

ORIGINAL ARTICLE

Essential role of macrophages in the initiation of allergic rhinitis in mice sensitized intranasally once with cedar pollen: regulation of class switching of immunoglobulin in B cells by controlling interleukin-4 production in T cells of submandibular lymph nodes

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ABSTRACT

The production of allergen-specific IgE antibodies (Abs) in allergen-sensitized patients or animals has a mutual relationship with the immunologic response leading to allergic rhinitis. We recently reported that, after an intranasal injection of cedar pollen into mice, an interleukin-4 (IL-4)-dependent increase in serum nonspecific IgE Abs was a prerequisite for the production of serum allergen-specific IgE Abs. Here, we explored which lymphoid organs were responsive to the intranasally injected allergen and how IL-4 and IgE Abs were produced in the lymphocytes. Time-dependent changes in the total cell numbers and in *in vitro* IgE Ab production in various lymphoid organs revealed that the submandibular lymph nodes were the main responsible organ. After treatment with allergen (for IgE production) or allergen and complete Freund's adjuvant (for IgG production), we separated submandibular lymph node cells into macrophage-, lymphocyte-, and granulocyte-rich populations by discontinuous Percoll density-gradient centrifugation. Unexpectedly, bulk cells, but not the lymphocyte- or macrophage-rich populations, produced significant amounts of IL-4, IgE, and IgG; whereas production was restored by addition of Mac-1⁺ cells from the macrophage-rich to the lymphocyte-rich fraction. Furthermore, a combination of the lymphocyte-rich population (for IgG [or IgE]) production and the macrophage-rich population (for IgE [or IgG]) production produced a large amount of IgE (or IgG). These results indicate that, in the initiation of allergic rhinitis, macrophages in the submandibular lymph nodes are essential not only for IL-4 or immunoglobulin production, but also for class switching of immunoglobulin in lymphocytes.

Key words Allergy, class switching, macrophage, rodent.

The adaptive immune response is a critical component of host defense against infection and is essential for normal health; however, it is also elicited by antigens (e.g.,

pollen, food, and drugs) not associated with infectious agents, thus causing atopic diseases (1). The prevalence of allergic rhinitis, a typical atopic disease, has recently been

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List of Abbreviation: Abs, antibodies; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; H and E, hematoxylin and eosin; HRP, horseradish peroxidase; Ig, immunoglobulin; *i.n.*, intranasal; IL-4, interleukin-4; *i.p.*, intraperitoneal; *i.v.*, intravenous; LPS, lipopolysaccharide; NALT, nasal-associated lymphoid tissue; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; *s.c.*, subcutaneous.

increasing worldwide (2). In sensitized subjects, production of specific IgE Abs directed toward an allergen is a prerequisite for the immunologic response leading to allergic rhinitis. Binding of allergen-specific IgE Abs to surface receptors on a wide variety of cells, mainly mast cells and eosinophils (3), and cross-linking of receptor-bound IgE with antigen result in the release of inflammatory mediators that lead to the symptoms of allergic diseases (4). However, several crucial questions regarding the mechanisms have not yet been fully answered. For example, what kind(s) of immune cells can recognize allergenic molecules as nonself? Are allergens recognized as nonself nonspecifically or specifically by the defense system? Why are IgE Abs rather than IgA, IgM or IgG Abs, produced towards an allergen?

After allergen (e.g., ovalbumin or *Schistosoma mansoni* egg antigen) sensitization and exposure, serum allergen-specific IgE concentrations are reportedly much higher in BALB/c mice than in C57BL/6 mice (5, 6). We have also found that BALB/c mice produce higher titers of serum IgE Ab than do C57BL/6 mice after treatment with Japanese cedar pollen allergen (Yamamoto *et al.*, unpublished data). Therefore, in the present study, we used BALB/c mice as the experimental animals. Recently, we reported that primary *i.n.* (or *i.p.*), but not *i.v.* (or *s.c.*), injections of cedar pollen extract without adjuvant induce allergenic activity in the form of an increase in serum IgE, but not IgA, IgG or IgM, Abs. We also found that IgE Abs did not react with the allergen, suggesting the increase was due to nonspecific IgE Abs in the serum (7). In addition, we showed that one or two more *s.c.* injections of the same allergen without adjuvant into *i.n.* (or *i.p.*) or *i.v.* (or *s.c.*) allergen-sensitized mice induce allergen-specific IgE Abs production (7). These results indicate that an increase in production of nonspecific IgE Abs without changes in IgA, IgG or IgM concentrations is a prerequisite for production of allergen-specific IgE Abs. Moreover, we found that the lymphocyte-rich fraction of PBMCs from mice sensitized once *s.c.* is ineffective in production of IL-4 and nonspecific IgE Abs and that addition of Mac-1⁺ cells from the monocyte-rich to the lymphocyte-rich fraction is essential for IL-4 and nonspecific IgE production (8). Therefore, one of the major goals of allergy research is finding a way to control IL-4-dependent production of nonspecific IgE Abs during the initial sensitization stage to ascertain how the immune system recognizes allergic molecules as nonself.

More recently, we reported that time-dependent changes in IgE⁺ cells in the spleen after 1st (*i.v.*) and 2nd (*s.c.*) injections of allergen correlate with changes in the concentrations of nonspecific IgE Ab in the serum, suggesting that the spleen is the main organ responsive to *i.v.* injected allergens (9). Although the nasal mucosa is

the first site of contact with inhaled antigens, the nature of local immune responses against allergens and the role of NALT in those responses have rarely been studied (10–13). Therefore, the next important question is whether NALT is responsive to an allergen injected *i.n.* into mice.

Since injections of allergen with adjuvant obscure the characteristics of injection sites, we previously injected cedar pollen without adjuvant *i.n.*, *i.p.*, *i.v.* or *s.c.* once into BALB/c mice to explore which lymphoid tissues (e.g., spleen, NALT, Peyer's patches, submandibular, axillary, inguinal, and mesenteric lymph nodes) are essential for production of nonspecific serum IgE Abs (7–9). In the present study, we injected cedar pollen with or without complete Freund's adjuvant *i.n.* once into BALB/c mice to induce IgG or IgE Abs efficiently with the same antigen. We found that submandibular lymph node, but not NALT, cells from mice sensitized with allergen alone *i.n.* once produced IL-4 and IgE Ab most efficiently. In addition, they most efficiently produced IgG Ab by sensitization with allergen and adjuvant *i.n.* once. Of particular interest, the lymphocyte-rich fraction alone was ineffective in production of IL-4 or IgE (or IgG) Abs; but the addition of Mac-1⁺ cells from the macrophage-rich to the lymphocyte-rich fraction was essential for production of these Abs. We also examined the cellular mechanisms for class switching of Ig in lymphocytes.

MATERIALS AND METHODS

Animals

Specific pathogen-free male BALB/c mice (7 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). After an *i.n.* injection of the test allergen with or without complete Freund's adjuvant (Sigma-Aldrich; St. Louis, MO, USA), the mice were housed in our animal facility under specific pathogen-free conditions in an air-conditioned room at 23 ± 2°C and ≈ 50% humidity for 1–3 weeks. The experiment was carried out in accordance with the Guidelines on Animal Experiments of Osaka Medical College and the Japanese Government Notification on Feeding and Safekeeping of Animals (Notification No.6 of the Prime Minister's Office). The experimental protocol was approved by the Review Committee for Animal Experiments of Osaka Medical College.

Allergic sensitization

Japanese cedar (*Cryptomeria japonica*) pollen crude extract-Cry j was purchased from Cosmo Bio, Tokyo, Japan. The cedar pollen extract was suspended in PBS and the suspension (100 µg/0.15–0.3 mL of PBS/mouse) was injected slowly into the area surrounding the nostrils

after *i.p.* injection of 0.25 mL of pentobarbital/ethanol/PBS (0.8 mL/2 mL/8 mL).

Antibodies

The following antibodies were used in this study: mouse IgE and IgG Abs from Zymed (San Francisco, CA, USA); rat anti-mouse IgE and IgG Abs from Biosource (Camarillo, CA, USA); HRP-labeled goat anti-mouse IgE and IgG Abs from Nordic (Tilburg, the Netherlands) and Cappel (Aurora, OH, USA); FITC-labeled rat anti-mouse CD14 (Sa2-8) Abs from eBioscience (San Diego, CA, USA); and Alexa Fluor 647-conjugated rat anti-mouse CCR3 (83103) Abs, PE-labeled rat anti-mouse CD3 (145-2C11), CD4 (RM4-4), CD11b/Mac-1 (M1/70), Ly-6G (1A8), CD45R/B220 (RA3-6B2), and IgM (R6-60.2) Abs and FITC-labeled rat anti-mouse Ly-6G (1A8), CD3 (145-2C11), CD8 (53-6.7), CD11b/Mac-1 (M1/70), and CD11c (HL3) Abs from PharMingen (San Diego, CA, USA).

Serum preparation

Blood samples were taken by cardiac puncture under chloroform anesthesia at various time intervals after *i.n.* injection of cedar pollen extract-Cry j with or without complete Freund's adjuvant. The whole blood was incubated in a CO₂ incubator at 37°C for 1 hr, stood overnight at 4°C, and then centrifuged at 440 g for 20 min. The supernatant fraction was stored in microtubes at -20°C prior to use.

Preparation of lymphocytes from nasal-associated lymphoid tissue or other lymphoid organs

Mice were anesthetized with chloroform and then bled from the inferior vena cava. After exsanguination, they were decapitated along the line between the upper and lower jaws. The facial skin was stripped from the head and the nose component separated from the rest of the head along the line of the eyeballs. A segment containing the tip of the nose and fore-teeth was severed from the rest of the specimen. After removal of the cheek muscles, cheek bones, and back teeth, NALT, which localize bilaterally on the posterior side of the palate, was separated from the rest of the nasal tissue by peeling the palate away. The excised palates were immediately placed into a 60 mm Petri dish containing stainless mesh on ice and 3 mL of ice-cold PBS with 5 mM EDTA. Using a dissection microscope (Nikon; Tokyo, Japan), the NALT was teased gently into the medium with syringe needles to release the cells, which were harvested by using siliconized Pasteur pipettes. Other lymphoid tissues such as submandibular, axillary, inguinal or mesenteric lymph nodes and Peyer's Patches were re-

moved aseptically; and single-cell suspensions prepared from them as described earlier (14).

Evaluation of lymphoid organ responsive to intranasally injected allergen

To evaluate lymphoid organ(s) responsive to *i.n.* injected allergen, we injected 2% Evans blue in PBS (2 mL/kg) *i.n.* and allowed it to permeate the neighboring lymphoid organs for 20 min. The mice were then anesthetized with chloroform and bled from the inferior vena cava. After exsanguination, NALT was separated from the rest of the nasal tissue and the skin covering the submandibular lymph nodes excised. The lymphoid organs stained by Evans blue were examined macroscopically.

Percoll density-gradient centrifugation of submandibular lymph node cells

Submandibular lymph node cells were suspended in 60% Percoll, and separated into lymphocyte-, macrophage-, and granulocyte-rich populations by discontinuous Percoll density-gradient (PBS/40%/50%/60% Percoll) centrifugation (1420 g, 15 min at 20°C), as previously described (9, 15). The cell types were assessed phenotypically by flow cytometry and morphologically after H and E-staining.

Flow cytometric analysis and sorting

For analysis, 10⁶ cells suspended in 50 µL of cold PBS containing 2% FCS were stained with a fluorescein-labeled rat anti-mouse Ab (1 µg) in the presence of 1.5 µL of rat whole serum and 1 µg of purified rat anti-mouse FcγRIII/II mAb (PharMingen) at 4°C for 20 min, washed, and analyzed by use of FACS. For sorting, the fluorescein-labeled or unlabeled cells were isolated by FACS in forward scattering/side scattering and FITC/PE modes, as described previously (16).

Cell number and viability

Cell numbers were determined by counting the cells in Turk's solution with a hemocytometer. Cell viability was assessed by the trypan blue exclusion method.

Cell culture

Cells in the lymphocyte-, macrophage- or granulocyte-rich fractions or various combinations of cells in the lymphocyte-rich fraction with specific antigen + or - cells were cultured for 6 days without cedar pollen in a 24- or 48-well plate containing RPMI 1640 medium containing 10% FCS. The culture media were stored in microtubes at -20°C prior to use.

Enzyme-linked immunosorbent assay for nonspecific IgE or IgG antibodies

The wells of enzyme immunoassay/radioimmunoassay (Costar #2592; Corning, NY, USA) were coated with 100 μ L of rat anti-mouse IgE or anti-IgG Ab (each 2 μ g/mL) at 4°C overnight. After three washes with PBS/Tween (PBS + 0.5% Tween 20), the wells were filled with 400 μ L of 1% BSA/PBS and then incubated for 2 hr at room temperature to block unsaturated protein binding sites. The plates were next washed three times with PBS/Tween; and 100 μ L of appropriately diluted serum samples in 1% BSA/PBS added to each of triplicate wells, after which the plates were incubated for 2 hr at room temperature. After three more washes with PBS/Tween, 100 μ L of HRP-labeled goat anti-mouse IgE or IgG Ab in 1% BSA/PBS was dispensed into each well, and the preparation allowed to react for 1 hr at room temperature. The wells were then washed thoroughly with PBS/Tween. Next, the antigen-Ab complex was incubated with 100 μ L of tetramethylbenzidine (Sigma-Aldrich) for 30 min at room temperature. The reaction was then stopped by the addition of 100 μ L of 1M H₂SO₄. Thereafter the OD_{450nm} of the solution in each well was read by a microplate reader SH-1000 (Corona Electric, Hitachinaka, Japan). For preparation of standard curves for serum Igs, the concentrations used in this study were as follow: 0.0039 μ g/mL, 0.0078 μ g/mL, 0.0156 μ g/mL, 0.0313 μ g/mL, 0.0625, and 0.125 μ g/mL for IgG and 0.00156 μ g/mL, 0.00313 μ g/mL, 0.00625 μ g/mL, 0.0125 μ g/mL, and 0.025 μ g/mL for IgE. To measure the Ig concentrations, serum samples were diluted 20-, 50-, and 100-fold for IgE and 50000-, 10,000-, and 20,000-fold for IgG. To measure the IgE or IgG concentrations in the culture medium, samples were diluted 1- and 2.5-fold or 10- and 25-fold, respectively. Microsoft Excel software was used to determine the amount of Ig based on the standard curve. The lower limit of the Abs assayed in this system was 0.5 to 1 ng/mL. The SD of the triplicate assay was less than 4% at 12.5 ng/mL and less than 2% at 25 ng/mL.

Submandibular lymph node cells, NALT cells or cells from other lymphoid tissues, all of which had been obtained on days 0–14 after an *i.n.* injection of the allergen, were cultured for 6 days, and the amount of IgE Ab in the culture medium assessed. When standard curves were constructed with fresh culture medium or PBS containing various amounts of IgE, most of these curves were linear up to 25 ng/mL; but the amounts of IgE in the culture media from NALT were always < 0 (data not shown), suggesting the presence of obstacles to ELISA assay of the medium after the 6 day culture period. Therefore, we cultured untreated cells from NALT, submandibular lymph nodes or

other lymphoid tissues for 6 days, and constructed a standard curve by adding 0–25ng/mL of IgE to the culture media.

Enzyme linked immunosorbent assay for interleukin-4

The amounts of IL-4 in the culture media were assessed by using a Cytoscreen ELISA kit (Biosource International, Camarillo, CA, USA). Fifty μ L of standard, control or experimental samples were added to each of several anti-IL-4 Ab-coated wells, and incubation carried out at 37°C for 1 hr. Fifty μ L of biotin-conjugated monoclonal Ab specific for IL-4 was added to each well, and the plates incubated at 37°C for 1 hr. After four washes with washing buffer, 100 μ L of streptavidin-HRP solution was added to each well and incubation continued at room temperature for 30 min. Following four more washes with washing buffer, the antigen-Ab complexes were incubated with 100 μ L of tetramethylbenzidine for 30 min at room temperature. The reaction was stopped by the addition of 100 μ L of stop solution, after which the absorbance of each well was read at 450 nm. A standard curve for IL-4 was prepared over the range of 0–1000 pg/mL.

Reverse transcriptase-polymerase chain reaction

Total RNAs were isolated from various kinds of cells by using TRIzol. The total RNAs were reverse transcribed to synthesize first-stranded cDNA by using SuperScriptII reverse transcriptase (Gibco-BRL, Cleveland, OH, USA). A mouse primer set for IL-4 cDNA (forward, 5'-ACG GAG ATG GAT GTG CCA AAC GTC-3'; reverse, 5'-CGA GTA ATC CAT TTG CAT GAT GC-3'; KURABO, Osaka, Japan) was used to amplify a 279 bp fragment; and 30 cycles of PCR were conducted in a GeneAmp PCR System apparatus (9700; PE Applied Biosystems, Foster City, CA, USA). A mouse β -actin primer set (forward, 5'-TGT GAT GGT GGG AAT GGG TCA G-3'; reverse, 5'-TTT GAT GTC ACG CAC GAT TTC C-3'; Kurabo, Osaka, Japan) was used to amplify a 514 bp fragment by 30 cycles of PCR. The PCR products were electrophoresed on 2% agarose gels (Funakoshi, Tokyo, Japan) and analyzed after ethidium bromide staining.

RESULTS

Preparation of nasal-associated lymphoid tissue lymphocytes from BALB/c mice

When we placed the excised palates with the nasal side facing up, we could clearly see two prominent strips of NALT with the naked eye (Fig. 1a). Fig. 1b shows H and E-stained

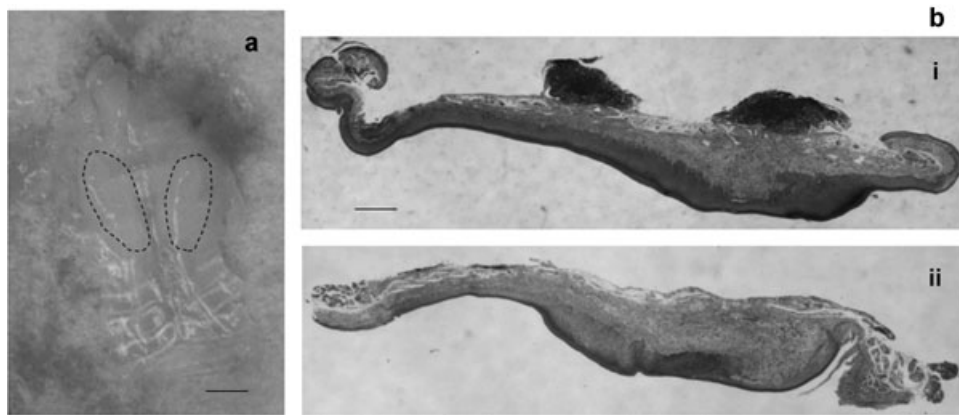


Fig. 1. Isolation of NALT. (a) Macroscopic view of the posterior surface of a palate fragment from the upper jaw of a normal BALB/c mouse. A pair of NALT (dotted lines) is shown on the surface of the palate as slightly elevated organs. Scale bar = 1 mm. (b) Histology of NALT of normal BALB/c mice shown by H and E-stained tissue sections. (i) Cross section of an isolated palate. A pair of NALT is shown on the surface of the palate as well-demarcated, slightly elevated organs. (ii) Cross section of a palate after the NALT has been gently teased from it. Scale bar = 100 μ m.

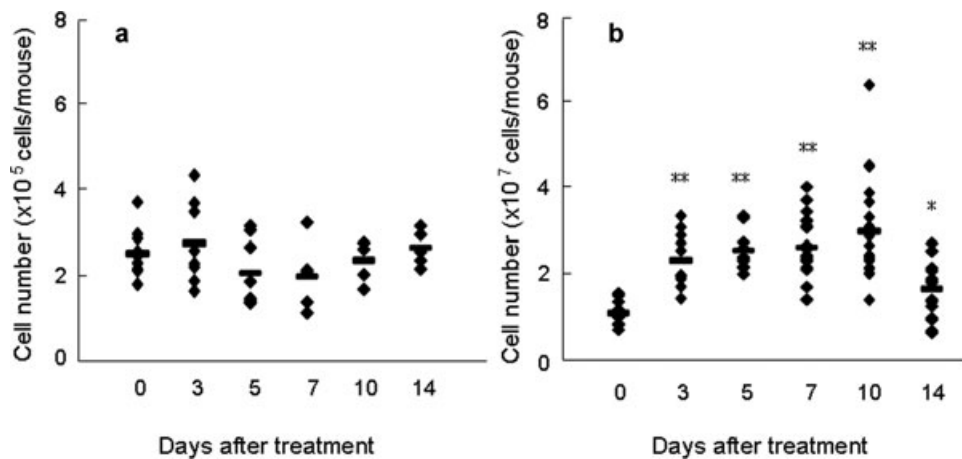


Fig. 2. Time-dependent changes in total number of cells in the NALT or submandibular lymph nodes after an *i.n.* injection of cedar pollen into BALB/c mice. On days 3–14 after *i.n.* sensitization (cedar pollen; 100 μ g/150 μ L of PBS), mono-dispersed cells were obtained from (a) the NALT ($n = 5$ –11) and (b) submandibular lymph nodes ($n = 11$ –20); and the total cell numbers determined. Each symbol and bar represent the total cell number from one mouse and the mean value, respectively. The difference between sample and control (days 0–14 after *i.n.* injection of PBS) is significant (*, $P < 0.01$; **, $P < 0.005$) according to Student's *t* test.

tissue sections of NALT from normal BALB/c mice before and after teasing. NALT cells were readily isolated, yielding approximately 2.5×10^5 viable cells per palate. Because we had exsanguinated the mice from the inferior vena cava, we noted few erythrocytes; thus more than 96% of the cells were the following immune cells: CD3⁺ cells ($53.5 \pm 3.8\%$; mean $\pm$ SD; $n = 3$); CD4⁺ cells ($38.6 \pm 2.6\%$; mean $\pm$ SD; $n = 3$); CD8⁺ cells ($17.5 \pm 2.5\%$; mean $\pm$ SD; $n = 3$); B220⁺ cells ($40.0 \pm 3.7\%$; mean $\pm$ SD; $n = 3$); Mac-1⁺ cells ($1.5 \pm 0.4\%$; mean $\pm$ SD; $n = 3$); CD11c⁺ cells ($0.6 \pm 0.0\%$; mean $\pm$ SD; $n = 3$); and Ly-6G⁺ cells ($0.3 \pm 0.1\%$; mean $\pm$ SD; $n = 3$). The cell yield from NALT and their phenotypic composition were essentially

the same as those reported previously (17, 18), showing that they had been accurately prepared.

Time-dependent changes in total number of cells in nasal-associated lymphoid tissue or submandibular lymph nodes after one intranasal injection of cedar pollen into BALB/c mice

Figure 2 shows the time-dependent changes in the total number of cells in NALT or submandibular lymph nodes of BALB/c mice after one *i.n.* injection of cedar pollen. The total number of NALT cells did not change significantly from days 0–14 after *i.n.* injection of the allergen (Fig. 2a);

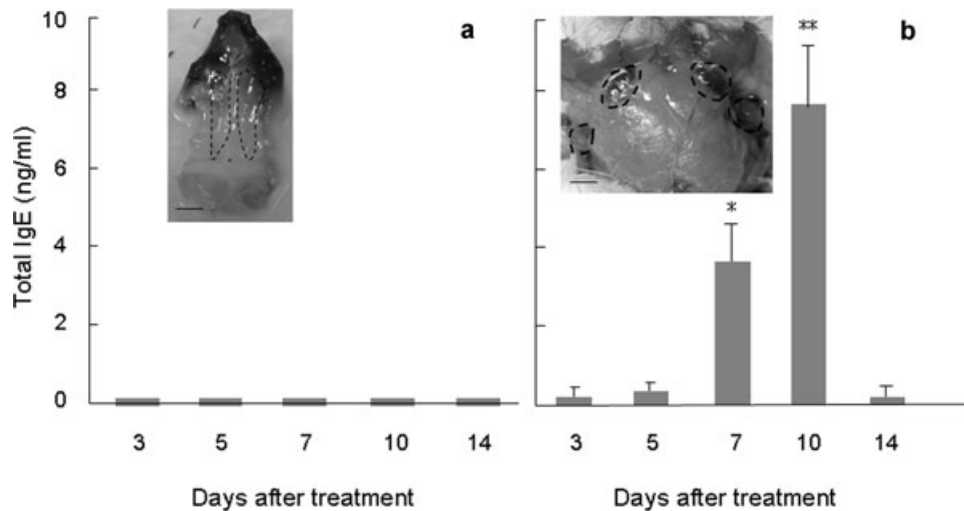


Fig. 3. *In vitro* IgE Ab production by bulk cells from NALT or submandibular lymph nodes from BALB/c mice allergen-sensitized *i.n.* once. On days 3–14 after *i.n.* sensitization (cedar pollen; 100 μ g/150 μ L of PBS), mono-dispersed cells were obtained from (a) NALT ($n = 12$) and (b) submandibular lymph nodes ($n = 30$); and the amounts of IgE Ab in the culture media determined after the cells (NALT [the cells from 5–6 mice were combined]: 1.5×10^6 cells/0.5 mL/48-well plate; submandibular lymph nodes: 3×10^6 cells/mL/24-well plate) had been cultured for 6 days. Data are expressed as the mean $\pm$ SEM. The difference between sample and control (PBS-injected: day 0) is significant (*, $P < 0.005$; **, $P < 0.001$) according to Student's *t* test. (Inset) Macroscopic views of the NALT (dotted lines in [a]) and submandibular lymph nodes (dotted lines in [b]) after *i.n.* injection of 2% Evans blue. Scale bar = 1 mm.

and the percentages of B220⁺, CD3⁺, Mac-1⁺, CD11c⁺, and Ly-6C⁺ cells were also unchanged (data not shown). In contrast, the total number of submandibular lymph node cells started to increase on day 3 after *i.n.* injection of the allergen, reached a peak ($\approx$ threefold that of the PBS-injected control) on day 10, and declined to the basal level by day 14 (Fig. 2b). Of particular interest, the percentage of B220⁺ cells on day 0 ($\approx 36\%$) started to increase from day 3 ($\approx 49\%$), reached a plateau on days 5–10 (54–55%), and decreased to the basal level by day 14 ($\approx 42\%$). In contrast, those of CD3⁺ cells, Mac-1⁺, CD11c⁺, and Ly-6C⁺ cells decreased time-dependently and returned to the basal level by day 14 (data not shown), suggesting that B220⁺ cells (e.g., B or pre-B cells) in the submandibular lymph nodes might be the cells that respond to *i.n.* injections of allergen.

***In vitro* IgE antibody production by bulk cells of nasal-associated lymphoid tissue or submandibular lymph nodes from BALB/c mice allergen-sensitized intranasally once**

Bulk cells from submandibular lymph nodes from mice that had been treated once *i.n.* with allergen produced a significant amount of IgE Ab on day 7 (mean $\pm$ SE, 3.8 ± 1.0 ng/mL; $n = 30$) with a peak on day 10 (7.8 ± 1.6 ng/mL; $n = 30$). The concentrations then decreased to the control level by day 14 (0.1 ± 0.1 ng/mL;

mean $\pm$ SEM; $n = 30$), demonstrating time-dependent changes in the amount of IgE Ab similar to the changes in total cell numbers. In contrast, the bulk cells from the NALT from mice that had been treated once *i.n.* with allergen did not produce significant amounts of IgE ($n = 12$) on days 0–14. The bulk cells of the axillary lymph nodes, Peyer's patches, inguinal lymph nodes, and mesenteric lymph nodes produced 1.8 ± 0.3 (mean $\pm$ SEM; $n = 15$), 1.3 ± 1.4 (mean $\pm$ SD; $n = 9$), 0.5 ± 0.3 (mean $\pm$ SD; $n = 9$), 0.1 ± 0.3 (mean $\pm$ SD; $n = 9$) ng/mL IgE on day 10, respectively (data not shown). When we injected 2% Evans blue in saline *i.n.*, submandibular lymph nodes, but not NALTs, were consistently clearly stained (Fig. 3, inset).

These results taken together demonstrate that the submandibular lymph nodes are the main organ that responds to *i.n.* injected allergens.

IgE or IgG antibody production in serum of BALB/c mice allergen- or allergen and adjuvant-sensitized intranasally once

To explore the mechanisms of IgG Ab production and compare them with those of IgE Ab production in submandibular lymph nodes, we injected the allergen with or without complete Freund's adjuvant *i.n.* once into BALB/c mice (Fig. 4). A significant amount of serum IgE (465.4 ± 111.6 ng/mL; mean $\pm$ SD; $n = 9$)

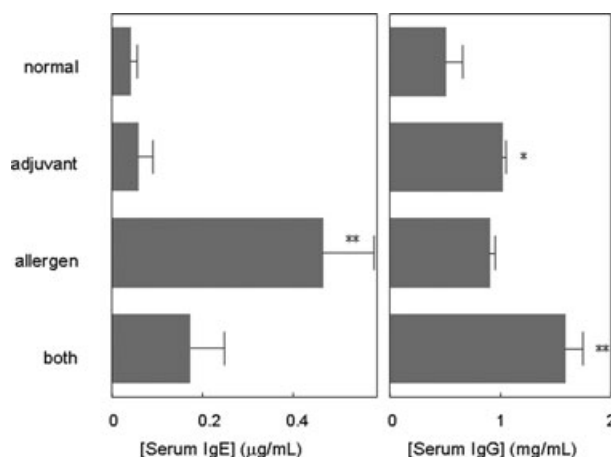


Fig. 4. The serum concentrations of IgE or IgG Abs of PBS-injected BALB/c mice or BALB/c mice adjuvant-, allergen- or adjuvant and allergen-sensitized *i.n.* once. The serum concentrations of IgE or IgG Abs were determined on day 10 after *i.n.* sensitization (adjuvant alone: 150 μ L of adjuvant and 150 μ L of PBS; allergen alone: 100 μ g of allergen in 300 μ L of PBS; adjuvant and allergen: 150 μ L of adjuvant and 100 μ g allergen in 150 μ L of PBS). Data are expressed as the mean $\pm$ S.D. ($n = 9$). The difference between sample and control (PBS-injected) is significant (*, $P < 0.05$; **, $P < 0.02$) according to Student's *t* test.

was induced by one *i.n.* injection of allergen alone. In contrast, one *i.n.* injection of the allergen with adjuvant induced a much smaller amount of serum IgE (172.5 ± 74.7 ng/mL; mean $\pm$ SD; $n = 9$). This was greater than that (57.6 ± 32.2 ng/mL; mean $\pm$ SD; $n = 9$) in mice treated with adjuvant alone or that (40.8 ± 14.8 ng/mL; mean $\pm$ SD; $n = 9$) in PBS-injected mice. In contrast, a large amount of serum IgG (1585.4 ± 161.0 μ g/mL; mean $\pm$ SD; $n = 9$) was induced by one *i.n.* injection of the allergen with adjuvant into mice; this amount of serum IgG was greater than that obtained after one *i.n.* injection of adjuvant (1018.2 ± 33.2 μ g/mL; mean $\pm$ SD; $n = 9$) or allergen (904.9 ± 51.2 μ g/mL; mean $\pm$ SD; $n = 9$) alone, both of which were greater than that (514.7 ± 161.8 μ g/mL; mean $\pm$ SD; $n = 9$) in PBS-injected mice.

These results indicate that one *i.n.* injection of allergen alone or with adjuvant is suitable for induction of serum IgE or IgG Ab, respectively.

Essential role of Mac-1⁺ cells in *in vitro* IgE production by submandibular lymph node cells from BALB/c mice allergen-sensitized intranasally once

To explore which population of cells in the submandibular lymph nodes is involved in the production of IgE Ab in response to the allergen, we separated the cells into macrophage-, lymphocyte-, and granulocyte-rich popula-

tions by Percoll density-gradient centrifugation. The yield of cells from the submandibular lymph nodes was 78–89% ($n = 9$). Fraction 3 (rich in lymphocytes) was the major ($93.5 \pm 7.2\%$; mean $\pm$ SD; $n = 9$) population, followed by fraction 2 (rich in macrophages; $1.2 \pm 0.1\%$; mean $\pm$ SD; $n = 9$) and fraction 4 (rich in granulocytes; $0.3 \pm 0.1\%$; mean $\pm$ SD; $n = 9$) in that order. Fraction 1 (rich in somewhat damaged cells) contained a small number of cells.

As we obtained the macrophage-, lymphocyte-, and granulocyte-rich fractions, we incubated various combinations of these cells for 6 days and then assessed the amounts of IgE Ab in the culture media (Fig. 5). Bulk submandibular lymph node cells from mice that had been treated with allergen once *i.n.* produced a significant amount of IgE Abs (6.2 ± 3.4 ng/mL; mean $\pm$ SD; $n = 9$); whereas the lymphocyte-rich (fraction 3) fraction of the lymph node cells did not (1.5 ± 0.8 ng/mL; mean $\pm$ SD; $n = 9$). The macrophage-rich (fraction 2) fraction was also inactive (1.1 ± 0.9 ng/mL; mean $\pm$ SD; $n = 9$). Of particular interest, IgE Ab production (4.6 ± 2.8 ng/mL; mean $\pm$ SD; $n = 9$) was restored by addition of the macrophage-rich fraction to the lymphocyte-rich fraction. In contrast, the amounts of IgE produced by cells in the damaged cell fraction (fraction 1; 1.5 ± 0.8 ng/mL; mean $\pm$ SD; $n = 9$) or granulocyte-rich fraction (fraction 4; 0.9 ± 0.5 ng/mL; mean $\pm$ SD; $n = 9$) were around the basal level. There was no additional effect after mixing either of them with the lymphocyte-rich fraction (data not shown). Furthermore, Mac-1⁺ or Mac-1[–] plus low+/CD4[–] cells, but not CD4⁺ cells, from the macrophage-rich fraction enhanced IgE Ab production when mixed with the lymphocyte-rich fraction. The Mac-1⁺ cells were phenotypically CD3[–]/IgM[–]/B220[–]/CD11c[–]/CD14[–]/Ly-6G[–]/CCR3[–] and morphologically mononuclear cells (Fig. 6), suggesting that they were macrophages.

These results suggest that cedar pollen might be recognized as an allergen by a mixture of lymphocyte- and macrophage-rich (*i.e.*, Mac-1⁺ or Mac-1^{low+}) fractions, resulting in release of IgE Abs from lymphocytes into the culture medium.

Essential role of Mac-1⁺ cells in *in vitro* IgG Ab production by submandibular lymph node cells from BALB/c mice allergen and adjuvant-sensitized intranasally once

Next, we incubated various combinations of macrophage- or lymphocyte-rich fraction in submandibular lymph node cells for 6 days and assessed the amounts of IgG Ab in the culture medium (Fig. 7). As expected, bulk submandibular lymph node cells from mice that

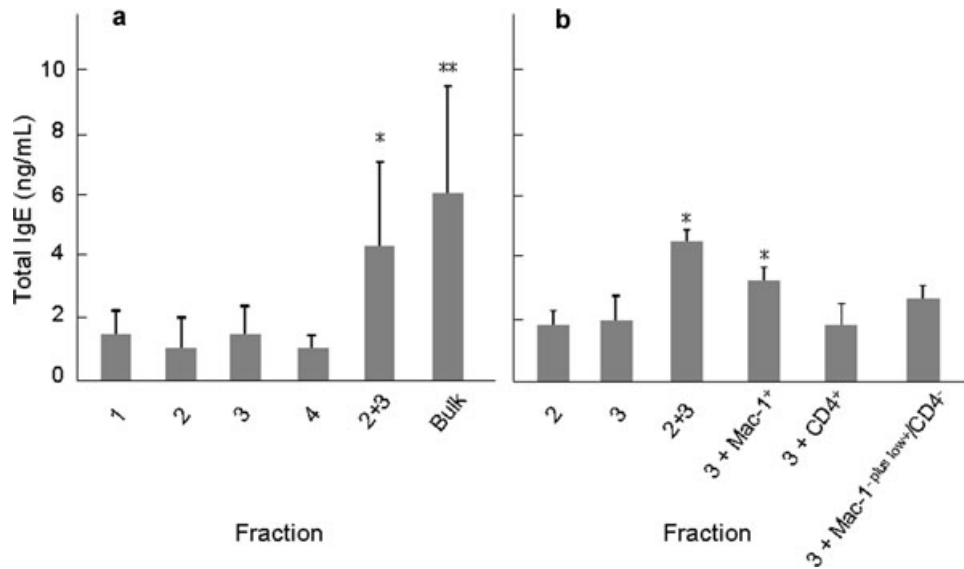


Fig. 5. IgE Ab released from various combinations of fractions of submandibular lymph node cells from mice allergen-sensitized *i.n.* once. On day 10 after *i.n.* sensitization (cedar pollen; 100 μ g/150 μ L of PBS), mono-dispersed cells were obtained from submandibular lymph nodes and separated into various fractions by Percoll density-gradient centrifugation. The concentrations of IgE Ab in the culture media were determined after the cells had been cultured for 6 days. (a) Total IgE produced by the various fractions. Fraction type and average number of cells in the cultures: 1, damaged cell-rich fraction (5.6×10^3 cells/well); 2, macrophage-rich fraction (6.4×10^5 cells/well); 3, lymphocyte-rich fraction (2.3×10^6 cells/well); 4, granulocyte-rich fraction (1.1×10^4 cells/well); bulk (3×10^6 cells/well). (b) The effects of adding specific antigen⁺ (e.g., Mac-1⁺) cells from fraction 2 to fraction 3 were also examined: Mac-1⁺ cells in fraction 2 (2.6×10^3 cells/well); CD4⁺ cells in fraction 2 (9.7×10^4 cells/well); CD4⁻/Mac-1⁻ plus low⁺ cells in fraction 2 (5.4×10^5 cells/well). Data are expressed as the mean $\pm$ S.D. ($n = 9$). The difference between sample and control (fraction 3 alone) is significant (*, $P < 0.05$; **, $P < 0.01$) according to Student's *t* test.

had been treated *i.n.* once with the mixture of allergen and adjuvant produced a significant amount of IgG Ab (629.2 ± 92.7 ng/mL; mean $\pm$ SD; $n = 15$). In contrast and unexpectedly, the lymphocyte-rich fraction (fraction 3) of the lymph node cells produced a small amount of IgG Ab (245.7 ± 59.0 ng/mL; mean $\pm$ SD; $n = 15$); and the macrophage-rich (fraction 2) fraction was almost inactive (154.2 ± 119.7 ng/mL; mean $\pm$ SD; $n = 15$). Of particular interest, IgG Ab production (477.0 ± 135.0 ng/mL; mean $\pm$ SD; $n = 15$) was restored by addition of the macrophage-rich fraction (fraction 2) to the lymphocyte-rich fraction (fraction 3). In contrast, the amounts of IgG produced by cells in the damaged cell (fraction 1; 104.0 ± 24.9 ng/mL; mean $\pm$ SD; $n = 15$)- or granulocyte (fraction 4; 0.0 ± 0.0 ng/mL; mean $\pm$ SD; $n = 15$)-rich fraction were around the basal level; and there was no additional effect after mixing either of them with the lymphocyte-rich fraction (data not shown). Furthermore, Mac-1⁺ or Mac-1⁻ plus low⁺/CD4⁻ cells, but not CD4⁺ cells, from the macrophage-rich fraction enhanced IgG production when mixed with the lymphocyte-rich fraction.

These results suggest that cedar pollen injected *i.n.* with complete Freund's adjuvant might be recognized as

a non-allergenic protein by a mixture of lymphocyte- and macrophage-rich (*i.e.*, Mac-1⁺ or Mac-1⁻ plus low⁺/CD4⁻) fractions, resulting in release of IgG Abs from lymphocytes into the culture medium.

Which fraction (lymphocyte- or macrophage-rich fraction) is required for class switching of Ig?

To explore which fraction (lymphocyte- or macrophage-rich fraction) is required for class switching of Ig, we injected the allergen alone or with complete Freund's adjuvant *i.n.* into BALB/c mice. We then prepared lymphocyte- and macrophage-rich fractions to produce IgE or IgG Abs, respectively; incubated various combinations of these cells for 6 days; and assessed the amounts of IgE or IgG Ab in the culture media (Fig. 8). The mixture of cells in the lymphocyte-rich and macrophage-rich populations from mice that had been treated *i.n.* once with allergen alone produced a significant amount of IgE (4.5 ± 1.2 ng/mL; mean $\pm$ SD; $n = 12$) and served as a positive control. A small amount of IgE (1.4 ± 0.4 ng/mL; mean $\pm$ SD; $n = 12$) was produced by the mixture of lymphocyte- and macrophage-rich populations from mice that had been

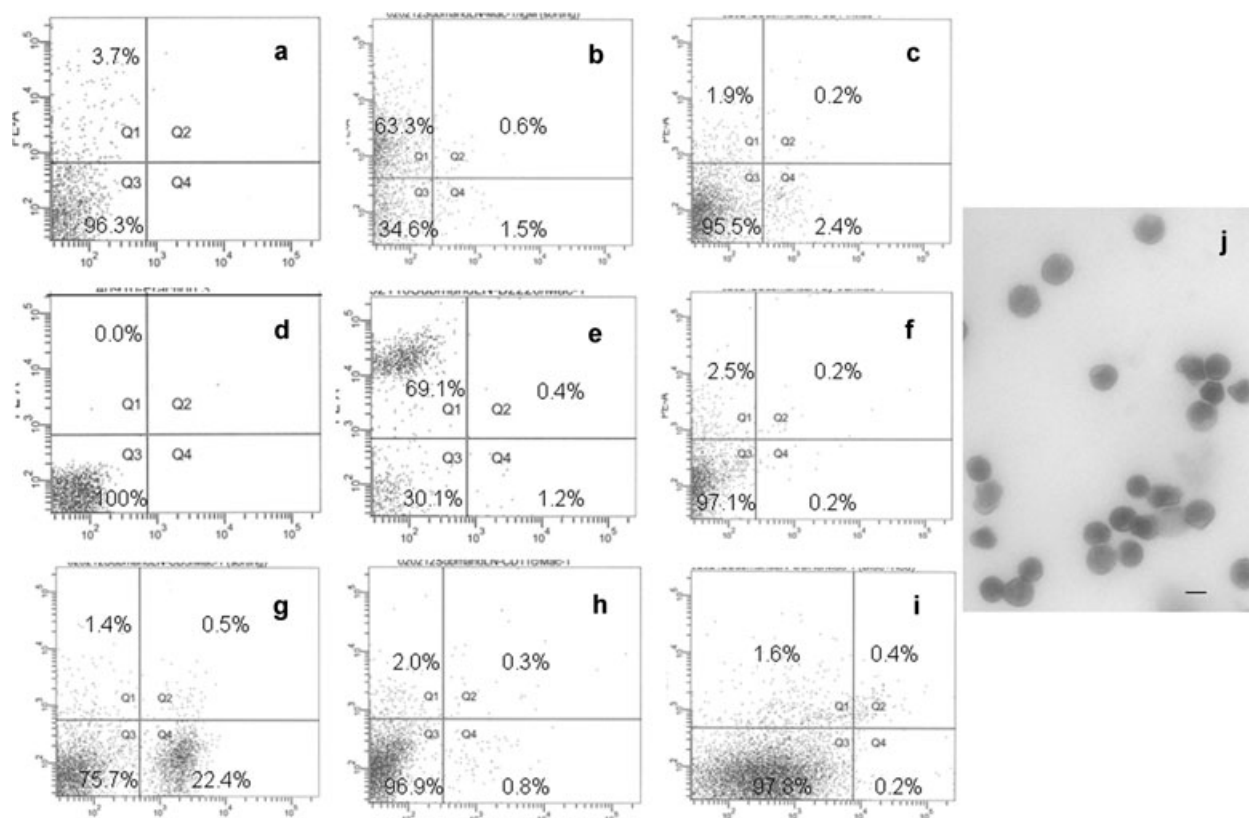


Fig. 6. Phenotypal and morphological characterization of Mac-1⁺ cells. The cells in (a) fraction 2 (macrophage-rich fraction) or (b) fraction 3 (lymphocyte-rich fraction) were stained with PE-labeled rat anti-mouse Mac-1 Ab. The nature of Mac-1⁺ cells in the fraction 2 was assessed phenotypically by FACS (Y-axis [PE]/X-axis [FITC or Alexa]; C-I) and morphologically after (j) H and E staining. (c), Mac-1/CD3; (d), IgM/Mac-1; (e), B220/Mac-1; (f), Mac-1/CD11c; (g), Mac-1/CD14; (h), Mac-1/Ly-6G; (i), Mac-1/CCR3. Each value represents % cell number. (j) H and E staining of Mac-1⁺ cells purified by FACS. Scale bar = 10 μ m.

treated once *i.n.* with a mixture of allergen and adjuvant and these served as a negative control. A combination of the lymphocyte-rich population (for IgG production) with the macrophage-rich fraction (for IgE production) produced a significant amount of IgE (3.3 ± 0.8 ng/mL; mean $\pm$ SD; $n = 12$). In contrast, a combination of the lymphocyte-rich population (for IgE production) with the macrophage-rich fraction (for IgG production) produced a small amount of IgE (1.1 ± 0.5 ng/mL; mean $\pm$ SD; $n = 12$). Similarly, a mixture of cells in the lymphocyte-rich and macrophage-rich populations from mice that had been treated *i.n.* once with a mixture of allergen and adjuvant produced a large amount of IgG (477.0 ± 135.0 ng/mL; mean $\pm$ SD; $n = 12$) Ab and served as a positive control. A small amount of IgG (9.4 ± 1.2 ng/mL; mean $\pm$ SD; $n = 12$) was produced by a mixture of lymphocyte- and macrophage-rich populations from mice that had been treated *i.n.* once with allergen and these served as a negative control. A combination of the lymphocyte-rich population

(for IgE production) with the macrophage-rich population (for IgG production) produced a large amount of IgG (359.5 ± 65.0 ng/mL; mean $\pm$ SD; $n = 12$). In contrast, a combination of the lymphocyte-rich fraction (for IgG production) with the macrophage-rich fraction (for IgE production) produced a small amount of IgG (181.6 ± 57.6 ng/mL; mean $\pm$ SD; $n = 12$).

These results taken together indicate that cells in the macrophage-rich population are involved in class switching to IgE or IgG after *i.n.* sensitization by allergen alone or with complete Freund's adjuvant, respectively.

Production of interleukin-4 by various combinations of submandibular lymph node cells from mice allergen- or allergen and adjuvant-sensitized intranasally once

Interleukin-4 is essential for *in vitro* or *in vivo* production of nonspecific IgE Abs in lymphocytes after sensitization with cedar pollen *i.n.* once (8). Therefore, we assessed the cellular source of IL-4 and its amount in the culture

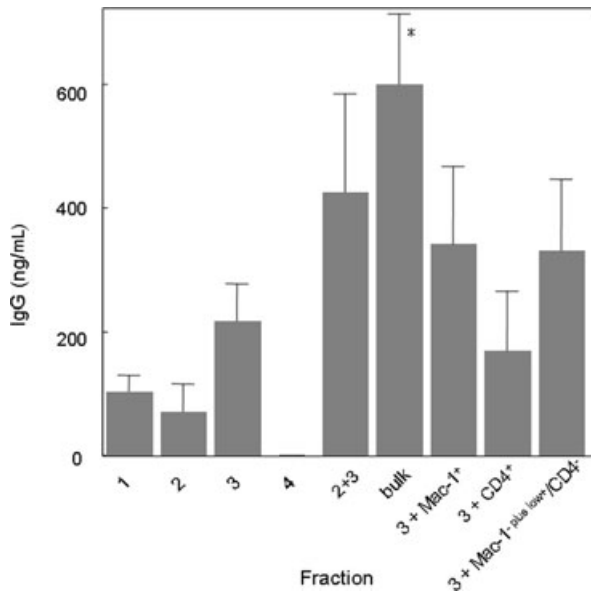


Fig. 7. Essential role of Mac-1⁺ cells in the *in vitro* production of IgG by submandibular lymph node cells from BALB/c mice allergen-sensitized *i.n.* once. On day 10 after *i.n.* sensitization (adjuvant and allergen: 150 μ L of complete Freund's adjuvant and 100 μ g of cedar pollen in 150 μ L of PBS), mono-dispersed cells were obtained from submandibular lymph nodes and separated into various fractions by Percoll density-gradient centrifugation. The concentrations of IgG Ab in the culture media were determined after the cells had been cultured for 6 days. The average number of cells added to the cocultures: 1, damaged cell-rich fraction (2.3×10^3 cells/well); 2, macrophage-rich fraction (5.0×10^5 cells/well); 3, lymphocyte-rich fraction (2.5×10^6 cells/well); 4, granulocyte-rich fraction (6.8×10^3 cells/well); bulk (3×10^6 cells/well). The effects of adding specific antigen⁺ (e.g., Mac-1⁺) cells from fraction 2 to fraction 3 were also examined: Mac-1⁺ cells in fraction 2 (2.0×10^3 cells/well); CD4⁺ cells in fraction 2 (7.7×10^4 cells/well); Mac-1⁺ plus low⁺ cells in fraction 2 (4.2×10^5 cells/well). Data are expressed as the mean $\pm$ S.D. ($n = 3$). The difference between sample and control (fraction 3 alone) is significant (*, $P < 0.02$) according to Student's *t* test.

medium (Fig. 9). Bulk submandibular lymph node cells from mice that had been injected once *i.n.* with allergen alone produced a large amount of IL-4 (96.1 ± 8.6 pg/mL; mean $\pm$ SD; $n = 9$). In contrast, the lymphocyte-rich (fraction 3) fraction of the lymph node cells produced a small amount (31.3 ± 10.9 pg/mL; mean $\pm$ SD; $n = 9$); and the macrophage-rich (fraction 2) fraction was almost inactive (20.1 ± 6.9 pg/mL; mean $\pm$ SD; $n = 9$). Surprisingly, mixed cultures of the lymphocyte-rich fraction with the macrophage-rich fraction produced a large amount of IL-4 (75.3 ± 9.9 pg/mL; mean $\pm$ SD; $n = 9$) that was released into the culture medium (Fig. 9a). In contrast, the amounts of IL-4 produced by cells in the damaged cell (fraction 1; 11.5 ± 2.7 pg/mL; mean $\pm$ SD; $n = 9$)- or granulocyte-rich (fraction 4; 5.4 ± 2.3 pg/mL;

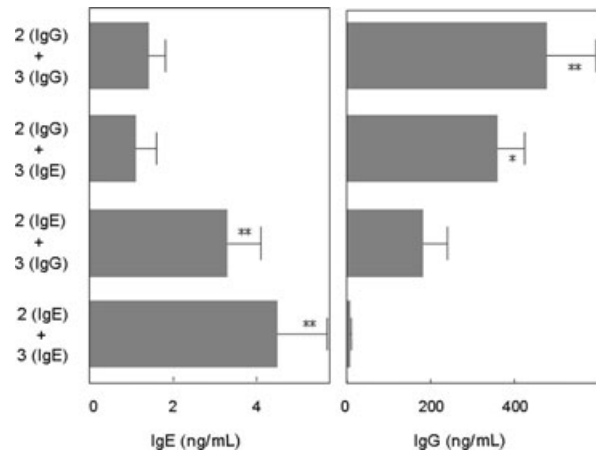


Fig. 8. Which fraction, lymphocyte- or macrophage-rich one, is essential for the class switching of Ig in B lymphocytes? On day 10 after BALB/c mice had been *i.n.* sensitized once with allergen alone (100 μ g of cedar pollen in 300 μ L of PBS) or with adjuvant and allergen (150 μ L of complete Freund's adjuvant and 100 μ g allergen in 150 μ L of PBS), the respective submandibular lymph node cells were separated into four fractions by Percoll density-gradient centrifugation; fractions 2 and 3 were used for the experiments. The concentrations of IgE or IgG Abs in the culture media were determined after the cells had been co-cultured in various combinations for 6 days. Average number of cells added to the co-cultures: fractions 2 (2 [IgE]: 5.5×10^5 cells/well) and 3 (3 [IgE]: 2.5×10^6 cells/well) from BALB/c mice sensitized *i.n.* once with allergen alone; fractions 2 (2 [IgG]: 5.3×10^5 cells/well) and 3 (3 [IgG]: 2.3×10^6 cells/well) from BALB/c mice sensitized *i.n.* once with adjuvant and allergen. Data are expressed as the mean $\pm$ S.D. ($n = 9$). The difference between sample and control: 2 (IgE) + 3 (IgE) for IgE production; 2 (IgE) + 3 (IgG) for IgG production is significant (*, $P < 0.05$; **, $P < 0.02$) according to Student's *t* test.

mean $\pm$ SD; $n = 9$) fraction were around the basal level; and there was no additional effect after mixing either of them with the lymphocyte-rich fraction (data not shown). On the other hand, bulk cells from mice that had been injected once *i.n.* with a mixture of allergen and complete Freund's adjuvant (Fig. 9b) produced almost no IL-4 (18.4 ± 6.9 pg/mL; mean $\pm$ SD; $n = 9$). The cells in their 2 + 3 fractions (macrophage-rich and lymphocyte-rich; 15.9 ± 6.9 pg/mL; mean $\pm$ SD; $n = 9$) or single (6.5–12.5 pg/mL; $n = 9$) fractions were also inactive, revealing that the cytokine IL-4 is crucial for class switching to IgE.

Of particular interest, a combination of the lymphocyte-rich population (for IgG production) with the macrophage-rich population (for IgE production) produced a large amount of IL-4 (73.3 ± 14.2 pg/mL; mean $\pm$ SD; $n = 12$). In contrast, a mixture of the lymphocyte-rich population (for IgE production) with the macrophage-rich population (for IgG production) produced a small amount of IL-4 (21.1 ± 6.1 pg/mL; mean $\pm$ SD; $n = 12$) (Fig. 8c), suggesting that

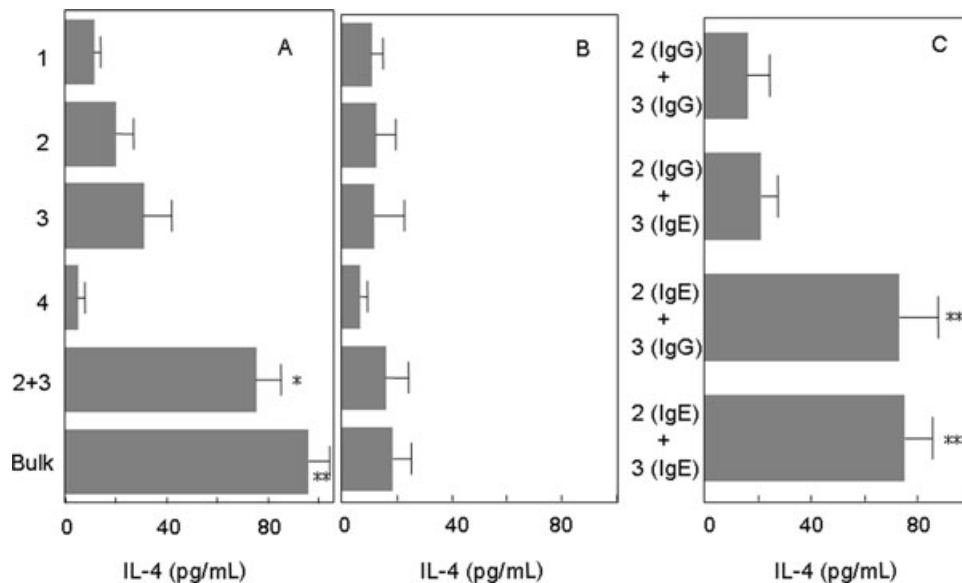


Fig. 9. Production of IL-4 by the cells in various fractions of submandibular lymph node cells from mice allergen- or allergen and adjuvant-sensitized *i.n.* once. On day 10 after *i.n.* sensitization with (a) allergen alone (100 μ g of cedar pollen in 300 μ L of PBS) or (b) with adjuvant and allergen (150 μ L of complete Freund's adjuvant and 100 μ g allergen in 150 μ L of PBS), mono-dispersed cells were obtained from submandibular lymph nodes and separated into four fractions by Percoll density-gradient centrifugation. The concentrations of IL-4 in the culture media were determined after the cells had been cultured in various combinations for 6 days. Average number of cells added to the cultures: (a) 1, damaged cell-rich fraction (4.4×10^3 cells/well); 2, macrophage-rich fraction (6.2×10^5 cells/well); 3, lymphocyte-rich fraction (1.4×10^6 cells/well); 4, granulocyte-rich fraction (9.9×10^3 cells/well). (b) 1, damaged cell-rich fraction (2.2×10^5 cells/well); 2, macrophage-rich fraction (5.0×10^5 cells/well); 3, lymphocyte-rich fraction (2.2×10^6 cells/well); 4, granulocyte-rich fraction (4.2×10^3 cells/well). (c) For example: 2 (IgE) + 3 (IgG) = fraction 2 of experiment (a) + fraction 3 of experiment (b). Data are expressed as the mean $\pm$ S.D. ($n = 9$). The differences between sample and control: fraction 3 for (a); 2 (IgG) + 3 (IgE) for (b) are significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$) according to Student's *t* test.

macrophage-rich fraction (for IgE production) plays a crucial role in production of IL-4.

Expression of interleukin-4 mRNA in CD3⁺ T cells of submandibular lymph nodes

We next studied which type of cells expresses IL-4 mRNA in submandibular lymph nodes. We obtained bulk cells of submandibular lymph nodes from BALB/c mice (day 10) that had been sensitized *i.n.* once with allergen alone, stained them with a panel of fluorescein-labeled Abs, and isolated CD3⁺ cells ($47.1 \pm 3.8\%$; mean $\pm$ SD; $n = 5$), B220⁺ cells ($50.6 \pm 4.2\%$; mean $\pm$ SD; $n = 5$), and Mac-1⁺ cells ($1.8 \pm 0.6\%$; mean $\pm$ SD; $n = 5$) by FACS. A PCR product of approximately 300 bp was clearly obtained from the RNA of the bulk or CD3⁺ cells, but not from that of the B220⁺ or Mac-1⁺ cells (Fig. 10). However, no PCR product was detected in the RNA of the CD3⁺ cells of submandibular lymph nodes from BALB/c mice (day 0 or 3) that had been sensitized once with allergen (data not shown). In contrast, the numbers of other types of cells, including mast cells, basophils, and eosinophils, in the submandibular lymph nodes on days 0–10 after

sensitization with cedar pollen *i.n.* once were too small (each less than 0.1%) to be analyzed by RT-PCR.

These results indicate that IL-4 is essential for IgE Ab production and is produced mainly in CD3⁺ T lymphocytes.

Discussion

In most previous animal models of pollen-induced allergic rhinitis, the allergic reactions were induced by repetitive pollen inhalation challenges to animals that had been sensitized by repeated instillation of the pollen extract plus adjuvant into their nostrils (19–21). Under these conditions, because leukocytes, especially eosinophils, migrate into the nasal cavity and induce edema in the mucosa; it has not been possible to determine precisely which reaction of the immune system to the allergen occurs first. Recently, it was reported that sensitization of mice by *i.n.* application of nine serial doses of Cry j 1 (0.2–5 μ g/5 μ L of PBS into each nostril) induced allergic rhinitis (22). The means of sensitization is clinically relevant; but it is unlikely that the amount of pollen extract inhaled or instilled is quantitatively related to the strength of the

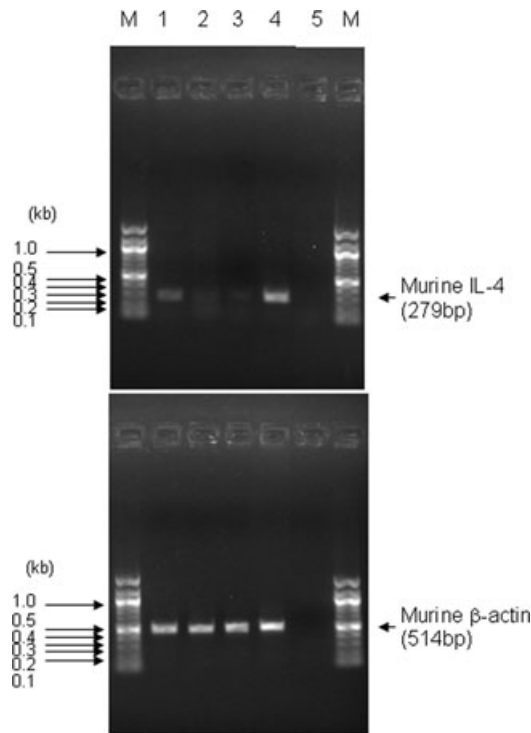


Fig. 10. RT-PCR analysis of mRNA expression of IL-4 in bulk, Mac-1⁺, B220⁺ or CD3⁺ cells of submandibular lymph nodes from BALB/c mice (day 10) that had been sensitized *i.n.* once with allergen alone. Results of agarose gel electrophoresis of PCR products after 30 cycles of amplification are illustrated. Upper panel: IL-4: M, DNA size standard marker (100 bp ladder); 1, bulk cells; 2, Mac-1⁺ cells; 3, B220⁺ cells; 4, CD3⁺ cells; 5, negative control (water). Lower panel: β -actin as a control.

reaction. In fact, instillation of a total amount of 16.6 μ g in 33.2 μ L of PBS of this allergen in five divided doses in one day into each of eight mice induced a significant increase in the serum concentrations of nonspecific IgE Ab on day 14 in only one mouse (Ogita-Nakanishi *et al.*, unpublished data). In contrast, an injection of the allergen into the area surrounding the nostrils (100 μ g in 0.15 mL of PBS) resulted in an increase ($\approx$ 10-fold of control) in serum IgE Ab production on day 10 (Fig. 4; references 7 and 8). Therefore, in the present study, we injected *i.n.* cedar pollen without adjuvant once into BALB/c mice to induce the initial stage of allergic rhinitis in various lymphoid organs, including the submandibular lymph nodes.

The histology of the palates, cell yields from the NALT, and their phenotypic composition (Fig. 1) were essentially the same as those reported previously (17, 18). However, the total cell numbers in the NALT did not change significantly on days 0–14 after *i.n.* injection of the allergen; and the bulk cells did not produce significant amounts of IgE

on days 0–14 (Figs. 2 and 3). Consistently, submandibular lymph nodes, but not the NALT, were clearly stained with *i.n.* injected Evans blue (Fig. 3, *inset*), suggesting that the NALT might not drain extracellular fluid containing *i.n.* injected allergen. Alternatively, it has been shown that *i.n.* immunization with a single dose of 1 μ L of PBS solution containing pathogens into each nostril can establish effective immunity against pneumococci, group A streptococci, influenza virus, *Bordetella pertussis*, herpes simplex virus or *Streptococcus mutans* in mice (18, 23–28). These results suggest that the once only application of pathogens in 1 μ L of PBS solution into each nostril is sufficient to reach both non-NALT lymphocytes and NALT lymphocytes. In contrast, application of even five times as much cedar pollen (3.32 μ g) in 6.64 μ L of PBS solution into each nostril might be insufficient to elicit or penetrate into the NALT or non-NALT lymphoid tissues (Ogita-Nakanishi *et al.*, unpublished data).

Previously, we reported that wild-type, IFN- γ $^{-/-}$, but not IL-4 $^{-/-}$, mice sensitized once (*i.n.* or *i.p.*) or twice (*s.c.* or *i.v.* and *s.c.*) showed a significant increase in nonspecific IgE Ab in their serum (8). In order to determine which population of PBMCs was involved in the *in vitro* production of nonspecific IgE Ab in that study, we separated PBMCs from mice sensitized *s.c.* once into three cell populations (*i.e.*, monocytes, lymphocytes, and granulocytes) by Percoll density-gradient centrifugation. (i) The lymphocyte- or monocyte-rich fraction alone does not produce of IL-4 and IgE Ab. (ii) Addition of the monocyte-rich fraction to the lymphocyte fraction is needed for production of IL-4 and IgE Abs. (iii) By using combined fractions from wild-type and IL-4 $^{-/-}$ mice we demonstrated that Mac-1⁺, but not CD4⁺ or CD4⁺/Mac-1⁺, cells are essential for IL-4 and IgE Ab production in lymphocytes. Also in the present study, Mac-1⁺/CD3⁺/IgM⁺/B220⁺/CD11c⁺/CD14⁺/Ly-6G⁺/CCR3⁺ cells in the macrophage-rich fraction were crucial for production of IL-4 and IgE Abs (Figs. 5 and 6) or IgG Abs (Fig. 7) by lymphocytes after *i.n.* sensitization with cedar pollen or this allergen and complete Freund's adjuvant. Although a large amount of IgE was induced by one *i.n.* injection of allergen alone (Fig. 4), the titer relative to high-titer IgE Ab was less than 0.00001 unit/mL (data not shown), revealing the increase to be due to nonspecific IgE Abs, as reported previously (7). Therefore, it is unlikely that the Mac-1⁺ mononuclear cells (Fig. 6) simply took up and processed protein antigens to present them to T cells.

It has been established that bacterial LPS, which can activate B cells independently of antigen, induce formation of a variety of Ig isotypes with the exception of IgE (29). However, when the same B cells are cultured for 5 days with LPS together with 100 to 500 units/mL of IL-4,

the result is the formation of IgE and selective enhancement of IgG1 formation (30), which is accompanied by a decrease in IgG2b and IgG3 formation. IL-4, essential for either conversion of Th0 to Th2 (31) or class switch of IgM to IgE (32), is produced by T cells, mast cells, basophils, eosinophils, and macrophages (33–36). In our mouse model system, CD3⁺ cells in the submandibular lymph nodes from mice that had been *i.n.* sensitized once with the allergen alone seemed to be the main producers of IL-4 (Fig. 10). However, the lymphocyte-rich fraction alone was inefficient in production of IL-4 or IgE (or IgG); addition of Mac-1⁺ cells from the macrophage-rich fraction to the lymphocyte-rich fraction was essential for this production (Figs. 5–7). Furthermore, a combination of the lymphocyte-rich population (for IgG [or IgE] production) with the macrophage-rich population (for IgE [or IgG] production) produced a large amount of IgE (or IgG). These results imply that Mac-1⁺ mononuclear cells might be involved in recognition of allergenic molecules as nonself (or allergen) and in class switching of Ig in B lymphocytes by controlling the amounts of IL-4 released from T lymphocytes.

Specific activation of an antigen-binding B cell (an antigen-presenting cell) by its cognate T cell leads to expression of CD40 ligand on the helper T-cell surface and to secretion of IL-4, IL-5, and IL-6, which drive proliferation and differentiation of B cells into antigen-specific Ab-secreting plasma cells (37). However, as reported previously (7, 8) and also in the present study, the IgE Ab produced by mice that have been injected once *i.n.* with allergen is not specific for that allergen: the titer relative to high-titer serum was less than 0.00001, suggesting that B cells might not act as antigen-presenting cells. Addition of Mac-1⁺ cells from the macrophage-rich fraction to the lymphocyte-rich fraction was essential for production of IL-4 and total IgE Abs in the lymphocytes (Figs. 5–7). Therefore, it is unlikely that cultured monocytes (8) or macrophages (present study) internalize and degrade allergen to present peptides from internal proteins to T cells, implying antigen-nonspecific activation of T cells by macrophages.

In conclusion: (i) the submandibular lymph nodes are the main organ responsive to *i.n.* injected cedar pollen; (ii) bulk cells in the submandibular lymph nodes from mice that have been treated *i.n.* once with allergen alone or with a mixture of allergen and complete Freund's adjuvant mainly produce IgE or IgG, respectively; and (iii) macrophages in the submandibular lymph nodes are essential for IL-4, IgE or IgG production by lymphocytes and are involved in class switching of Ig in B lymphocytes by controlling the amounts of IL-4 released from CD3⁺ T lymphocytes.

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DISCLOSURE

The authors have no financial conflicts of interest.

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