

Allergen-induced CD11b⁺ CD11c^{int} CCR3⁺ macrophages in the lung promote eosinophilic airway inflammation in a mouse asthma model

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Abstract

Although the recruitment of macrophages to the lung is a central feature of airway inflammation, its function in ongoing T_H2 cell-mediated eosinophilic airway inflammation remains controversial. Here, we have demonstrated that the allergen-induced CD11b⁺ CD11c^{int} macrophage expressing CC chemokine receptor 3 (CCR3) in the lung performs a crucial function in the induction of eosinophilic asthma in a murine model. In the lungs of normal mice, residential cells evidencing high granularity phenotypically evidenced CD11b^{int} CD11c⁺ or CD11b⁺ CD11c^{int} cells, appearing at a 2:1 ratio. After allergen challenge, however, this reverses dramatically, up to a ratio of one to six. Approximately 91% of increased CD11b⁺ CD11c^{int} cells evidenced the expression of the CCR3 eotaxin receptor, but not other chemokine receptors, such as CCR5 and CXCR4. Interestingly, the CD11b⁺ CD11c^{int} cells purified from the lungs of OVA (ovalbumin)-sensitized and challenged mice evidenced higher antigen-presenting activity than was observed in CD11b^{int} CD11c⁺ cells. In order to investigate the *in vivo* function of CD11b⁺ CD11c^{int} cells, the cells were isolated from the lungs of OVA-sensitized and challenged mice and then adoptively transferred prior to the allergen challenge of normal mice. In the CD11b⁺ CD11c^{int}-transferred mice airway hyperresponsiveness, eosinophilic inflammation in the lung and T_H2 cytokine secretion in the bronchoalveolar lavage fluids were significantly enhanced as the result of OVA challenge, as compared with the mice that received OVA-primed CD90⁺ T cells or CD11b^{int} CD11c⁺ cells. These findings show that CD11b⁺ CD11c^{int} macrophages expressing CCR3 as key pro-inflammatory cells are both necessary and sufficient for allergen-specific T cell stimulation during ongoing eosinophilic airway inflammation.

Introduction

Asthma is a chronic inflammatory disease of the airways, which is characterized by recurrent episodes of airway obstruction and wheezing (1, 2). Asthma has a variety of causes, including exposure by inhalation to allergens, including microbial and viral components, although in many cases the causative agents are unknown (3–5). Generally, the pathology and the course of disease are similar regardless of the cause, thus indicating that the inflammatory response and airway pathology are common responses of the respiratory tract to injury (3–5).

A predominant T_H2 response to inhaled allergens, which results in airway infiltration by mast cells, lymphocytes, eosi-

nophils, neutrophils and T_H2-type cytokines, may play a crucial role in the induction of airway inflammation (6, 7). CD4 T_H2 cells are believed to initiate and perpetuate disease. Generally, these cells comprise a small percentage of the total leukocytes in the lung (8–10), but the levels of these cells are increased markedly in the airways of asthmatic individuals. This result suggests that CD4 T_H2 cells promote allergic diseases of the airways, and the study of these cells has helped to define the pathways by which T_H2 cells and T_H2 cytokines effect these changes (6–12). T_H2 cytokines, including IL-4, IL-5 and IL-13, have been definitively associated with allergic asthma (6–12). Persistent T_H2 cytokine production

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ultimately induces significant pathological changes, which occur via multiple pathways. Among them, IL-4 and IL-13 are involved specifically in Ig class switching to IgE, whereas IL-4 and IL-10 perform a crucial function in T_H2 cell commitment (12–14). In addition to the T_H2 response, the T_H1 response against allergens is involved in airway inflammation. A recent study has shown that T_H1- and T_H2-associated cytokines play opposing roles in the pathogenesis of allergic asthma (15). T_H1 cytokines, including IFN- γ and IL-12, exert a protective effect against the development of asthma in children after respiratory viral infections (15). Other cytokines, including tumor necrosis factor, have been demonstrated to amplify the effects of asthmatic inflammation (16–18). Recently, regulatory T cells (CD4⁺CD25⁺ cells and NK T cells) have been suggested to play a role in the control of asthma and allergy, but the specific mechanisms underlying this phenomenon remain unknown (19, 20).

In addition to T_H2 lymphocytes, resident or recruited alveolar macrophages (AMs) exist predominantly in the alveolar spaces and conducting airways, and are known to be important to the regulation of immune reactions against allergens derived from microbial and viral components (21–23). Several reports have suggested that resident AMs contribute to the suppression of antigen-specific activation and the antigen-presentation activities of dendritic cells (24–27). Mice that received AMs from non-sensitized mice were protected against the development of airway hyperresponsiveness (AHR), whereas the AMs from sensitized mice participate in the inflammatory response (26). However, it remains difficult to distinguish original resident AMs from more recently recruited AMs. Tissue-recruited macrophages also function as key regulators in cells undergoing local activation in response to a variety of inflammatory and immune stimuli (23). Enhanced macrophage recruitment may result in the accumulation of tissue macrophages, which are activated by T_H2 cytokines, including IL-4 and IL-13 (23). IL-4 and IL-13 up-regulate the expression of the mannose receptor and MHC class II molecules by macrophages, which in turn stimulate endocytosis and antigen presentation, and induce the expression of selective chemokines, including macrophage-derived chemokines, which are believed to be involved in cell recruitment (23). Nevertheless, the characteristics and functions of recruited or increased macrophages during airway inflammation remain unclear. The principal objective of the present study was, therefore, to study macrophages infiltrating into the lungs of sensitized and challenged mice and to determine whether the cells can promote airway inflammation when adoptively transferred into normal mice.

Methods

Mice

Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA), housed under pathogen-free conditions at the animal facility of the Asan Institute for Life Science (Seoul, Korea) and maintained on an ovalbumin (OVA)-free diet. All experimental procedures were approved by the Animal Ethics committee of the Asan Institute for Life Science.

Sensitization and aerosol challenge with OVA

At the age of 6–7 weeks, the mice were sensitized to OVA via two intra-peritoneal injections of 20 μ g OVA (Grade VI; Sigma, St Louis, MO, USA) emulsified with 50 μ g alum (Sigma-Aldrich) in PBS in a total volume of 100 μ l on days 1 and 14. The control mice received a saline injection rather than the OVA/alum solution. On days 21, 22 and 23, the mice were exposed to aerosol challenge with 30 μ g ml⁻¹ OVA in PBS for 30 min. The aerosol challenge was performed in a Plexiglas exposure chamber coupled to a Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment, Richmond, VA, USA; particle size 2–3 μ m) driven by compressed air at a flow rate of 6 l min⁻¹. Control mice inhaled PBS alone under the same conditions as used for the experimental group.

Measurement of AHR

Twenty-four hours after the final aerosol challenge with OVA, AHR was assessed in conscious and unrestrained mice via whole-body plethysmography. Each mouse was placed in a plastic chamber and exposed to aerosolized normal saline for 3 min followed by increasing concentrations of aerosolized methacholine solutions (2.5, 5, 10 and 20 mg ml⁻¹; Sigma-Aldrich), each of which was administered for 3 min. Ten seconds after each aerosol exposure, 3-min enhanced pause (Penh) readings, which were calculated automatically on the basis of the mean pressure generated in the plethysmography chamber during inspiration and expiration combined with the time of each phase, were recorded and averaged.

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) fluid samples were centrifuged for 10 min at 170 $\times g$, and each cell pellet was re-suspended in 0.5 ml of sterile PBS. The total number of viable cells was determined in a Neubauer counting chamber using trypan blue solution. Differential cell counts were conducted using cytocentrifuge preparations of BAL fluid stained with Diff-Quik solution (Dade Diagnostics of Puerto Rico Inc., Aguada, PR, USA). For cytokine measurement, the BAL fluid was centrifuged for 10 min at 170 $\times g$ and the cell pellet was re-suspended in 0.5 ml of sterile PBS. Cytokine measurements were conducted simultaneously. Levels of IL-4, IL-5, IFN- γ and IL-13 were measured via ELISA using matched antibody pairs (R&D Systems Inc., Minneapolis, MN, USA).

Preparation of cells infiltrated into the lungs and flow cytometry analysis

The lungs were sliced into small cubes and incubated for 30 min in 5 ml of RPMI 1640 solution containing 0.1% collagenase (Type IV; Sigma-Aldrich), 0.01% hyaluronidase (Sigma-Aldrich) and 0.002% DNase (Sigma-Aldrich). Infiltrated cells were separated via centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK). For staining, one million cells were incubated on ice for 30 min with FITC-conjugated anti-CD11c (HL3), Alexa Fluor-conjugated anti-CC chemokine receptor (CCR3) (83103), PE-conjugated anti-CCR5

(C34-3448), PE-conjugated anti-CXCR4 (2B11/CXCR4) and Allophycocyanin (APC)-conjugated anti-CD11b (M1/70) antibodies. The reagents used in this study were purchased from PharMingen (San Diego, CA, USA). In some experiments, PE-conjugated anti-F4/80 (eBioscience), FITC-conjugated anti-CD205 (AbD Serotec) and PE-conjugated anti-CD11c (BD Biosciences) antibodies were used. The cells were washed three times with PBS buffer and analyzed with a FACScaliber apparatus (Becton Dickinson, Franklin Lakes, NJ, USA).

Isolation of CD90⁺ T cells from spleen, CD11b^{int} CD11c⁺ cells and CD11b⁺ CD11c^{int} cells from lungs

The lungs were sliced into small cubes and then incubated for 30 min in 5 ml RPMI 1640 solution containing 0.1% collagenase (Type IV; Sigma-Aldrich), 0.01% hyaluronidase (Sigma-Aldrich) and 0.002% DNase (Sigma-Aldrich). Infiltrated cells were separated via centrifugation on Percoll (GE Healthcare). CD11c⁺ or CD11b⁺ cells from infiltrated cells were isolated using Auto-MACS (Miltenyi Biotec, Auburn, CA) with anti-CD11c or CD11b antibody conjugated to magnetic beads in accordance with the manufacturer's instructions (Miltenyi Biotec). Enriched CD11b⁺ or CD11c⁺ cells were further stained with anti-CD11b and anti-CD11c antibodies to sort CD11b^{int} CD11c⁺ and CD11b⁺ CD11c^{int} cells. The stained cells were gated into CD11b^{int} CD11c⁺ and CD11b⁺ CD11c^{int} cells according to the expression of CD11b or CD11c molecules and then sorted using the FACSvantage system (Becton Dickinson). The purity of >96% CD11b^{int} CD11c⁺ cells and CD11b⁺ CD11c^{int} cells could be obtained thusly. In the case of CD11b⁺ CD11c^{int} cells, approximately 91% of the cells expressed the CCR3 eotaxin receptor. For the sorting of the CD90⁺ T cells, splenocytes were prepared from control mice treated with PBS or OVA-sensitized and challenged mice. CD90⁺ (Thy-1) cells were isolated from the cells using Auto-MACS (Miltenyi Biotec) with anti-CD90 antibody conjugated with magnetic beads in accordance with the manufacturer's instructions (Miltenyi Biotec). CD90⁺ T cells (>96%) could be obtained thusly.

Cell proliferation assay and measurement of secreted T_H2 cytokines

Different numbers of purified cells (1.5×10^5 , 0.75×10^5 and 0.014×10^5), CD11b^{int} CD11c⁺ and CD11b⁺ CD11c^{int} cells, were cultured with 5×10^5 CD90⁺ cells in 96-well round-bottom tissue-culture plates (Corning, NY, USA) in the presence or absence of $30 \mu\text{g ml}^{-1}$ OVA for 96 h. Prior to the harvesting of the cells, the cultures were pulsed with $1 \mu\text{Ci } ^3\text{H-thymidine}$ for 8 h, and harvested with a Tomcat Mach II harvester (Wallac, Turku, Finland) onto filter plates, which were read with a β -counter. Each experiment was conducted in triplicate. For the measurement of secreted T_H2 cytokines, 5×10^5 CD90⁺ cells were cultured with 1.5×10^5 CD11b⁺ CD11c^{int} cells in 96-well round-bottom tissue-culture plates in the presence or absence of $30 \mu\text{g ml}^{-1}$ OVA for 72 h. Levels of IL-4, IL-5 and IL-13 in the culture supernatants were measured via ELISA using matched antibody pairs (R&D Systems Inc.).

Adoptive transfer of CD90⁺ T cells, CD11b^{int} CD11c⁺ cells and CD11b⁺ CD11c^{int} cells

CD90⁺ T cells, CD11b^{int} CD11c⁺ cells and CD11b⁺ CD11c^{int} cells of OVA-sensitized and challenged mice were isolated as described above. CD90⁺ T cells (5×10^6), 2.5×10^6 CD11b^{int} CD11c⁺ cells and 2.5×10^6 CD11b⁺ CD11c^{int} cells were intravenously transferred into Balb/c recipient mice on day 0. On days 2, 3 and 4, the mice were exposed to aerosol challenge with $30 \mu\text{g ml}^{-1}$ OVA in PBS for 30 min, which was performed in a Plexiglas exposure chamber coupled to a Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment; particle size 2–3 μm) driven by compressed air at a flow rate of 6 l min⁻¹. Control mice inhaled PBS alone under the same conditions as employed for the experimental group.

Lung histology assay

For histopathologic assessment, the lungs were fixed overnight in formalin, embedded in paraffin, sectioned to 4 μm and stained with hematoxylin and eosin. The degree of lung inflammation was evaluated via the previously described method (28). In brief, lung lesions, including alveolar septal infiltrates, perivascular infiltrates and peribronchial infiltrates, were subjectively graded on a numeric scale of 1–4, corresponding to minimal, mild, moderate and marked severity by 2 independent pathologists who were both blinded to the treatments. Overall histopathologic scores were generated from the average of the individual lesion scores for each mouse.

Statistical analysis

All data are expressed as the means $\pm$ standard error of means. For comparisons between groups, Mann–Whitney and Kruskal–Wallis tests were used. Statistical significance was defined as $P < 0.05$ or $P < 0.01$.

Results

Increase in the infiltration of CD11b⁺ CD11c^{int} macrophages expressing CCR3 into the lungs

Macrophages are a heterogeneous population of resident and recruited cells that are found in all organs, and are involved in tissue homeostasis and defense, as well as inflammation (23). During inflammation, tissue-infiltrated macrophages derived from the peripheral system undergo local activation via surface ligands and cytokines, induce a spectrum of pro- and anti-inflammatory states. In this study, we attempted to evaluate the characteristics of macrophages infiltrated into the lung tissues after allergen challenge in a murine experimental asthma model (29). OVA-sensitized and challenged mice were prepared in accordance with the experimental protocol depicted in Fig. 1(A). In order to confirm the model, we measured airway responsiveness to methacholine 24 h after the final challenge. Enhanced pause was greater in the OVA-sensitized/challenged mice than in the control mice (PBS/PBS), by up to 3-fold in the mice treated with 5 mg ml^{-1} of methacholine (Fig. 1B, open circles). Moreover, in OVA-sensitized and challenged mice, the levels of T_H2 inflammatory cytokines,

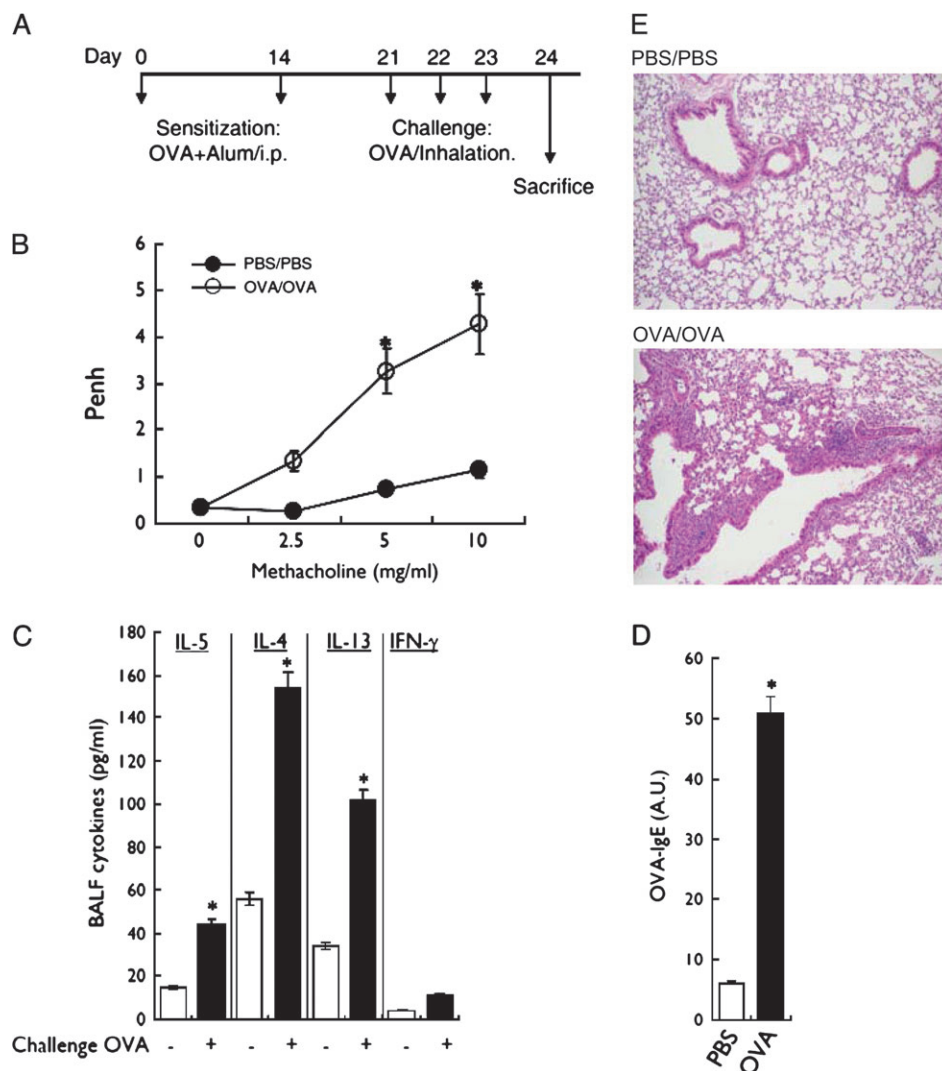


Fig. 1. Macrophages and eosinophils markedly increase in the BAL fluids of OVA-sensitized and challenged mice. (A) Balb/c mice ($n = 6$) were sensitized to OVA by two intra-peritoneal injections of 20 μg of OVA emulsified with 50 μg of alum in PBS in a total volume of 100 μl on days 0 and 14. Control mice ($n = 6$) received a saline injection rather than the OVA/alum solution. On days 21, 22 and 23, the mice were exposed to aerosol challenge with 30 $\mu\text{g ml}^{-1}$ of OVA in PBS for 30 min, as described in Methods. (B–D) Twenty-four hours after OVA inhalation, AHR to methacholine (2.5 mg, 5 mg and 10 mg) was assessed (B). BAL fluids were harvested, and the concentrations of IL-5, IL-4, IL-13 and IFN- γ were assayed by ELISA (C). * $P < 0.01$ compared with control mice. For measurement of OVA-specific IgE in serum, mice were bled, the sera were collected and OVA-specific IgE was quantified by ELISA (D). * $P < 0.01$ compared with control mice. (E) For lung histology, the mice were sacrificed and their lungs were removed, fixed in 10% buffered formalin and processed for routine histology in paraffin. Tissue sections were stained with hematoxylin/eosin and then examined by light microscopy by a board-certified pathologist (DGR). Digital images were taken of each sample.

including IL-4, IL-5 and IL-13, and the level of OVA-specific IgE were increased significantly (Fig. 1C and D). In addition, histological analyses indicated increased infiltrations of inflammatory cells in the OVA-sensitized/challenged mice (Fig. 1E).

To investigate the function of macrophages infiltrated into the lung, infiltrated cells were isolated with Percoll, in accordance with the following experimental procedures, which were also described in Methods. Isolated cells were stained with anti-CD11b, anti-CD11c and anti-CCR3, anti-CCR5 or anti-CXCR4 antibodies, after which phenotypic analysis was conducted via flow cytometry. As is shown in Fig. 2(A), high granularity cells were gated by Forward Scatter (FSC) versus Side Scatter (SSC) characteristics and characterized further via the expression of CD11b or

CD11c molecules. In the control mice, CD11b⁺ macrophages resident in the lung phenotypically manifested as CD11b^{int} CD11c⁺ or CD11b⁺ CD11c^{int} cells, appearing in a 2:1 ratio (Fig. 2B). Interestingly, upon OVA allergen challenge, their proportions were reversed dramatically up to a 1:6 ratio. CD11b^{int} CD11c⁺ cells decreased by up to 3-fold ($24 \pm 2.1\%$ to $7 \pm 1.4\%$), whereas CD11b⁺ CD11c^{int} cells increased by up to 3.5-fold ($13 \pm 1.8\%$ to $44 \pm 2.7\%$) (Fig. 2B). Moreover, the absolute numbers of CD11b⁺ CD11c^{int} cells were significantly higher in the ovalbumin immunized and ovalbumin challenged (OVA/OVA) mice than in the PBS/PBS specimens (Fig. 2C). In order to determine whether increased CD11b⁺ CD11c^{int} cells are macrophages, we conducted further staining with anti-F4/80 antibody for

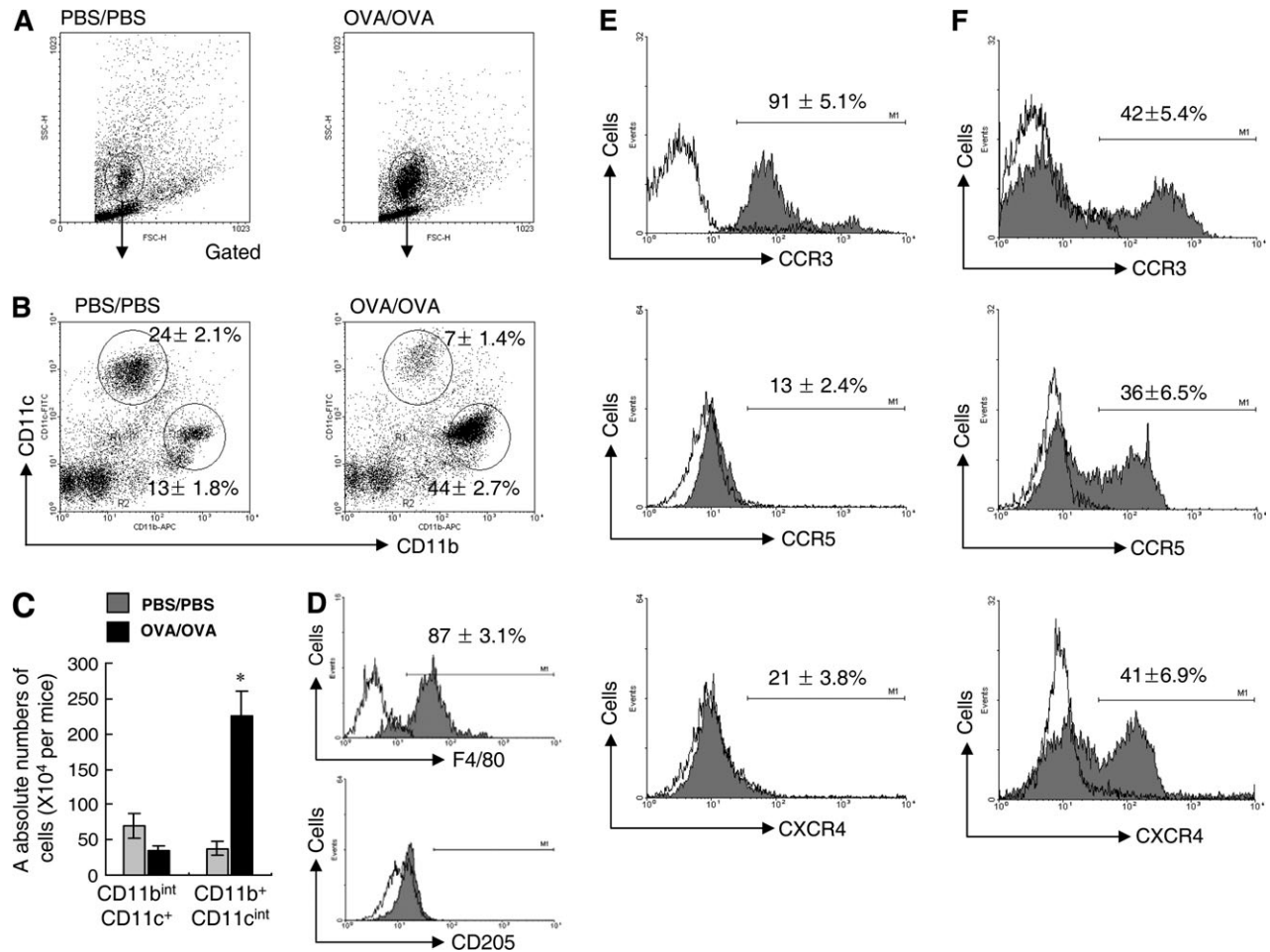


Fig. 2. CD11b⁺ CD11c^{int} cells expressing CCR3 markedly increase in the lung tissue of OVA-sensitized and challenged mice. The mice were challenged with OVA in sensitized mice, as described in the legend for Fig. 1. The lungs were isolated, sliced into small cubes and then incubated for 30 min in 5 ml RPMI 1640 solution containing 0.1% collagenase, 0.01% hyaluronidase and 0.002% DNase. Infiltrated cells were separated via centrifugation on Percoll. Cells (10⁶) were incubated on ice for 30 min with anti-CD11c, anti-CCR3, anti-CCR5, anti-CXCR4 and anti-CD11b antibodies. (A) High granularity cells were gated in the FSC/SSC plot. (B) Within the set gate, the expressions of CD11b and CD11c molecules were analyzed. (C) The absolute numbers of CD11b^{int} CD11c⁺ and CD11b⁺ CD11c^{int} cells in the lung tissue were shown. The data were expressed as the means ± standard error (SEs); *n* = 6 for each group; **P* < 0.05 as compared with saline-challenged mice (PBS/PBS). (D–F) Infiltrated cells were isolated from OVA-sensitized and challenged mice. After logical gating was conducted via a high granularity cell gate in (A) and CD11b⁺ CD11c^{int} cell gate in (B), expression of F4/80 antigen and CD205 antigen was evaluated in the set gate (D). After logical gating was conducted, expression of CCR3, CCR5 or CXCR4 receptors were evaluated in CD11b⁺ CD11c^{int} cells (E) or CD11b⁺ CD11c⁺ cells (F). Open histogram indicates isotypic control corresponding to each antibody. The data were expressed as the means ± SEs; *n* = 6 for each group.

macrophages and anti-CD205 antibody for dendritic cells. As shown in Fig. 2(D), 87% of CD11b⁺ CD11c^{int} cells strongly expressed F4/80 antigen, but not CD205, thereby indicating that CD11b⁺ CD11c^{int} cells are phenotypically macrophages. More interestingly, approximately 91% of the CD11b⁺ CD11c^{int} cells expressed the CCR3 eotaxin receptor, whereas no significant expressions of other chemokine receptors, including CCR5 and CXCR4, could be detected (Fig. 2E). However, the CD11b⁺ CD11c⁺ cells contained some CCR3⁺ (up to 42%), CCR5⁺ (up to 36%) or CXCR4⁺ (up to 41%) cells (Fig. 2F). Although we are unable to define clearly the origin of the CD11b⁺ CD11c^{int} CCR3⁺ cells, the observed increase in CD11b⁺ CD11c^{int} CCR3⁺ cells may be the result of allergen challenge and may perform an essential function in ongoing airway inflammation. Therefore, we further investigated the functions of CD11b⁺ CD11c^{int} cells expressing CCR3 *in vitro* and *in vivo*.

CD11b⁺ CD11c^{int} cells have the potential ability to induce the proliferation of allergen-specific T cells

Although it has been well established that the immunological function of macrophages is generally associated with the ability of antigen presentation and the secretion of inflammatory cytokines (30, 31), previous reports have shown that, in cases of allergic inflammation, AMs can suppress the activation of T cells as well as the antigen presentation activity of dendritic cells (24–27). Therefore, we attempted to determine the immunological function of allergen-induced CD11b⁺ CD11c^{int} cells in the context of antigen presentation. CD11b⁺ CD11c^{int} cells, CD11b^{int} CD11c⁺ cells and CD90⁺ T cells were isolated from control mice (PBS/PBS) and OVA-sensitized and challenged mice (OVA/OVA), respectively, in accordance with the following experimental procedures,

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which were also described in Methods. Different numbers of $CD11b^+ CD11c^{int}$ cells and $CD11b^{int} CD11c^+$ cells as stimulator cells were co-cultured for 96 h with $CD90^+$ T cells as responder cells in the presence or absence of OVA antigen. The proliferation activity of antigen-specific T cells in those conditions was assessed via ^3H -incorporation assays. As had been expected, $CD11b^+ CD11c^{int}$ cells or $CD11b^{int} CD11c^+$ cells derived from control mice did not induce the proliferation of $CD90^+$ T cells derived from control mice, even in the presence of OVA antigen (Fig. 3A). However, $CD11b^+ CD11c^{int}$ cells or $CD11b^{int} CD11c^+$ cells derived from OVA-sensitized and challenged mice (OVA/OVA) significantly induced the proliferation of $CD90^+$ T cells derived from OVA/OVA mice in an antigen-dependent manner (Fig. 3B). More interestingly, the ability to induce T cell proliferation was sig-

nificantly higher in the $CD11b^+ CD11c^{int}$ cells than in the $CD11b^{int} CD11c^+$ cells. In order to determine whether the proliferation activity was restricted on OVA-primed $CD90^+$ T cells, we conducted additional cell proliferation assays by using $CD11b^+ CD11c^{int}$ cells purified from OVA/OVA mice and $CD90^+$ T cells purified from PBS/PBS mice. As is shown in Fig. 3(C), $CD11b^+ CD11c^{int}$ cells purified from OVA/OVA mice induce marginal activity to induce the proliferation of $CD90^+$ T cells purified from PBS/PBS mice, even in the presence of OVA antigen, when compared with the results shown in Fig. 3(B). These results strongly suggest that, at least in this murine model, allergen-induced $CD11b^+ CD11c^{int}$ cells expressing CCR3 can potentially induce the proliferation of antigen-specific T cells. Furthermore, the cells significantly induced the secretion of T_H2 cytokines, including IL-4, IL-5

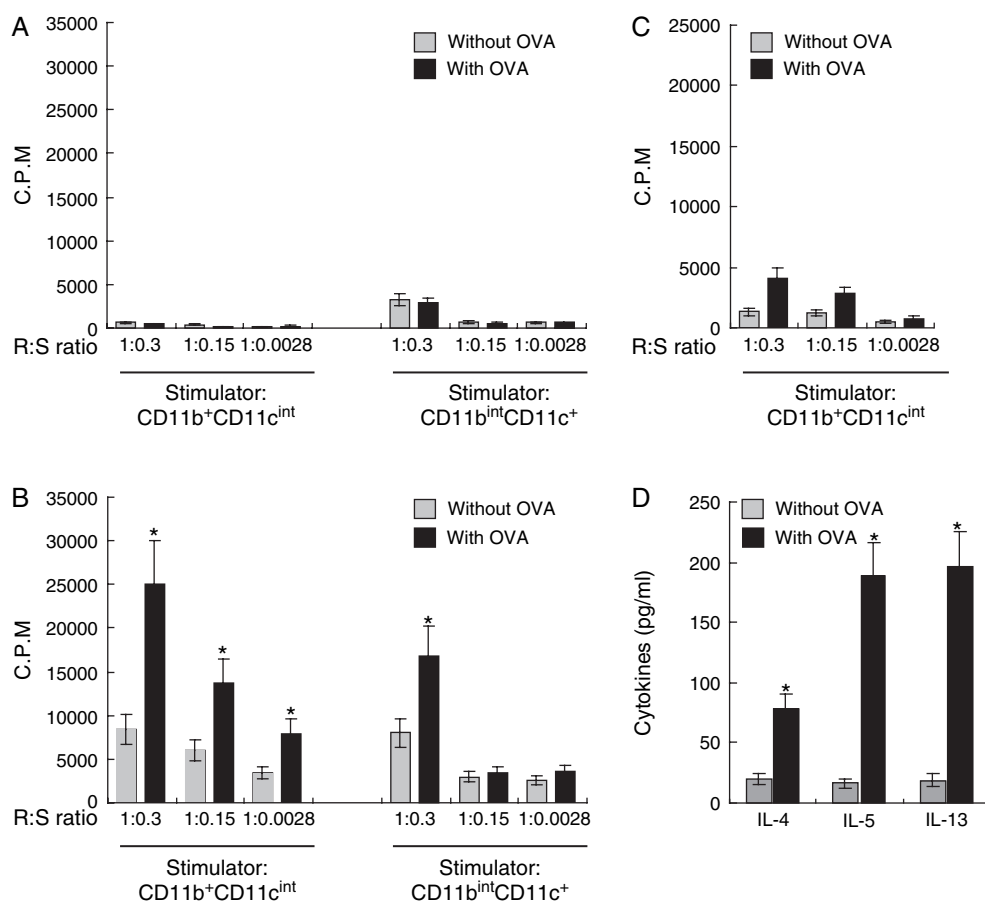


Fig. 3. $CD11b^+ CD11c^{int}$ cells derived from OVA-sensitized and challenged mice induce proliferation of OVA-specific T cells. $CD90^+$ T cells as responder cells (R) were isolated from mice treated with PBS (A) and OVA-sensitized and challenged mice (B), as described in Methods, respectively. $CD11b^{int} CD11c^+$ and $CD11b^+ CD11c^{int}$ cells as stimulator cells (S) were isolated from mice treated with PBS (A) and OVA-sensitized and challenged mice (B), as described in Methods, respectively. Different numbers of purified cells (1.5×10^5 , 0.75×10^5 and 0.014×10^5), $CD11b^{int} CD11c^+$ and $CD11b^+ CD11c^{int}$ cells were cultured with 5×10^5 $CD90^+$ cells in 96-well round-bottom tissue-culture plates in the presence (filled squares) or absence (hatched squares) of $30 \mu g ml^{-1}$ OVA for 96 h. (C) $CD90^+$ T cells and $CD11b^+ CD11c^{int}$ cells were isolated from PBS-treated mice or OVA-sensitized and challenged mice, respectively. Different numbers of purified $CD11b^+ CD11c^{int}$, as indicated, were cultured with 5×10^5 $CD90^+$ cells in 96-well round-bottom tissue-culture plates in the presence (filled squares) or absence (hatched squares) of $30 \mu g ml^{-1}$ OVA for 96 h. Before the harvesting of the cells, the cultures were pulsed for 8 h with $1 \mu Ci$ ^3H -thymidine, harvested and read with a β counter. Each experiment was conducted in triplicate. The data were expressed as the means $\pm$ standard error (SEs); * $P < 0.05$ as compared with that of without OVA. (D) $CD90^+$ T cells and $CD11b^+ CD11c^{int}$ cells were isolated from OVA-sensitized and challenged mice. $CD90^+$ T cells (5×10^5) were cultured with 1.5×10^5 $CD11b^+ CD11c^{int}$ cells in 96-well round-bottom tissue-culture plates in the presence (filled squares) or absence (hatched squares) of $30 \mu g ml^{-1}$ OVA for 72 h. Levels of IL-4, IL-5 and IL-13 cytokines in supernatants were measured via ELISA. Each experiment was conducted in triplicate. The data were expressed as the means $\pm$ SEs; * $P < 0.05$ as compared with that of without OVA.

and IL-13, from OVA-specific T cells (Fig. 3D), thereby indicating a potential role in enhancing T_H2 response by antigen presentation.

CD11b⁺ CD11c^{int} cells induce AHR *in vivo*

Based on the above results, we attempted to further determine whether allergen-induced CD11b⁺ CD11c^{int} cells can contribute to the induction of eosinophilic inflammation *in vivo*. To this end, we utilized the adoptive transfer method. CD11b⁺ CD11c^{int}, CD11b^{int} CD11c⁺ and CD90⁺ T cells were isolated from the lungs of OVA-sensitized and challenged mice, as described in Methods. CD90⁺ T cells (5×10^6), 2.5×10^6 CD11b^{int} CD11c⁺ cells or 2.5×10^6 CD11b⁺ CD11c^{int} cells were intravenously transferred into normal mice prior to OVA challenge, as depicted in Fig. 4(A). In this study, mice that received OVA-primed CD90⁺ T cells were utilized as a positive control to compare AHR and airway inflammation with that of mice that had received CD11b^{int} CD11c⁺ cells or CD11b⁺ CD11c^{int} cells, as several reports had suggested that OVA-specific T cells can induce AHR and airway inflammation when adoptively transferred into normal mice prior to allergen challenge (32–34). Twenty-four hours after the final challenge, we measured AHR to metha-

choline in these mice. As expected, mice that received OVA-primed CD90⁺ T cells evidenced significant increases in the levels of Penh (Fig. 4B, open circles), thereby indicating that allergen-specific T cells are sufficient to induce AHR. This result was basically consistent with the previous results (32–34). In addition, mice receiving CD11b^{int} CD11c⁺ cells or CD11b⁺ CD11c^{int} cells also evidenced increases in the levels of Penh in a dose-dependent manner as the result of methacholine treatment. Interestingly, Penh levels were significantly higher in mice receiving CD11b⁺ CD11c^{int} cells (Fig. 4B, open triangles) than in mice receiving OVA-primed CD90⁺ T cells (Fig. 4B, open circles) or CD11b^{int} CD11c⁺ cells (Fig. 4B, closed triangles). These results indicate that allergen-induced CD11b⁺ CD11c^{int} cells, as well as allergen-specific T cells, are involved in the induction of AHR *in vivo*.

CD11b⁺ CD11c^{int} cells induce eosinophilic inflammation via an increase in eosinophil infiltration into BAL fluids and the secretion of T_H2 cytokines such as IL-5 and IL-13 into BAL fluids

We then attempted to determine whether the increase in AHR in mice receiving CD11b⁺ CD11c^{int} cells was associated with eosinophilic airway inflammation. Analyses of the

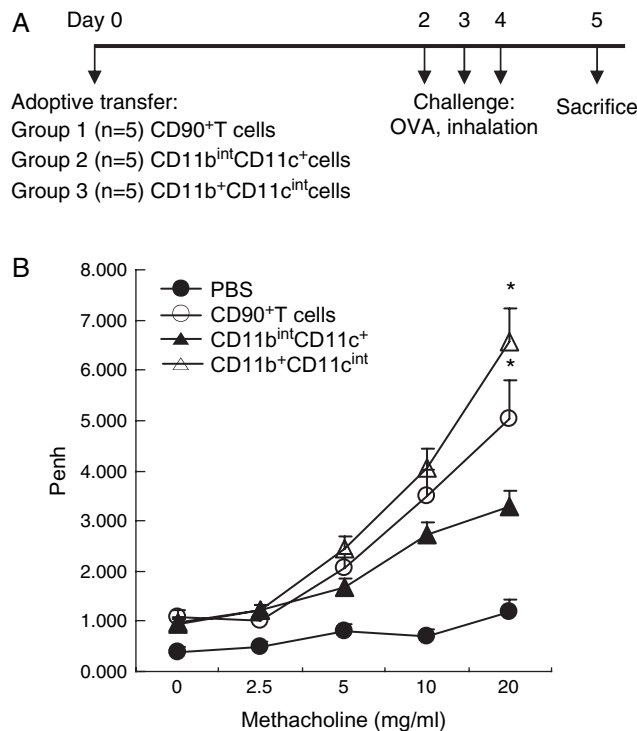


Fig. 4. A experimental protocol for adoptive transfer and AHR. CD90⁺ T cells, CD11b^{int} CD11c⁺ and CD11b⁺ CD11c^{int} cells were purified from OVA-sensitized and challenged mice ($n = 25$), as following experimental procedures described in Methods. (A) CD90⁺ T cells (5×10^6), 2.5×10^6 CD11b^{int} CD11c⁺ cells and 2.5×10^6 CD11b⁺ CD11c^{int} cells were transferred intravenously into Balb/c recipient mice on day 0. On days 2, 3 and 4, the mice were exposed to aerosol challenge with $30 \mu\text{g ml}^{-1}$ OVA in PBS for 30 min, performed in a Plexiglas exposure chamber coupled to a Jet nebulizer driven by compressed air at a flow rate of 6 l min^{-1} . Control mice ($n = 5$) inhaled PBS alone under the same conditions as used for the experimental group. (B) At 24 h after the final aerosol challenge with OVA, AHR was assessed in conscious and unrestrained mice via whole-body plethysmography. Each mouse was placed in a plastic chamber and exposed to aerosolized normal saline for 3 min followed by increasing concentrations of aerosolized methacholine solutions, each of which was administered for 3 min. Ten seconds after each aerosol exposure, 3-min Penh readings, calculated automatically on the basis of the mean pressure generated in the plethysmography chamber during inspiration and expiration combined with the time of each phase, were recorded and averaged. The data were expressed as the means $\pm$ standard errors; $n = 5$ for each group; * $P < 0.05$ as compared with mice that received with CD11b^{int} CD11c⁺ cells.

BAL fluids of mice that received OVA-primed CD90⁺ T cells revealed an increased number of total BAL cells, lymphocytes and eosinophils (Fig. 5, filled squares), as compared with those observed in PBS-treated control mice (Fig. 5, open squares). More interestingly, analyses of the BAL fluids of mice that received CD11b⁺ CD11c^{int} cells revealed a markedly increased number of total BAL cells, macrophages and eosinophils (Fig. 5, checked box). CD11b⁺ CD11c^{int} cells, in particular, significantly enhanced the infiltration of eosinophils, as compared with that observed with OVA-specific CD90⁺ T cells or CD11b^{int} CD11c⁺ cells. By way of contrast, mice receiving CD11b^{int} CD11c⁺ cells evidenced only a weak induction of the infiltration of inflammatory cells (Fig. 5, hatched box). These results were very consistent with those of the AHR observed in Fig. 4(B). In accordance with this, histological analysis also revealed significant increases in peribronchial and perivascular inflammatory infiltrates in the lung tissues of mice that had received OVA-primed CD90⁺ T cells (Fig. 6B) or CD11b⁺ CD11c^{int} cells (Fig. 6D), as compared with mice that had received CD11b^{int} CD11c⁺ cells (Fig. 6C) or were treated with PBS (Fig. 6A). In order to compare the degree of lung inflammation, histopathologic analysis was performed (28). Lung lesions from each animal, including alveolar septal infiltrates, perivascular infiltrates and combined bronchus-associated lymphoid tissue hyperplasia and peribronchiolar infiltrates were subjectively graded on a severity scale of minimal (1), mild (2), moderate (3) and marked (4). Lesion scores were expressed as average mean values ($n = 5$). As shown in Fig. 6(E), Lesion scores were 2.8 ± 0.44 in CD90⁺ T cell-treated mice, 1.8 ± 0.44 in CD11b^{int} CD11c⁺ cell-treated mice and 3.6 ± 0.54 in CD11b⁺ CD11c^{int} cell-treated mice, respectively. These results strongly suggest that CD11b⁺ CD11c^{int} cells as well as OVA-specific T cells are

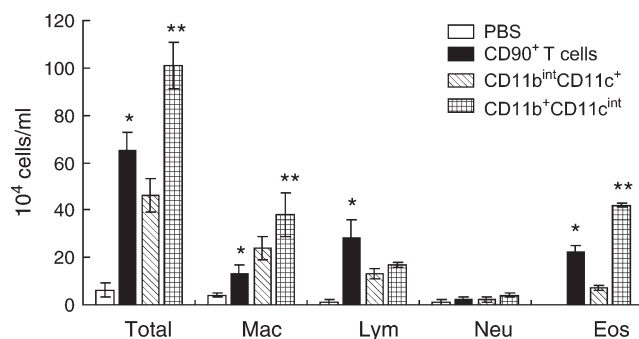


Fig. 5. CD11b⁺ CD11c^{int} cells induce inflammatory cell infiltration in the BAL fluids of adoptively transferred mice. The number of total cells and differential cellular components of BAL fluids from mice treated with PBS (open squares), mice that received CD90⁺ T cells (filled squares), mice that received CD11b^{int} CD11c⁺ cells (hatched box) and mice that received CD11b⁺ CD11c^{int} cells (checked box) were counted 24 h after the final challenge with OVA. Differential cell counts were conducted on a minimum of 400 cells in each of four different random locations to identify macrophages (Mac), lymphocytes (Lym), neutrophils (Neu) and eosinophils (Eos). The data were expressed as the means $\pm$ standard errors; $n = 5$ for each group; * $P < 0.05$ as compared with saline-challenged mice (PBS), ** $P < 0.05$ as compared with mice that received with CD90⁺ T cells.

relevant to allergen-dependent eosinophilic inflammation via the modulation of eosinophil infiltration. Eosinophilic airway inflammation and AHR are also associated with T_H2 cytokines, including IL-4, IL-5 and IL-13 (6–12) derived from T_H2 cells. IL-4 regulates allergic inflammation via the promotion of T_H2 cell differentiation, IgE synthesis and mucus hypersecretion; IL-5 promotes eosinophilic inflammation and infiltration into the airways; and IL-13 promotes B cell differentiation and is capable of inducing isotype switching in B cells, thereby generating IgE (6–12). As is shown in Fig. 3, CD11b⁺ CD11c^{int} cells potently induced the proliferation of OVA-specific T cells and T_H2 cytokine secretion. Therefore, we also attempted to determine whether adoptively transferred CD11b⁺ CD11c^{int} cells could also enhance the levels of these T_H2 cytokines *in vivo*. We determined the concentrations of IL-4, IL-5 and IL-13 in BAL fluids. The transfer of OVA-primed CD90⁺ T cells induced significant increases in IL-4 and IL-5 concentrations, whereas IL-13 production was relatively marginal (Fig. 7, filled squares). Interestingly, mice treated with CD11b⁺ CD11c^{int} cells evidenced increases in the concentrations of all three cytokines in their BAL fluids (Fig. 7, checked box). Although CD11b⁺ CD11c^{int} cells purified from OVA/OVA mice induced marginal activity to induce the proliferation of CD90⁺ T cells purified from PBS/PBS mice in *in vitro* (Fig. 3C), mice that received CD11b⁺ CD11c^{int} cells significantly enhanced secretion of T_H2 cells upon the allergen challenge. These results suggest at least two possibilities. First, *in vitro* experimental condition to be done in Fig. 3(C) may be different with *in vivo* condition that is immunocompetently facilitated by adoptively transferred CD11b⁺ CD11c^{int} cells upon the allergen challenge in terms of status of immune response. Second, allergen challenge to the mice that received CD11b⁺ CD11c^{int} cells may lead lung environment to more favorable conditions capable of stimulating or inducing either naive CD4 T cells or primed CD4 T cells by allergen challenge. More interestingly, IL-13 levels in these mice were increased significantly, indicating that CD11b⁺ CD11c^{int} cells may preferentially induce IL-13 cytokines from T lymphocytes and other inflammatory cells such as eosinophils. Collectively, these results indicate that allergen-induced CD11b⁺ CD11c^{int} cells perform an essential role in the induction of eosinophilic airway inflammation, thereby increasing the infiltration of eosinophils and the production of T_H2 cytokines, including IL-4, IL-5 and IL-13.

Discussion

A number of studies have demonstrated the importance of inflammatory cells, including mast cells, lymphocytes, eosinophils and neutrophils, in addition to T_H2-type cytokines, in asthma (6, 7). Among these cells, CD4 T_H2 cells are believed to initiate and perpetuate disease via cooperation with cytokines (8–10). The CD4⁺ T cells of the airways of asthmatic patients express several cytokines, including IL-3, IL-4, IL-5, IL-10, IL-13 and granulocyte-macrophage colony-stimulating factor (35–37), thereby demonstrating that these T lymphocytes perform central roles in the pathogenesis of asthma. Although the roles of T_H2/CD4 T cells, which secrete IL-4, IL-5 and IL-13, have been well established in the

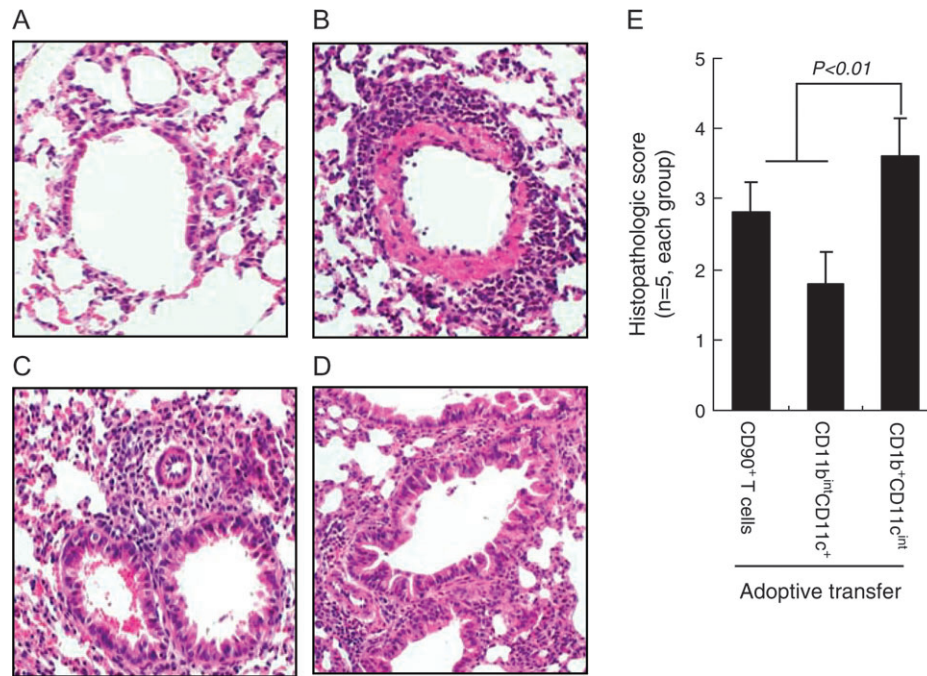


Fig. 6. Lung histology in mice that received CD90⁺ T cells, CD11b^{int} CD11c⁺ cells and CD11b⁺ CD11c^{int} cells. Lungs were isolated from mice treated with PBS (A), mice that received CD90⁺ T cells (B), mice that received CD11b^{int} CD11c⁺ cells (C) and mice that received CD11b⁺ CD11c^{int} cells (D). Lung tissue was fixed in 10% buffered formalin, and processed for routine histology in paraffin. Tissue sections were stained with hematoxylin/eosin and examined via light microscopy by a board-certified pathologist (DGR). Digital images were obtained of each sample, the entire histologic section on the glass slide was selected and areas were quantified with National Institutes of Health ImageJ software. Histopathologic analysis was performed (E). Lung lesions for each animal, including alveolar septal infiltrates, perivascular infiltrates, and combined bronchus-associated lymphoid tissue hyperplasia, and peribronchiolar infiltrates were subjectively graded on a severity scale of minimal, mild, moderate and marked [corresponding to numbers 1 (minimal) to 4 (marked)]. Lesion scores were represented as average mean values ($n = 5$).

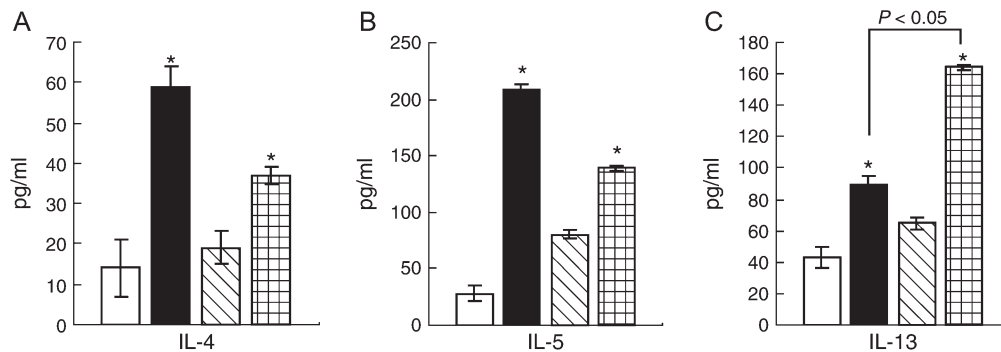


Fig. 7. CD11b⁺ CD11c^{int} cells induce T_H2 cytokines in BAL fluids of adoptively transferred mice. At 24 h after the final OVA inhalation, BAL fluids were harvested from mice treated with PBS (open squares), mice that received CD90⁺ T cells (filled squares), mice that received CD11b^{int} CD11c⁺ cells (hatched box) and mice that received CD11b⁺ CD11c^{int} cells (checkered box). The BAL fluid was centrifuged at 170 × g for 10 min. Cytokine measurements were conducted simultaneously. Levels of IL-4, IL-5 and IL-13 were measured via ELISA. The data were expressed as the means ± standard errors; $n = 5$ for each group. * $P < 0.05$ as compared with saline-challenged mice (PBS).

context of allergic disease, the function of CD8⁺ T cells in allergen-induced AHR and inflammation remains controversial. However, the results of a recent study suggested that antigen-primed CD8⁺ T cells are required for the full development of AHR and airway inflammation, and this appears to be associated with the production of IL-13 from these primed T cells (38). As a result, we determined that allergen-specific T cells play a crucial role in airway inflammation.

The transfer of OVA-specific T cells, including CD4 and CD8 T cells, dramatically enhanced AHR, the infiltration of eosinophils and macrophages and the generation of T_H2 cytokines, including IL-4, IL-5 and IL-13 in the BAL fluids. These findings reveal that allergen-specific CD4⁺ and CD8⁺ lymphocytes may perform an essential role in the induction of eosinophilic airway inflammation. These results were partially consistent with previous results (38).

In addition, macrophages, including AMs, are the most abundant cells in the alveoli, distal airspaces and conducting airways (21–23). The function of macrophages in ongoing T_H2 cell-mediated eosinophilic airway inflammation is currently a matter of some controversy. Previous studies have demonstrated that the infiltration of macrophages into airway environments increases markedly as the result of challenge with allergens (21–27). Moreover, several clinical reports have demonstrated that the AMs from atopic asthmatic subjects, but not from atopic non-asthmatic subjects, perform critical functions in airway pathogenic immunity, as the result of an increase in T_H2 cytokine production (39). This indicates that the recruited macrophages may play an important role in asthmatic diseases. However, several animal studies with AMs have reported conflicting results (26, 27). A recent report showed that the transfer of non-sensitized AMs 24 h prior to OVA challenge administered to AM-depleted, sensitized rats resulted in the abrogation of AHR. By way of contrast, the transfer of sensitized AMs to AM-depleted, unsensitized rats did not significantly alter airway responsiveness (26). The authors speculated that the protective role of AMs in asthmatic disease is unrelated to a reduction in inflammatory cell recruitment, but may instead involve other mechanisms, in which other AM mediators are involved in the modulation of AHR. However, two crucial questions remain unanswered. One of these questions concerns the different characteristics of AM derived from sensitized and non-sensitized animals. We propose that, as compared with resident macrophages in non-sensitized mice, the recruited macrophages may prove to differ critically in terms of phenotype and function, and may also perform a crucial role in asthma pathogenesis. The other crucial question is why, if resident or recruited macrophages do indeed exert protective effects against the development of airway inflammation, do so many recruited macrophages in experimental asthma models exert no protective effects against AHR? This may, however, be the consequence of functional differences between resident and recruited macrophages. Considering these questions, the present study was conducted in order to determine the precise role of recruited macrophages infiltrated into the lungs during airway inflammation.

In the present study, we characterized allergen-induced macrophages infiltrated into lung tissues. When defined by the expression of the CD11c molecule, residential cells evidencing a high degree of granularity in the lungs of normal mice phenotypically manifested as CD11b^{int} CD11c⁺ or CD11b⁺ CD11c^{int} cells at a 2:1 ratio. Allergen challenge, interestingly, resulted in a dramatic reversal, with a ratio of 1:6 (CD11b^{int} CD11c⁺ cells versus CD11b⁺ CD11c^{int} cells), thereby indicating that the change might be the consequence of allergen challenge. When the cells were further characterized with antibodies specific for the CCR3 eotaxin receptor, CCR5 and CXCR4 chemokine receptors, approximately 91% of allergen-induced CD11b⁺ CD11c^{int} cells expressed the CCR3 eotaxin receptor, but not other chemokine receptors, such as CCR5 and CXCR4. In the *in vitro* study, we determined that allergen-induced CD11b⁺ CD11c^{int} cells purified from the lungs of asthmatic mice evidenced a potential ability to induce the proliferation of

OVA-specific T cells. These results indicate that CD11b⁺ CD11c^{int} cells expressing CCR3 may harbor the ability to present antigens to antigen-specific T cells. In the *in vivo* study, we also determined that mice treated with CD11b⁺ CD11c^{int} cells or OVA-specific CD90⁺ T cells manifested increases in eosinophilic infiltration and T_H2 cytokines, including IL-4, IL-5 and IL-13, in the BAL fluids. More importantly, we determined that mice treated with CD11b⁺ CD11c^{int} cells evidenced a dramatic increase in IL-13 levels in the BAL fluids. IL-13 is critically associated with the features of inflammatory lung disorders, including eosinophil-rich inflammatory cell infiltration, airway hyperreactivity and airway remodeling (40). Moreover, recent reports have suggested that, in IL-13-mediated airway disease, eosinophils and eosinophil-signaling molecules (e.g. eotaxins and CCR3) may play a key role in eosinophilic airway inflammation (40). Although IL-13 is predominantly produced by T_H2-polarized CD4⁺ T cells, moreover, recent studies also suggest that IL-13 is produced by numerous non-T cell populations such as mast cells, basophils and eosinophils and also facilitates the recruitment of inflammatory cells including T cells (41, 42). Consistent with the findings of previous reports, our findings clearly indicate that CD11b⁺ CD11c^{int} cells induced by allergens promote eosinophilic airway inflammation via increases in eosinophil infiltration and the production of T_H2 cytokines, particularly IL-13. Although our results did not definitively demonstrate the *in vivo* fate of adoptive transferred cells with regard to migration or localization, the cells may migrate to airway environments, especially into the lung, via the lymphatic pathway. This possibility is supported, in part, by a previous report in which the adoptive transfer of OVA-specific CD4⁺ T cells was shown to result in migration from the vessels into the airway wall (43). After OVA challenge, the migrated cells may provoke airway inflammation and AHR via the induction of infiltration of other inflammatory cells and the production of T_H2 cytokines.

In summary, our data show that CD11b⁺ CD11c^{int} cells expressing CCR3 accumulate to a significant degree in the lung tissue upon OVA challenge in sensitized mice. These cells evidence a potent antigen presentation ability, allowing for the stimulation of allergen-specific T cells. Moreover, in the *in vivo* tests, the cells may be implicated in the promotion of eosinophilic airway inflammation via the modulation of eosinophil infiltration and T_H2 cytokine production. Collectively, the findings of this study indicate the presence of an important functional role for allergen-induced CD11b⁺ CD11c^{int} cells expressing CCR3 not only in the stimulation of allergen-specific T cells but also in the promotion of eosinophilic airway inflammation.

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Abbreviations

AHR	airway hyperresponsiveness
AM	alveolar macrophage

BAL	bronchoalveolar lavage
CCR3	CC chemokine receptor
OVA	ovalbumin
OVA/OVA	ovalbumin immunized and ovalbumin challenged
Penh	enhanced pause

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