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Role of VLA-4 in the development of allergic conjunctivitis in mice

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Purpose: The severity of allergic conjunctivitis (AC) correlates with the degree of eosinophil infiltration into the conjunctiva, which is believed to be mediated by chemokines and adhesion molecules. The adhesion molecule very late antigen (VLA)-4 and its ligand, vascular cell adhesion molecule (VCAM)-1, are known to play important roles in eosinophil infiltration. However, the expression and function of VLA-4 in AC have not been investigated in detail. We sought to characterize VLA-4-expressing cells in the conjunctivas of mice that are developing experimental AC (EC) and to determine whether the interaction between VLA-4 and VCAM-1 is needed for the infiltration of eosinophils into the conjunctiva in AC.

Methods: EC was induced in Balb/c mice by active immunization with ragweed (RW) or adoptive transfer of RW-primed splenocytes, followed by RW challenge. Twenty-four hours after RW challenge, the conjunctivas were harvested. The conjunctivas from naive mice or mice developing EC were evaluated for VLA-4 and VCAM-1 expression by immunohistochemistry and immunofluorescent analyses. To investigate whether the interaction between VLA-4 and VCAM-1 is needed for the genesis of AC, mice developing EC were treated with anti-VLA-4 or anti-VCAM-1 antibodies two h before and after RW challenge. As a control, EC-developing mice were treated with normal rat IgG. Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis.

Results: Upon induction of EC, VLA-4-expressing cells infiltrated the conjunctiva but the constitutive VCAM-1 expression around conjunctival vessels was not augmented. Immunofluorescent analyses demonstrated that most of the T cells infiltrating the conjunctiva expressed VLA-4 but only half of the infiltrating eosinophils expressed it. Treatment with both anti-VLA-4 and anti-VCAM-1 antibodies significantly suppressed the infiltration of eosinophils into the conjunctiva that was induced by either active immunization or splenocyte transfer.

Conclusions: These results confirm that VLA-4-expressing cells infiltrate the conjunctiva and that the interaction between VLA-4 and VCAM-1 is needed for the development of EC.

Allergic conjunctivitis (AC) is initiated by the interaction between an allergen and allergen-specific IgE that binds to the Fc receptor on the surface of mast cells [1]. The mast cells then release inflammatory mediators such as histamine, which induce allergic symptoms in the conjunctiva [2]. Eosinophils are also thought to be important effector cells in AC, since the number of infiltrating eosinphils into the conjunctiva rises as the severity of AC increases [3]. Although the mechanism behind the infiltration of eosinophils into the conjunctiva has not been fully elucidated, our group has demonstrated that interleukin (IL)-4-producing Th2-type cells are involved [4,5]. This led to the hypothesis [6] that infiltrating antigen-specific Th2 cells produce IL-4 and that this activates conjunctival fibroblasts; these cells in turn produce eosinophil-attracting chemokines, such as eotaxin. This hypothesis is largely consistent with observations made in experimental animal studies [4-6] and human clinical studies [7,8].

Along with chemokines, the migration of eosinophils from vessels requires interactions between eosinophils and vascular endothelium followed by the rolling, reversible or firm adhesion, transmigration and lodging of the eosinophils. These interactions are mediated by adhesion molecules [9]. Of the known adhesion molecules, very late antigen-4 (VLA-4: $\alpha 4\beta 1$ integrin) has been demonstrated to be involved in the infiltration of eosinophils into inflammatory sites [10-12]. VLA-4 interacts with vascular cell adhesion molecule-1 (VCAM-1, CD106) and fibronectin, which are expressed on the activated vascular endothelial cells [13]. Several groups have demonstrated that in experimental allergic lung inflammation, the interaction between VLA-4 and VCAM-1 is important for the recruitment of leukocytes into the airway by various methods to block the interaction between VLA-4 and VCAM-1 [11,12,14-16]. With regard to experimental AC, however, only one paper has addressed this issue, and this study only employed anti-VLA-4 antibody to demonstrate the involvement of this adhesion event in guinea pig experimental AC [17]. Moreover, the expression of VLA-4 in the conjunctiva has not been examined previosuly [17]. To understand the roles VLA-4 and VCAM-1 play in the development of experimental AC in more detail, we induced experimental AC (experimental immune-mediated blepharoconjunctivitis, EC) in mice and investigated (i) the expression of VLA-4 in the conjunctiva and (ii) the effect of anti-VLA-4 antibody, anti-VCAM-1 antibody, and a combination of both antibodies on the development of EC.

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METHODS

Mice: Inbred wild-type Balb/c mice were purchased (Japan SLC Inc., Hamamatsu, Shizuoka, Japan) and kept in specific

pathogen-free conditions at the animal facility of Kochi Medical School. Female 6- to 12-week-old mice were used for experiments. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents: Short ragweed pollen (RW) was purchased from Polysciences, Inc. (Warrington, PA). RW extract was obtained from LSL CO. LTD. (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, MO). The hybridomas producing rat anti-mouse integrin α4 chain (PS/2, rat IgG2b) and rat anti-mouse VCAM-1 (M/K-2.7, rat IgG1) antibodies were purchased from ATCC (Rockville, MD). Anti-α4 integrin antibody reacts with α4β1 (VLA-4, CD49d) and α4β7 (CD49d/β7). For in vivo treatments, these antibodies were purified from ascites by using protein G columns; these preparations contained less than 100 pg/ml endotoxin. Normal rat IgG (nrIgG) was purchased from MP Biomedicals Inc. (Aurora, OH). Rat anti-mouse CD3 (CT-CD3) was purchased from Caltag (Burlingame, CA). Rabbit anti-mouse major basic protein (MBP) was kindly provided by Dr. James J. Lee (Mayo Clinic, Scottsdale, AZ). Biotinylated rabbit anti-rat immunoglobulins were obtained from Dako (Copenhagen, Denmark). Rat anti-mouse CD4 (L3T4) and biotinylated goat anti-rabbit immunoglobulins were purchased from BD Pharmingen (San Diego, CA). Streptoavidin-Tex Red was obtained from Molecular Probes (Eugene, OR).

Experimental allergic conjunctivitis induction by active immunization and treatment with antibodies: RW adsorbed on alum was injected into the left hind-footpad and the tail base. Fifty μl of the emulsion (50 μg of RW and 2 mg of alum) was injected into each site. Ten days later, the eyes of the immunized mice were challenged with RW in phosphate-buffered saline (PBS; 2 mg in 10 μl per eye). Two h before and after the challenge, the mice were intraperitoneally injected with 500 μg of each antibody (each mouse therefore received a total of 1 mg of antibody). When the mice were treated with both anti-VLA-4 and anti-VCAM-1 antibodies, the mixture of 250 μg of each antibody was injected twice. Twenty-four h later, mice were sacrificed and the eyes, including the conjunctivas, were harvested for histological analysis to evaluate eosinophil infiltration. For immunohistochemical and immunofluorescent analyses, conjunctivas from EC-developing mice that had not been treated with any antibodies were used. Each group consisted of 10 mice.

Experimental allergic conjunctivitis induction by adoptive transfer of in vitro-stimulated short ragweed pollen-primed splenocytes and treatment with antibodies: Naive Balb/c mice were immunized with RW in both their left hind-footpad and tail base and were not treated with any antibodies. Ten days later, splenocytes harvested from the mice were cultured with RW extract at final concentrations of 5 μg/ml in 75 cm^2 flasks at a concentration of 10^7 cells per ml in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan Co., Tokyo, Japan), 2-mercaptoethanol (2-ME, $5x10^{-5}$ M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO_2, $2x10^7$ splenocytes were intraperitoneally injected into syngeneic naive Balb/c mice. Four days after the splenocyte transfer, the mice were challenged with RW in PBS (2 mg in 10 μl per eye). Two hours before and after the challenge, the mice were intraperitoneally injected with antibodies as described above. Twenty-four hours after the challenge, the eyes were harvested for histological analysis to evaluate eosinophil infiltration. Each group consisted of 10 mice.

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 two blind observers. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. Because counted area varies depending on the severity of inflammation (when inflammation is severe, thickness of lamina propria mucosae increases), data of cell counting are shown as infiltrating eosinophil number divided by area (mm^2) measured by Scion Image (Scion Corporation, Frederick, MD). The data are presented as averages±SEM of all the mice examined.

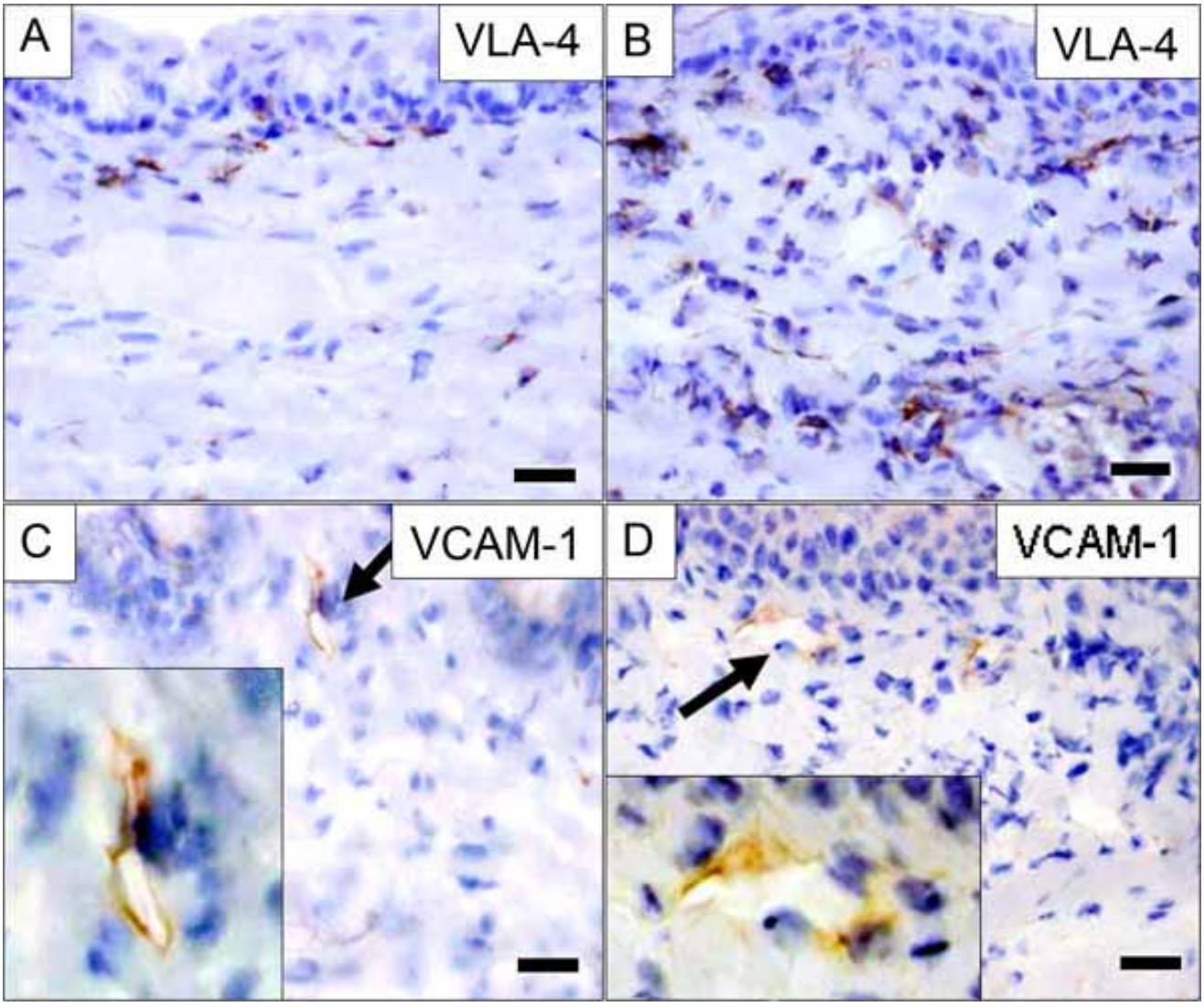
Immunohistochemistry: Sections were prepared for immunohistochemistry by following the method previously described in the literature [18]. In brief, the eyes from naive or actively immunized mice that were developing EC were immediately frozen in 3% carboxymethyl cellulose (CMC) gel. Sections (four μm thick) 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 then exposed to the primary antibodies (anti-VLA-4 and anti-VCAM-1) for 30 min and subsequently to biotinylated anti-rat antibodies for another 30 min. All slides were subjected to an Avidin-Biotin-Complex kit (Vector Laboratories, Inc., Burlingame, CA) and then developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma).

Immunofluorescent analyses: Sections prepared as previously described were incubated with FITC-labeled anti-VLA-4 antibody for 1 h. To reduce lipofuscin autofluorescence, the sections were then treated with a solution of Sudan Black B (Allied Chemical, New York, NY) in 70% methanol for 1 min [19]. Next, these sections were stained with unlabeled rat anti-mouse CD3, CD4, and rabbit anti-mouse MBP antibodies for 1 h and then incubated for another 1 h with biotin-conjugated rabbit anti-rat IgG and biotin-conjugated goat anti-rabbit immunoglobulins, respectively. The sections were further incubated with streptoavidin-Tex Red for another 1 h. The sections were examined with a BX50 microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis: Differences in the VLA-4+ cell numbers between naive mice and mice developing EC and in the infiltrating eosinophil numbers of the nrIgG-treated group and the antibody-treated groups were tested for significance by Student's t-test. An α level of 0.05 was chosen.

RESULTS

Immunohistochemical staining of the conjunctiva: To examine the expression of VLA-4 and VCAM-1 in the conjunctivas of mice with EC, we subjected the conjunctivas from naive mice and actively immunized mice that were developing EC to immunohistochemical staining. In naive mice, VLA-4-expressing cells were present in the lamina propria (Figure 1A), and weak staining of VCAM-1 was observed around vessels (Figure 1C). In mice developing EC, the numbers of VLA-4-expressing cells were significantly increased by 2.4 fold (Figure 1B,E). However, the VCAM-1 expression levels were similar to those in naive mice (Figure 1D). Thus, infil-



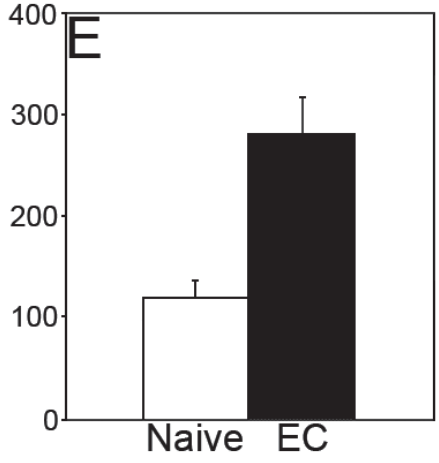


Figure 1. Expression of VLA-4 and VCAM-1 in conjunctivas of actively induced EC. Conjunctivas from naive mice (**A**,**C**) or actively immunized mice that were developing experimental allergic conjunctivitis (EC; **B**,**D**) were subjected to immunohistochemical analysis using anti-VLA-4 antibody (**A**,**B**) or anti-VCAM-1 antibody (**C**,**D**). Insets show the portions of the slides indicated by arrows at higher magnification. The scale bars in the images represent 20 μm. One representative section from five mice is shown. **E**: VLA-4+ cells in the lamina propria of naive and EC-developing mice were counted, and the data are presented as the number of VLA-4+ cells/mm^2. Error bars represent the standard error of the mean. Note that while the number of VLA-4-expressing cells increased when EC was induced, VCAM-1 expression was not changed.

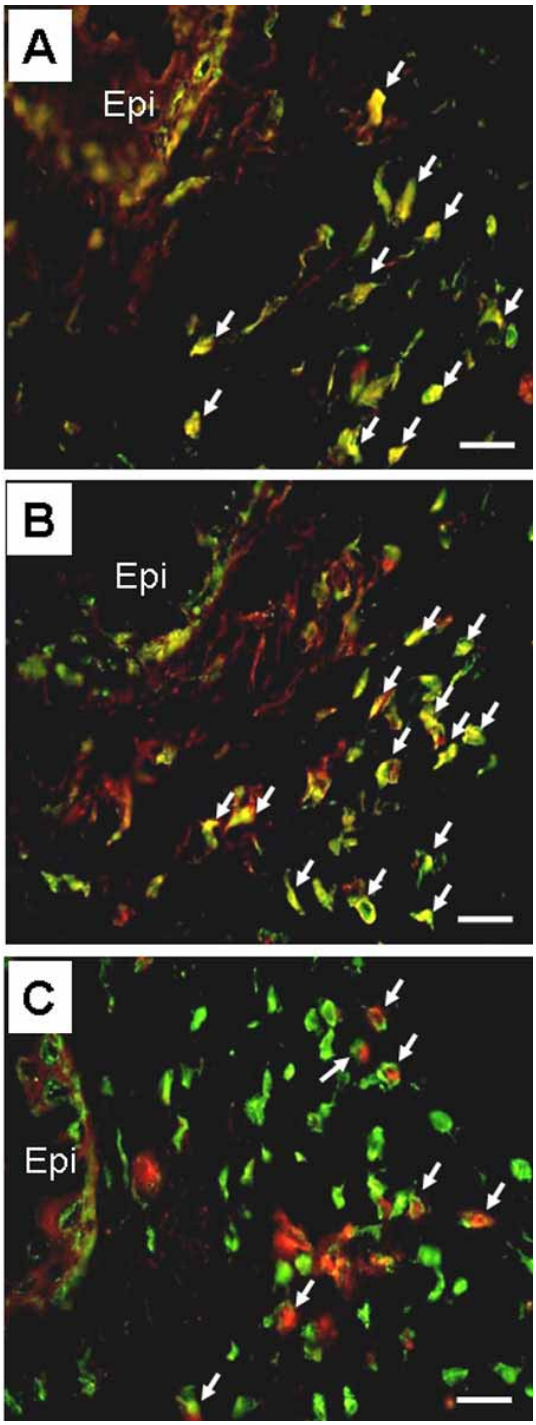


Figure 2. Immunofluorescent analyses of the cells infiltrating conjunctivas of actively immunized mice. Conjunctivas from actively immunized mice that were developing experimental allergic conjunctivitis (EC) were harvested for immunofluorescent analyses. The serial sections were first stained with FITC-labeled anti-VLA-4 antibody and then with anti-CD3 (**A**), anti-CD4 (**B**), or anti-MBP (**C**) antibodies, followed by development with Texas Red. Note that most of the CD3+ (**A**) and CD4+ (**B**) cells expressed VLA-4, whereas VLA-4 colocalized with MBP in only half of the eosinophils (**C**). Arrows indicate the cells that were stained with two different antibodies and the epithelium (Epi) is identified. The scale bars represent 20 μm. The sections shown are representative of those from five different mice.

tration of VLA-4+ cells is induced by the induction of EC.

Immunofluorescent microscopic analyses of VLA-4+ cells in the conjunctiva: Next, we sought to define the phenotypes of the infiltrating VLA-4+ cells in the conjunctiva by immunofluorescent microscopic analyses. As shown in Figure 2A,B, VLA-4 and CD3 (Figure 2A) or CD4 (Figure 2B) clearly colocalized. In contrast, VLA-4 and MBP colocalized to a lesser extent, and only about half of the MBP+ cells were also VLA-4+. Furthermore, the pattern of colocalization differed from CD3+ and CD4+ cells, because the VLA-4 signal surrounded the MBP signal (Figure 2C, arrows) while the CD3/CD4 signal merged closely with the VLA-4 signal. Thus, while most of the infiltrating T cells expressed VLA-4, only half of the MBP+ eosinophlils did so.

Treatment with anti-VLA-4 and anti-VCAM-1 antibodies suppresses the infiltration of eosinophils into the conjunctiva induced by active immunization: Although we found that VLA-4-expressing cells infiltrate the conjunctiva, it is not clear whether VLA-4 is actually necessary for the infiltration event. Therefore, we next sought to examine whether blocking VLA-

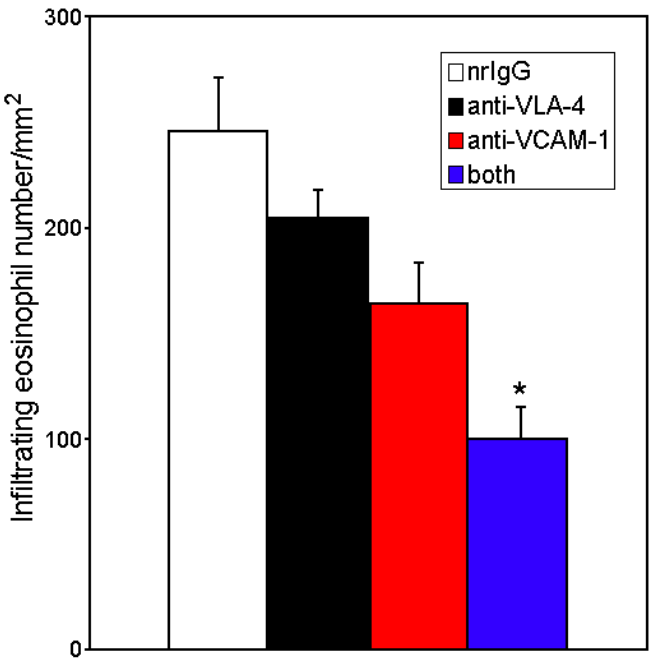


Figure 3. EEffects of anti-VLA-4 and/or anti-VCAM-1 antibodies on actively induced EC. Actively immunized mice developing experimental allergic conjunctivitis were treated with anti-VLA-4 or anti-VCAM-1 antibodies 2 h before and after short ragweed pollen challenge. As the control, the mice were treated with nrIgG. Twenty-four h after the challenge, conjunctivas were harvested for histological analysis and the eosinophils were visualized by Giemsa staining. The eosinophils in the lamina propriae were counted, and the data are presented as the mean number of eosinophils/mm^2. Note that compared to the nrIgG treatment, the number of eosinophils was significantly decreased by treatment with both anti-VLA-4 and anti-VCAM-1 antibodies (indicated as "both" in the image). The asterisk indicates a significant difference for the comparison of "both" treatments to nrIgG treatments ($p<0.05$). Error bars represent the standard error of the mean.

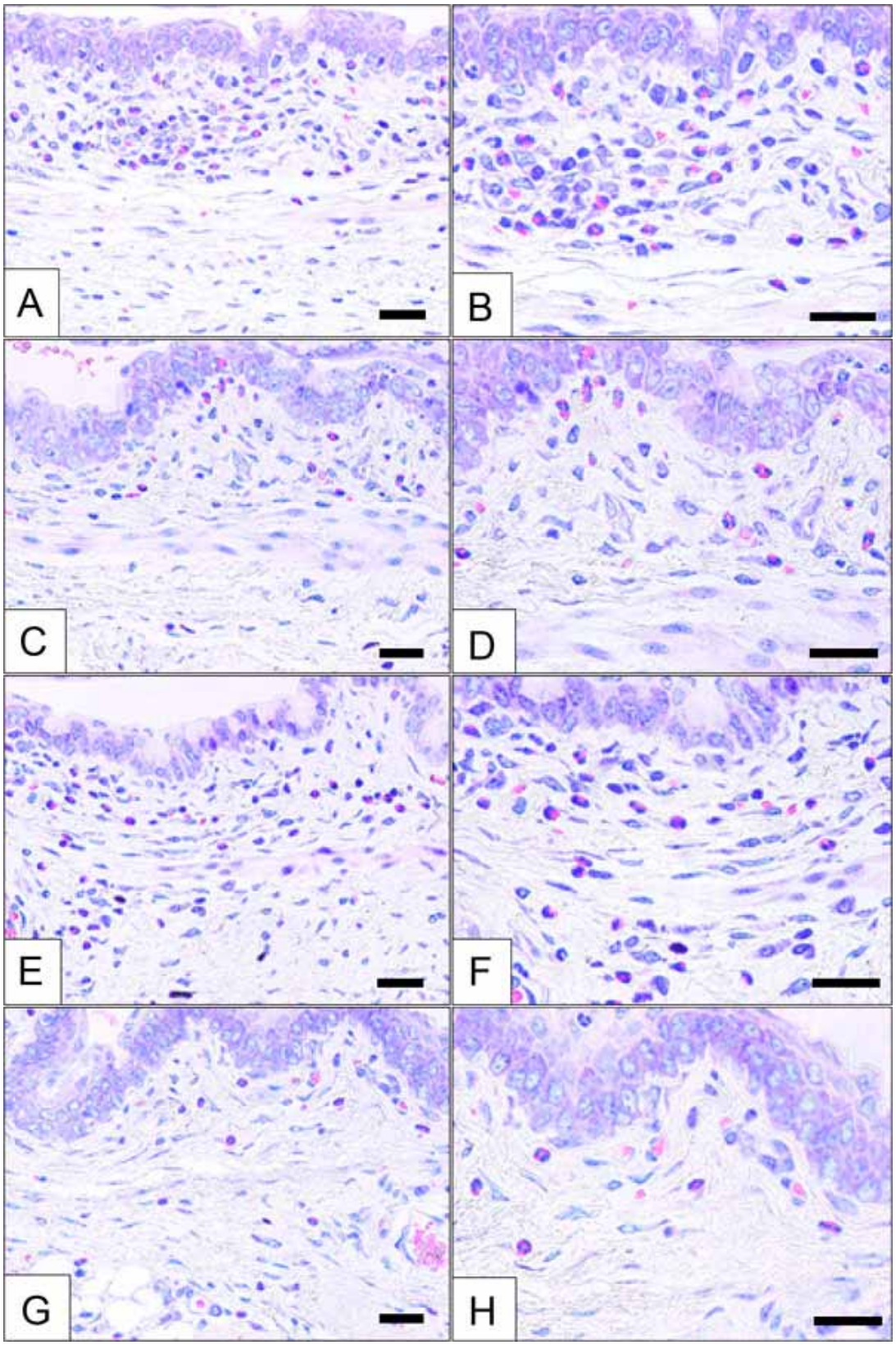


Figure 4. Effects of anti-VLA-4 and anti-VCAM-1 antibodies on passively induced EC: microphotographic evidence. Passively immunized mice developing experimental allergic conjunctivitis were treated with anti-VLA-4 antibody (**C**,**D**), anti-VCAM-1 antibody (**E**,**F**), and both antibodies (**G**,**H**) 2 h before and after short ragweed pollen challenge. Twenty-four h after the challenge, the conjunctivas were harvested for histological analysis, and eosinophils were detected by Giemsa staining. As a control, the passively immunized mice were treated with nrIgG (**A**,**B**). The scale bars represent 20 μm. The sections shown are representative of those from five different mice.

4 in vivo by using a VLA-4-specific monoclonal antibody can suppress the infiltration of eosinophils into the conjunctiva. We also examined the effect of blocking VCAM-1 (the ligand for VLA-4) on the conjunctival infiltration of eosinophils. To this aim, we stained the paraffin-embedded sections with Giemsa and evaluated the infiltration of eosinophils. Infiltration of eosinophils into the conjunctiva was slightly less by treatment with anti-VLA-4 and anti-VCAM-1 antibodies, compared to the treatment with nrIgG (Figure 3). Significant suppression was noted only when the mice were treated with both antibodies (Figure 3).

Treatment with anti-VLA-4 and anti-VCAM-1 antibodies suppresses the infiltration of eosinophils into the conjunctiva induced by short ragweed pollen-primed splenocyte transfer: We have demonstrated previously that RW-primed splenocyte transfer induces more eosinophil infiltration into the conjunctiva compared with active immunization [20]; this infiltration is predominantly mediated by RW-primed CD4+ T cells. Furthermore, we found here that T cells express VLA-4 more frequently than MBP+ eosinophils. Consequently, we asked whether the anti-VLA-4 and anti-VCAM-1 antibodies can also suppress the more severe eosinophil infiltration that is observed in passively immunized mice by splenocyte transfer. We found the antibodies even suppressed the eosinophil infiltration in this situation (Figure 4, Figure 5). Infiltration of eosinophils into the conjunctiva was slightly less by treatment with anti-VCAM-1 antibody (Figure 4E,F) than treatment with nrIgG (Figure 4A,B), but the difference was not statistically significant (Figure 5). Moreover, unlike the case with the actively immunized mice, treatment with anti-VLA-4 antibody alone apparently inhibited infiltration of eosinophils (Figure 4C,D) and this suppression was statistically significant (Figure 5). Similar to active immunization, suppression was most remarkable when the mice were treated with both antibodies (Figure 4G,H, Figure 5).

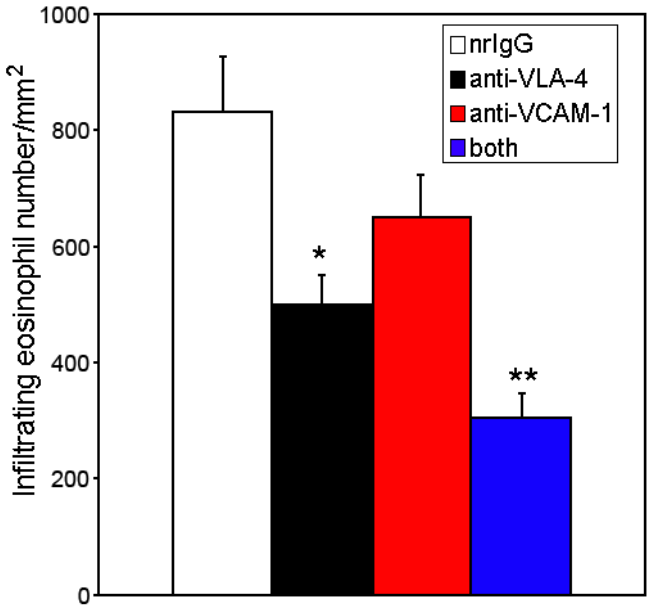


Figure 5. Effects of anti-VLA-4 and anti-VCAM-1 antibodies on passively induced EC: eosinophil counting. The eosinophils in the lamina propriae shown in Figure 4 were counted, and the data are presented as the mean number of eosinophils/mm^2. Note that compared to the nrIgG treatment, the number of eosinophils was significantly decreased by the treatment with anti-VLA-4 antibody alone and both anti-VLA-4 and anti-VCAM-1 antibodies (indicated as "both" in the Figure). The asterisk indicates a significant difference for the comparison of anti-VLA-4 treatments to nrIgG treatments ($p<0.05$); the double asterisks indicate a significant difference for the comparison of "both" treatments to nrIgG treatments ($p<0.01$). Error bars represent the standard error of the mean.

DISCUSSION

VLA-4 has been reported to be expressed in various types of inflammatory cells, including eosinophils and T cells [13], and the interaction between VLA-4 and its ligand VCAM-1 is considered to be important for the infiltration of inflammatory cells [21]. Here, we aimed to investigate the involvement of VLA-4 in the development of EC, a murine experimental AC.

First, to confirm that the infiltrating cells in EC express VLA-4, immunohistochemical analysis was performed. Induction of EC significantly increased the number of VLA-4+ cells in the conjunctiva. We then examined the phenotypes of the infiltrating VLA-4+ cells by immunofluorescent analyses. These revealed that while most of the infiltrating CD3+ and CD4+ cells expressed VLA-4, only half of the MBP+ eosinophils did so; moreover, the colocalization pattern of VLA-4 differed between the T cells and eosinophils. This latter is likely to be due to the fact that MBP is predominantly present in the cytoplasmic granules [22], while VLA-4 [23] and the T-cell markers are expressed on the cell surface. That only the half of the MBP+ eosinophils expressed VLA-4 is unexpected since many previous studies have shown that eosinophils express VLA-4 [24,25]. To exclude the possibility of technical problems, we performed immunocytological analysis of peripheral blood leukocytes from naive WT mice. This confirmed that most of the MBP+ eosinophils in the peripheral blood of Balb/c mice express VLA-4 (data not shown). Thus, while most eosinophils in the peripheral blood express VLA-4, only half of the MBP+ eosinophils in the conjunctivas of mice developing EC do so.

There are several possible explanations for this unexpected result. One is that infiltrating eosinophils express low levels of VLA-4 and therefore the colocalization of MBP and VLA-4 was below detection by immunofluorescent analysis. This low expression of VLA-4 may be because infiltrating eosinophils are activated in the inflamed conjunctiva and therefore VLA-4 expression may have been downregulated [26,27]. Second, the cell surface membrane of eosinophils in the conjunctiva may have been destroyed by the activation of the cells; as a result, VLA-4 expression on the cell surface may not be detected. It has often been observed that the cell surface membrane of degranulated eosinophils is damaged, probably due to the cytotoxic effects of MBP and eosinophil peroxidase [28]. Third, some of the infiltrating MBP+ eosinophils actually do not express VLA-4. If the latter is true, and given the conjunctivas were harvested 24 h after RW challenge, it is likely that

the infiltration of eosinophils may not be dependent on the interaction between VLA-4 and VCAM-1; instead, it may be dependent on other adhesion molecules or chemokines. Finally, our technique may not be enough to completely detect VLA-4 expression, based on the fact that VLA-4 expression was detected in most, but not all, of the peripheral blood eosinophils.

To examine whether the interaction between VLA-4 and VCAM-1 is needed for the infiltration of eosinophils into the conjunctiva, we treated mice that were developing EC with blocking antibodies against VLA-4 and/or VCAM-1. In the case of EC induced by active immunization, treatment with both anti-VLA-4 and anti-VCAM-1 antibodies significantly inhibited the infiltration of eosinophils. This indicates that the interaction between VLA-4 and VCAM-1 is needed for the infiltration of eosinophils into the conjunctiva. Compared to the report of experimental airway inflammation [14], inhibition of recruitment of leukocytes was less in EC (74% inhibition in experimental airway inflammation and 60% inhibition in EC). This may be partly due to the difference of treatment protocol (24 h before challenge in experimental airway inflammation and 2 h before and after challenge in EC). Therefore, to achieve more complete inhibition of eosinophil infiltration, it is necessary to optimize treatment timing.

In Balb/c mice, eosinophil infiltration can be induced by transferring RW-primed splenocytes or T cells, followed by RW challenge [29]. Compared to active immunization, the transfer of RW-primed splenocyes induces more severe eosinophil infiltration [22]. Furthermore, the EC induced by splenocyte transfer is predominantly mediated by cellular immunity, especially by RW-primed CD4+ T cells [30]. In addition, we found here that most of the T cells infiltrating the conjunctiva expressed VLA-4. Therefore, we next examined whether the anti-VLA-4 and anti-VCAM-1 antibodies also suppress eosinophil infiltration when EC is induced by splenocyte transfer. Significant inhibition of eosinophils was noted by treatment with anti-VLA-4 antibody alone or in combination with anti-VCAM-1 antibody. The inhibition was less remarkable when the amount of treating antibodies was one-fifth of that used previously (100 μg per treatment), which indicates that the effects of the antibodies were dose-dependent (data not shown). Suppression of eosinophil infiltration by treatment with both antibodies was more remarkable in EC by splenocyte transfer. In addition, significant suppression of eosinophil infiltration by treatment with anti-VLA-4 antibody was observed only when EC was induced by splenocyte transfer. The reason for these differences is not clear but one possibility is that the infiltration of eosinophils in the nrIgG-treated mice was more severe in EC induced by splenocyte transfer and therefore, significance is more easily obtained. The other is that IgE-mediated eosinophil infiltration, which is involved in EC induced by active immunization but not in that induced by splenocyte transfer [22], is less dependent on the interaction between VLA-4 and VCAM-1 and thus the suppressive effects of the antibodies were more marked in passively induced EC.

Analyses of samples from vernal keratoconjunctivitis (VKC), the most severe form of AC, show that compared to normal controls, VLA-4+ cells were significantly increased and VCAM-1 expression was induced in the conjunctiva [31,32]. These data are basically in accord with our findings. Furthermore, our data showing that treatment with antibodies against VLA-4 and/or VCAM-1 suppresses the infiltration of eosinophils into the conjunctiva confirms that these molecules are indeed involved in the pathogenesis of allergic reactions in the conjunctiva. This suggests that inhibiting the interaction between VLA-4 and VCAM-1 in the conjunctiva may be a valuable therapeutic tool for treating AC characterized by eosinophil infiltration such as VKC.

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