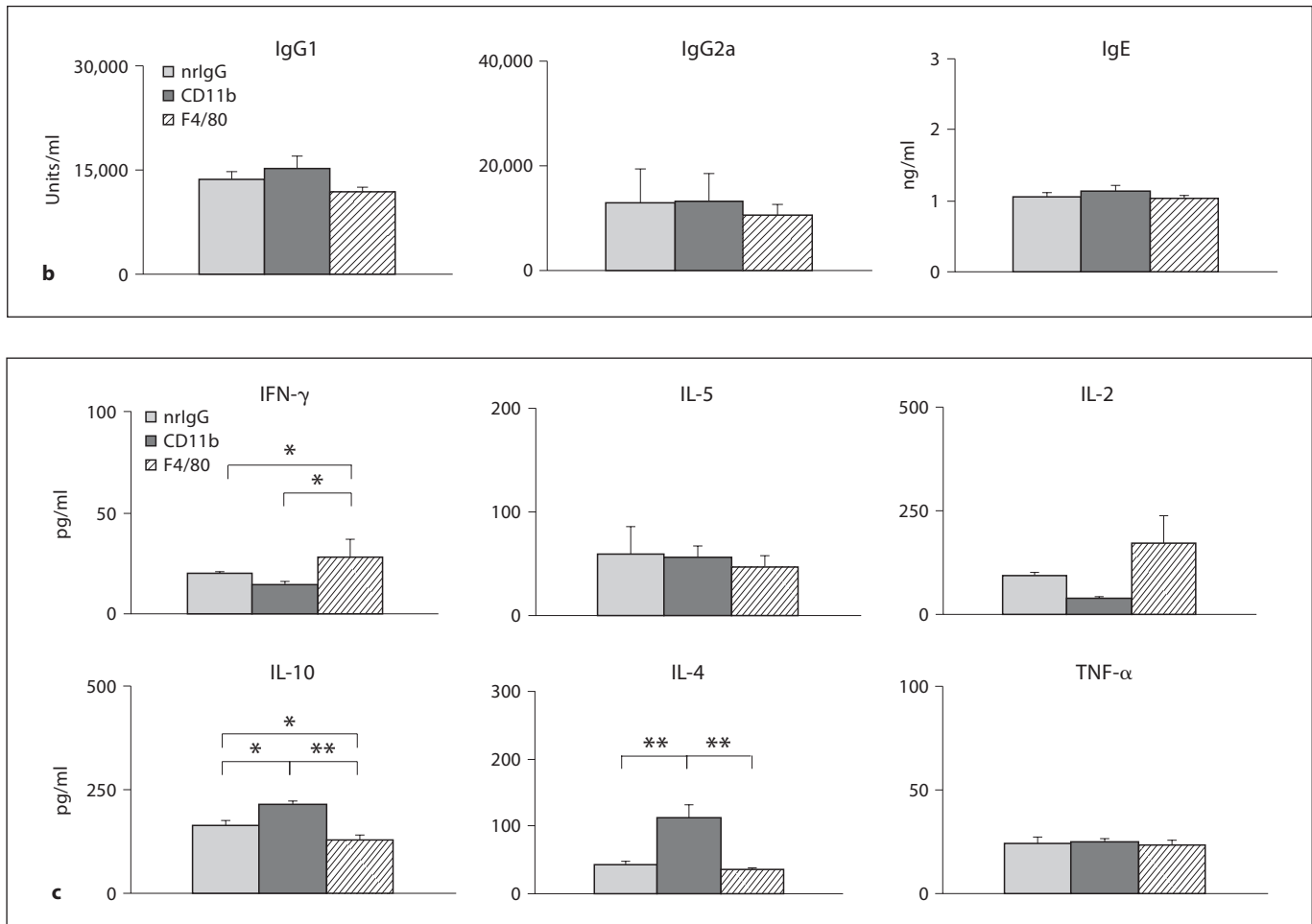


Fig. 2. Effect of systemic treatment with anti-CD11b and anti-F4/80 Abs during the induction phase of EC. BALB/c mice were immunized with RW, injected i.p. 5 times every other day starting on the day of immunization with 200 μ g anti-CD11b Ab, anti-F4/80 Ab or nrlgG and challenged on day 10 with RW in eye drops (n = 10/group). Twenty-four hours later, eyes were harvested for histological analysis to evaluate conjunctival eosinophil numbers (a), blood was collected for measurement of RW-specific Ig levels in the serum (b) and spleens were harvested for cytokine assays (c). The data are presented as units/ml (IgG1 and IgG2a) or ng/ml (IgE; b), or pg/ml (c). * $p < 0.05$, ** $p < 0.01$.



mice were injected intraperitoneally with anti-CD11b and anti-F4/80 Abs, after which eotaxin was injected subconjunctivally. The anti-CD11b Ab-treated group had significantly fewer eosinophils than the nrlgG-treated group (fig. 5). While the anti-F4/80 Ab treatment had a similar effect, this effect did not achieve statistical significance (fig. 5).

Discussion

Here, we demonstrate that the systemic blocking of CD11b and F4/80 molecules by intraperitoneal injection of anti-CD11b and anti-F4/80 Abs during the effector phase suppressed the development of EC. However, the severity of conjunctival eosinophil infiltration in EC was

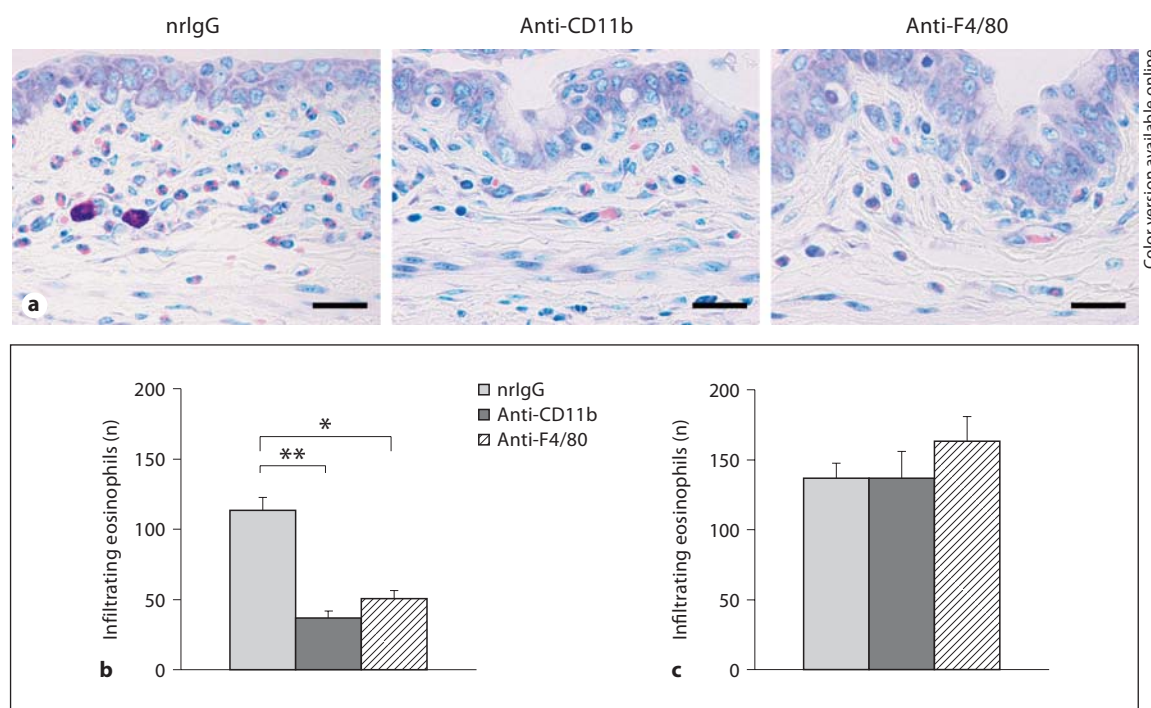


Fig. 3. Effect of treatment with anti-CD11b and anti-F4/80 Abs during the effector phase of EC. BALB/c mice were immunized with RW. Ten days later, they were injected once i.p. with 200 μ g anti-CD11b Ab, anti-F4/80 Ab or nrlgG and challenged 2 h later with RW in eye drops (n = 10/group; **a, b**). Alternatively, on day 10, anti-CD11b Ab (n = 5), anti-F4/80 Ab (n = 5) or nrlgG (n = 4)

Abs (10 μ g in 10 μ l) were subconjunctivally injected 2 h prior to RW challenge (**c**). Twenty-four hours after RW challenge, the eyes were harvested for histological analysis. **a** Microphotographs of Giemsa staining. Scale bar = 20 μ m. **b, c** Conjunctival eosinophil numbers. * $p < 0.05$, ** $p < 0.01$.

not affected by the local blocking of CD11b and F4/80 molecules by subconjunctival injection of the blocking Abs. Since the subconjunctivally injected Abs would block the CD11b and F4/80 molecules on the surface of conjunctiva-resident macrophages, local CD11b and F4/80 molecules are less likely to participate in EC-associated conjunctival eosinophilia. Therefore, it could be considered that systemically circulating CD11b+ and F4/80+ cells, rather than conjunctival CD11b+ and F4/80+ cells, participate in the conjunctival eosinophil infiltration.

This leads to the question: which systemically circulating cells express CD11b and F4/80 molecules? CD11b and F4/80 are known to be expressed by monocytes/macrophages [8, 9], and it has also been reported that human and murine eosinophils express these molecules [14–16]. To examine the latter possibility, the IL-5 gene was introduced into murine muscles by electroporation to increase the eosinophil compartment. Using flow cytometric analysis, we defined the eosinophil compartment on the basis of its high forward and side scatters, as previously

reported [11, 12]. We found that most of these cells expressed CD11b and F4/80. We also observed that eotaxin-induced conjunctival eosinophil infiltration was inhibited by the intraperitoneal injection of anti-CD11b and anti-F4/80 Abs. These findings strongly suggest that the CD11b and F4/80 molecules expressed by eosinophils participate in the conjunctival eosinophil infiltration associated with the development of EC. Recently, it was reported that in dextran sodium sulfate-induced intestinal epithelial injury, eotaxin-1 expression is restricted to the intestinal CD11b+ F4/80+ macrophages, while in pediatric ulcerative colitis patients, eotaxin-1 expression is restricted to CD68+ intestinal macrophages and the basolateral compartment of intestinal epithelial cells [17]. In both of these diseases, eosinophils accumulated in the colon. These observations indicate that intestinal macrophages participate in eosinophil infiltration into the colon. A similar mechanism may also play a role in AC. Thus, it will be of interest to investigate whether CD11b+ F4/80+ macrophages participate in the conjunctival eosinophil infiltration associated with EC.

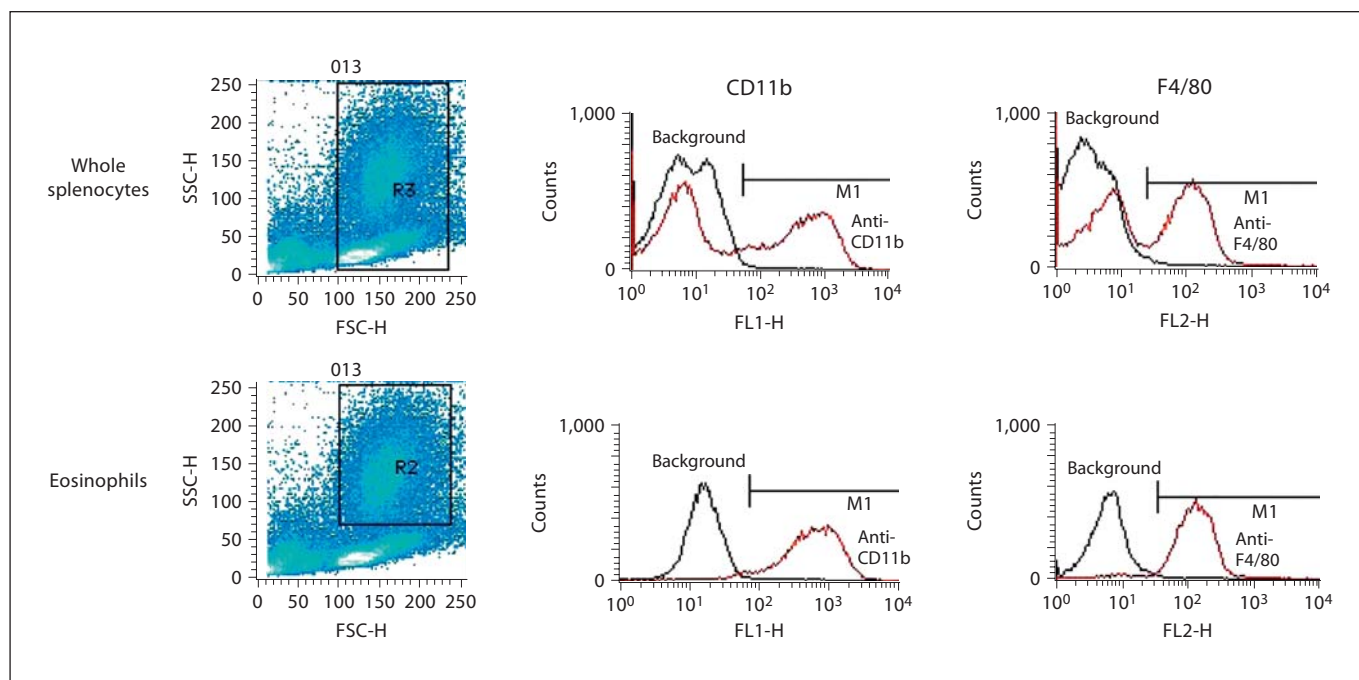


Fig. 4. Expression of CD11b and F4/80 by splenocytes from IL-5 gene-transduced mice. The IL-5 gene was electroporated into the muscles of BALB/c mice and the spleens were harvested 14 days later for flow cytometric analysis. The splenocytes were gated into R2 (eosinophils, lower lane) or R3 (whole splenocytes, upper lane). Representative data of 1 of 3 mice are shown as dot-plots and histograms. On the histogram data, the background line represents

background staining with control IgG and the other line indicates staining with anti-CD11b or anti-F4/80 Ab, as appropriate. The percentage of cells in R2 relative to the total number of cells in R3 was $35.9 \pm 2.1\%$. In R2, $93.6 \pm 0.5\%$ and $90.9 \pm 1.1\%$ of the cells expressed CD11b and F4/80, respectively. M1 indicates the cells positively stained with the Ab.

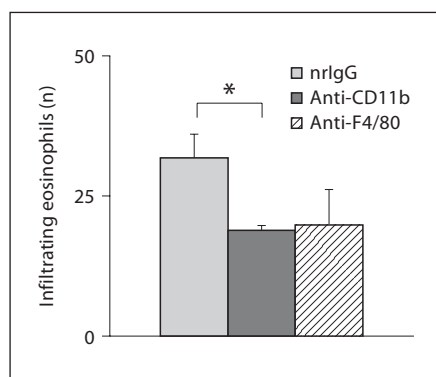


Fig. 5. Effects of systemic treatment with anti-CD11b and anti-F4/80 Ab on conjunctival eosinophilia induced by subconjunctival injection of eotaxin. Naïve BALB/c mice were injected i.p. with 200 μ g of anti-CD11b Ab, anti-F4/80 Ab or nrlgG ($n = 3/\text{group}$) and then injected subconjunctivally 2 h later with recombinant mouse eotaxin (0.1 μ g in 10 μ l). Twenty-four hours later, the conjunctivas were harvested for histological analysis to evaluate conjunctival eosinophil numbers. * $p < 0.05$.

With regard to the role of CD11b in the allergic disease models, it has been reported that Mac-1 (CD11b/CD18)-deficient mice show enhanced eosinophilic inflammation in the lung in response to allergic Ag challenge [18]. In that report, the authors also investigated the effect of anti-Mac-1 Ab on allergic lung inflammation. However, unlike the effect of Mac-1 deficiency, anti-Mac-1 Ab treatment during the effector phase (before intranasal Ag challenge) significantly reduced the airway eosinophilia [18]. This is consistent with our observation that systemic treatment with anti-CD11b and anti-F4/80 Abs during the effector phase suppressed the conjunctival eosinophilia associated with EC. It was also shown in the study described above that eotaxin challenge induced similar levels of airway eosinophilia in wild-type and Mac-1-deficient mice [18]. This is contrary to our observation that eotaxin-induced conjunctival eosinophilia was suppressed by systemic anti-CD11b and anti-F4/80 Ab treatment. While the reason for this discrepancy between the previous study [18] and our own is unclear, it is possible that Mac-1 deficiency leads to upregulation of compen-

sating pathways such as the upregulation of very late antigen (VLA)-4/vascular cell adhesion molecule-1 (VCAM-1). There are also other possibilities, namely, the previous report employed C57BL/6 mice, while we used BALB/c mice and the target tissue being examined differed (lung vs. conjunctiva). To clarify this issue, it will be necessary to investigate whether Mac-1 deficiency affects conjunctival eosinophilia.

We have previously shown that VLA-4/VCAM-1 interaction plays a role in the conjunctival eosinophil infiltration associated with the development of EC [19]. However, colocalization of VLA-4 was more remarkable on T cells than on eosinophils in EC-developing conjunctiva and the effect of systemic anti-VLA-4 Ab treatment was more remarkable in T cell transfer-induced EC than in

active immunization-induced EC [19]. Thus, VLA-4/VCAM-1 interaction may be important for the conjunctival eosinophil infiltration that is predominantly mediated by T cells. Furthermore, while the blocking of VLA-4 alone slightly, but not significantly, suppressed conjunctival eosinophil infiltration [19], the blocking of CD11b and F4/80 during the effector phase of EC significantly suppressed the conjunctival eosinophil infiltration. These observations, together with the finding that CD11b and F4/80 are expressed by eosinophils themselves, indicate that CD11b and F4/80 may be attractive therapeutic target molecules, as their inhibition may ameliorate the conjunctival eosinophil infiltration in severe forms of AC.

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