Splenic Dendritic Cells Induced by Oral Antigen Administration Are Important for the Transfer of Oral Tolerance in an Experimental Model of Asthma

Katsuya Nagatani, Makoto Dohi, Yasuo To, Ryoichi Tanaka, Katsuhide Okunishi, Kazuyuki Nakagome, Kayo Sagawa, Yudo Tanno, Yoshinori Komagata and Kazuhiko Yamamoto

J Immunol 2006; 176:1481-1489; doi: 10.4049/jimmunol.176.3.1481
http://www.jimmunol.org/content/176/3/1481

References  This article cites 45 articles, 20 of which you can access for free at: http://www.jimmunol.org/content/176/3/1481.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Splenic Dendritic Cells Induced by Oral Antigen Administration Are Important for the Transfer of Oral Tolerance in an Experimental Model of Asthma

Katsuya Nagatani,1 Makoto Dohi, Yasuo To, Ryoichi Tanaka, Katsuhide Okunishi, Kazuyuki Nakagome, Kayo Sagawa, Yudo Tanno,2 Yoshinori Komagata,3 and Kazuhiko Yamamoto

Peripheral tolerance can be induced after the feeding of Ag, which is referred to as oral tolerance. We demonstrated in this study that the oral administration of OVA induced tolerance in an experimental model of asthma in mice, and investigated which cells function as the regulatory cells in the transfer of this oral tolerance. In OVA-fed mice, the percentage of eosinophils in bronchoalveolar lavage fluid, serum IgE levels, airway hyperresponsiveness, and mRNA levels of IL-13 and eotaxin were significantly lower than found in nonfed mice. Histological examination of lung tissue showed a suppression of the accumulation of inflammatory cells in the peribronchial area of OVA-fed mice. Feeding after the first immunization or between the first and the second immunization suppressed these findings, whereas feeding just before the airway Ag challenge did not. The suppression of disease in OVA-fed mice was successfully transferred by injection of whole spleen cells of OVA-fed mice. When CD11c⁺ dendritic cells (DCs) were removed from splenocytes, this transfer of suppression was completely abolished. The injection of splenic DCs purified from OVA-fed mice alone transferred the suppression, whereas the injection of splenic DCs from naïve mice that were cocultured with OVA in vitro did not. These data suggest that not only CD4⁺ T cells, but also CD11c⁺ DCs induced by Ag feeding are important for the active transfer of oral tolerance in this murine experimental model of asthma. The Journal of Immunology, 2006, 176: 1481–1489.

oral tolerance is one of the approaches to inducing Ag-specific peripheral tolerance. Although the therapeutic applications of oral tolerance to the treatment of Th1-driven autoimmune diseases such as experimental allergic encephalomyelitis and collagen-induced arthritis are relatively well documented, the mechanisms of suppression of Th2-driven allergic inflammation such as bronchial asthma are not yet well understood (1). The induction of oral tolerance is an immunologic process that is mediated by more than one mechanism and depends on dose and regimen. For the therapeutic application of oral tolerance to patients with allergies or autoimmune diseases, it is important to estimate the most effective conditions for these factors (2). In particular, the feeding dose has been regarded as an important factor in the induction of oral tolerance. It was previously reported that low-dose feeding of Ag induced active cellular regulation, which was adoptively transferred in vivo (3, 4). In contrast, high-dose feeding of Ag induced clonal anergy and/or deletion that was not transferable (5–8). Some studies have shown that high- or low-dose feeding of Ag prevents Th2-driven allergic inflammation such as bronchial eosinophilia (9–11) and airway hyperresponsiveness (12, 13). However, there have been few reports on whether feeding dose and regimen influence the effects of oral tolerance in Ag-induced lung inflammation in mice (14), which models certain aspects of human asthma. We demonstrated that the oral administration of Ag induced tolerance in an experimental model of asthma, and we evaluated whether the feeding protocol exerted an influence on the induction of tolerance in this model.

Regarding the regulatory cells induced in oral tolerance, there have been conflicting findings. Initially, it was reported that the regulatory cells induced by Ag feeding were CD8⁺ cells in Lewis rat models of experimental allergic encephalomyelitis (15) and uveitis (16). Yet, it has also been reported that CD8⁻ cells but not CD4⁻ deficient or depleted mice cells had induced oral tolerance in murine models of collagen-induced arthritis (17), experimental allergic encephalomyelitis (18), and pulmonary eosinophilia (9). It is now generally accepted that CD4⁻ T cells are major cells in the induction of oral tolerance. We performed the adoptive transfer of spleen cells depleted of a specific subpopulation, including APC, to determine which cells were regulatory cells in this transfer of oral tolerance, and we showed that dendritic cells (DCs) were important for the transfer. Then, we found that the injection of CD11c⁺ DCs from OVA-fed mice could transfer the tolerance.

A recent study has shown that the adoptive transfer of pulmonary DCs from mice exposed to respiratory Ag induced Ag-specific unresponsiveness in recipient mice (19), suggesting that DCs play a critical role in the induction of the tolerance. In addition, it has been demonstrated that DCs are the major APC in gut-associated lymphoid tissue, and that DCs control the immune response to fed Ag. In the

1 Current address: Department of Community Health and Medicine, Research Institute, International Medical Center of Japan, 7-3-1 Toyama, Bunkyo-ku, Tokyo 113-8655, Japan.
2 Current address: Division of Nephrology and Hypertension, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan.
3 Address correspondence and reprint requests to Dr. Yoshinori Komagata, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail address: komagata-tky@umin.ac.jp

4 Abbreviations used in this paper: DC, dendritic cell; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff.
present study, bronchial eosinophilia was actively suppressed by the transfer of splenic DCs from Ag-fed mice. Our report indicates the major role of splenic DCs in the transfer of active suppression by oral tolerance in a murine experimental model of asthma.

Materials and Methods

Mice

Male BALB/c mice (6 wk of age) were purchased from Japan SLC. The mice were maintained under specific pathogen-free conditions. All of the animal experiments conducted in this study were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo (Tokyo, Japan).

Immunization of mice

Five to ten mice per group were immunized according to a previously described method (20, 21). Briefly, mice were immunized i.p. with 2 μg of OVA (grade V; Sigma-Aldrich) in 2 mg of aluminum hydroxide (alum). This immunization was repeated after a 10-day interval (on days 0 and 10). Control mice received a saline injection instead of the OVA/alum solution. Seven days after the immunization, sensitized mice inhaled an aerosolized solution of 3% OVA dissolved in PBS for 10 min. OVA inhalation was conducted for 3 days in a row (days 18, 19, and 20). Control mice inhaled PBS alone under the same conditions as used for the experimental group.

Induction of oral tolerance

Before the first i.p. immunization, mice were fed 1 mg (low-dose) or 30 mg (high-dose) of OVA or water only (nonfed) every other day from 10 to 2 days before the first immunization (five feedings in total) by gastric intuba-

tion with a stainless steel animal feeding needle. To examine the inhibi-
tory effect of feeding on ongoing immunization, mice were fed 30 mg of OVA only before the first i.p. immunization (pre-i.p.), only after the second i.p. immunization (post-i.p.), or between the first and the second immuni-

zation (intermediate i.p.). For post-i.p. feeding, we fed OVA every other day from the day of the second immunization to 8 days after the immuni-

zation. For intermediate i.p. feeding, we fed OVA every other day from 1 to 9 days after the first immunization.

Measurement of airway hyperresponsiveness

At 24 h after the final immunization (day 21), airway hyperresponsiveness was assessed by methacholine-induced airflow obstruction in the conscious mice, as previously described. Briefly, the mice were exposed for 2.5 min to nebulized physiologic saline (Otsuka Pharmaceutical), followed by incre-

dental doses (1–30 mg/ml) of nebulized methacholine. These mice were placed in a whole-body plethysmograph for 2.5 min following nebu-

lization, and enhanced pause (Penh) was measured using Biosystem XA

WB system (Buxco Electronics). “Penh” represents pulmonary airflow

obstruction and was calculated using the formula: Penh = ((Te − Tr)/ (Tr × PEF/PIF)), where Penh = enhanced pause (dimensionless), Te = expiratory time (seconds), Tr = relaxation time (seconds), PEF = peak expiratory flow (milliliters per second), and PIF = peak inspiratory flow (milliliters per second). Penh was measured and averaged approximately every 5 s, and the cumulative values were averaged as the Penh value for each time point. Airway hyperresponsiveness was expressed as PC20Mch (200% provacative concentration of methacholine), which is the concentra-

tion of methacholine that doubled the baseline Penh value.

Analysis of bronchoalveolar lavage fluid (BALF)

After the measurement of airway hyperresponsiveness, bronchoalveo-

lar lavage samples were obtained. The mice were anesthetized by i.p. injection of sodium pentobarbital (Dainippon Chemicals), and then the lungs were lavaged with 0.5 ml of saline four times. The lavage fluid was centrifuged and the cells were resuspended in 1 ml of saline with 1% BSA (Wako Pure Chemical). Total cell numbers were counted using a hemocytometer. Cy-

tosin samples were prepared by centrifuging the suspensions at 300 rpm for 5 min. To clearly distinguish the eosinophils from the neutrophils, three different stains were applied: Diff-Quick, May-Grünwald-Giemsa, and Hansel (eosin) stains. At least 300 leukocytes were differentiated by light microscopy based on the standard morphologic criteria. The level of IL-13 in BALF was detected by ELISA kit (R&D Systems) following the man-

ufacturer’s instructions.

Measurement of serum total IgE and OVA-specific Ig

On day 21, blood samples were obtained from the inferior vena cava with a 25-gauge needle under anesthesia. After the samples had fully coagu-

lated, they were centrifuged, and the sera were collected and stored at −80°C until use. Total IgE was assayed by ELISA using paired Abs (BD Pharmingen) according to the manufacturer’s instructions. To measure OVA-specific IgE, IgG1, and IgG2a in sera, plates were coated with 2 μg/ml OVA instead of capture Abs for OVA-specific IgE, IgG1, and IgG2a, and these samples were assayed as described earlier. The titers of the samples were calculated by comparison with internal standards, which were obtained from the sera of OVA-sensitized mice on day 18. These standards were calculated as 500 U/ml.

Histological examination of lung tissue

After bronchoalveolar lavage samples were obtained, in some of the ex-

periments the lungs were perfused with physiologic saline and were re-

sected from the mice. The lungs were fixed with neutralized buffered for-

malin (WAKO) and embedded in paraffin. Sections (3-μm thick) were

stained with H&E or periodic acid-Schiff (PAS). The intensity of histo-

logical changes in the lungs was evaluated with four grading scores (0, no

inflammation; 1, slight/mild; 2, moderate; and 3, severe), according to the
distribution and intensity of the following findings, as previously reported (20, 21): 1) epithelial shedding or undulation of the nuclei of bronchi-

al epithelial cells, 2) increase in the number of goblet cells, 3) infiltration of inflammatory cells from vessels into the mucosal and submucosal area of the bronchus and peribronchial interstitium, and 4) hypertrophy and thick-

eening of the smooth-muscle cell layer.

RT-PCR for analysis of cytokine and chemokine gene expression in the lung

In some experiments, the lungs were removed after perfusion with physi-

ologic saline, and total RNA was extracted using ISOGEN (Nippon Gene) according to the manufacturer’s instructions. Total RNA (10 μg) was re-

verse-transcribed using oligo(dT)15 primer (Promega) and SuperScript II

RNase H-reverse transcriptase (Invitrogen Life Technologies) at 42°C for

2 h. To ensure that each sample contained the same amount of CDNA, the

β-actin cDNA concentration of each sample was first determined using

β-actin-specific primers. These samples were amplified for the appropriate number of cycles, such that the amount of PCR product remained on the linear part of the amplification curve. The PCR products were electrophore-

sed in a 2% agarose gel and were visualized by ethidium bromide stain-

ing. We then determined the levels of IL-13, eotaxin, IL-10, IFN-γ, and

TGF-β using the following specific primer sets. The sense primer for β-ac-

tin was 5’-AGCAGATGGAGAAGATCTGG-3’t, and the antisense primer was

5’-TCTGATAGGGCCACAGTGTG-3’t. The sense primer for IL-13

was 5’-TTCTGCTTTGCTTGGTGCTTCG-3’t, and the antisense primer was

5’-GATGGCATATGCAATTTCTGGTGTTTG-3’t. The sense primer for eotaxin was 5’-GGCAGAATCTCCATGTCTCC-3’t, and the anti-

sense primer was 5’-CACCTCTCTTGGGAGTCAGC-3’t. The sense primer for

IL-10 was 5’-TACCTGGTAGAGGTAGTGC-3’t, and the antisense primer was

5’-TCGGTGAAGGACTACATGAGT-3’t. The sense primer for TGF-β was 5’-

CTTAAAGAGGACCTGGTTT-3’t, and the antisense primer was 5’-CAGG

AGGCACAAATCAGTGT-3’t.

Positive and negative selection of spleen cells and adoptive cell transfer

BALB/c mice were fed 30 mg of OVA every other day for a total of five feedings. Two days after the last feeding, the spleen of each mouse was digested with 0.1% collagenase (Sigma-Aldrich) at 37°C for 20 min. In some experiments, single-cell suspensions of whole spleen cells were pre-

pared and cultured with Con A (2 μg/ml; Sigma-Aldrich) for 48 h. Cells were counted, and 106 cells were adoptively transferred i.v. into naive BALB/c mice. For negative selection, CD4+, CD8+, CD11c+, CD19+, and CD11b- cells were depleted from the whole spleen cells using magnetic beads (MACS; Miltenyi Biotec) with biotinylated anti-mouse CD4, CD8, CD11c, CD19, and CD11b mAb (BD Pharmingen), according to the man-

ufacturer’s instructions. The efficiency of depletion was examined by flow cyto-

metry (>99%). For positive selection, CD4+ or CD11c+ cells were purified using anti-mouse CD4+ or CD11c+ microbeads (MACS) follow-

ing the manufacturer’s instructions. The purity of positively selected cells was >93%, which was checked using flow cytometry. For cell transfer experiments, cells were transferred into naive BALB/c mice from the tail veins just before the first immunization or just after the second immuni-

zation. The number of transferred cells was 107 for whole spleen cells, subpopulation-depleted spleen cells, or positively selected CD4+ cells or 108 for positively selected DCs.
Preparation of OVA-pulsed DCs

Single cell suspensions of whole spleen cells were prepared from naive BALB/c mice, and CD11c+ cells were purified using anti-mouse CD11c microbeads (MACS) as described earlier. Purified 1 × 10^7/ml CD11c+ cells were cultured with OVA at 1 mg/ml for 24 h. Cultured cells were collected, and 10^6 cells were adoptively transferred i.v. into naive BALB/c mice and immunized as described.

Statistical analysis

The results are expressed as the mean ± SEM. Statistical evaluation was performed with one-way ANOVA. Values of p < 0.05 were considered statistically significant.

Results

Both low- and high-dose feeding prevented not only bronchial eosinophilia but also airway hyperresponsiveness

Bronchial eosinophilia is reduced by the oral administration of OVA in the murine model of OVA-induced lung inflammation (14). We first examined whether the oral administration of OVA prevented not only bronchial eosinophilia, but also airway hyperresponsiveness, and we evaluated the feeding dosage required for the induction of tolerance in this model. BALB/c mice were immunized twice i.p. with OVA/alum, and before the first i.p. immunization, we fed the mice 1 mg (low-fed) or 30 mg (high-fed) of OVA, or water only (nonfed) once every other day for a total of five feedings. Following OVA inhalation after the second immunization, the number of total cells or eosinophils in the BALF, serum IgE levels, and airway hyperresponsiveness were examined.

In the OVA-fed mice, the total cell number in the BALF (Fig. 1A), the percentage of eosinophils in the BALF (Fig. 1B), and serum total IgE levels (Fig. 1C) were remarkably lower than those found in nonfed mice. Moreover, in the OVA-fed mice, PC_{250Mch}, which reflects airway hyperresponsiveness in this murine model of asthma, was also significantly higher than that in nonfed mice (Fig. 1D). Histological examination of the lung tissue showed a dramatic suppression of the accumulation of inflammatory cells in peribronchial and perivascular areas in low-dose (Fig. 1E, b and e) and high-dose (Fig. 1E, c and f) OVA-fed mice, as compared with nonfed mice (Fig. 1E, a and d). At high magnification, dense mononuclear cell infiltration was present in the peribronchial and perivascular areas of the tissue in the nonfed mice; this infiltration consisted primarily of eosinophils and lymphocytes (Fig. 1E, a and d). High-dose feeding was more effective than low-dose feeding in all of these observations (Fig. 1).

OVA feeding before the i.p. immunization prevented the disease, but feeding after the i.p. immunization failed to suppress it

It is important in terms of clinical application whether Ag feeding has suppressive efficacy on ongoing immune responses. To determine whether the timing of administration affected the induction of tolerance, we fed OVA to the mice not only before the immunization, but also after the second i.p. immunization or between the first and the second i.p. immunization; we then evaluated the mice for the suppression of several parameters of the disease. High-dose feeding before the i.p. immunization (pre-i.p.) suppressed the percentage of eosinophils in BALF (Fig. 2A), the level of serum total IgE (Fig. 2B), and airway hyperresponsiveness (Fig. 2C). The feeding between the first and the second i.p. immunization (intermediate i.p.) also suppressed the percentage of eosinophils in BALF, the level of serum total IgE, and airway hyperresponsiveness (Fig. 2F). However, the feeding after i.p. immunization (post-i.p.) failed to induce suppression of the disease.

Th2 cytokines such as IL-13 and chemokines such as eotaxin can be regarded as essential mediators for the development of bronchial eosinophilia and/or airway hyperresponsiveness. To examine whether the suppression of bronchial eosinophilia or airway hyperresponsiveness was consistent with the local cytokine and chemokine patterns in the lung, we checked the gene expression of IL-13 and eotaxin in the whole lung tissue using RT-PCR. We also checked the expression of IFN-γ as a Th1 cytokine and of IL-10 and TGF-β as regulatory cytokines to examine the mechanism of suppression. As shown in Fig. 2D, the levels of IL-13 and eotaxin in the lung remarkably decreased in OVA-fed mice when we fed the mice before i.p. immunization, as compared with the nonfed mice. However, the feeding after the i.p. immunization failed to induce suppression of IL-13 or eotaxin. The levels of TGF-β, IL-10, and IFN-γ in the lung did not change in the OVA-fed mice (Fig. 2D). When we checked the expression level of other Th2 cytokines such as IL-4 and IL-5, the levels of these cytokines were sometimes suppressed in the group fed before i.p. immunization. However, the results often fluctuated from experiment to experiment (data not shown). We also measured the protein level of Th2 cytokines in BALF. In parallel with the gene expression, IL-13 in BALF almost completely disappeared in the group fed before i.p. immunization (Fig. 2E). However, the protein levels of IL-4 and IL-5 were not significantly lower in this group (data not shown).

Whole spleen cells of OVA-fed mice transferred suppression of bronchial eosinophilia before and after i.p. immunization

Oral tolerance is an active immunologic process that is mediated by multiple mechanisms. One such mechanism is active suppression by Ag-specific regulatory cells. To examine whether the bronchial eosinophilia in this model is actively suppressed by the cell transfer, we adoptively transferred spleen cells of high-dose OVA-fed mice. In this model, 10^7 whole spleen cells were adoptively transferred i.v. into naive BALB/c mice, and then the mice were immunized with OVA/alum and subjected to OVA inhalation to induce bronchial eosinophilia. In this experiment, we cocultured the whole spleen cells with Con A for 48 h before the transfer to expand T cells. Whole spleen cell transfer from high-dose OVA-fed mice before the first i.p. immunization remarkably suppressed bronchial eosinophilia (Fig. 3A). Whole spleen cell transfer from low-dose OVA-fed mice also suppressed bronchial eosinophils (data not shown). Next, to evaluate whether the suppression of bronchial eosinophilia could be transferred into mice in which immunization was in progress, we transferred whole spleen cells into the mice after the second immunization with OVA/alum. Whole spleen cell transfer from high-dose OVA-fed mice after the second i.p. immunization significantly suppressed bronchial eosinophilia, compared with the whole spleen cell transfer from nonfed mice (Fig. 3B). These results indicate that the bronchial eosinophilia in this model was actively suppressed by whole spleen cell transfer from OVA-fed mice, even in cases in which the immunization was already ongoing.

CD11c+ DCs are essential for the transfer of disease suppression by oral tolerance

To identify the regulatory cells in this transfer system of oral tolerance, we examined whether the adoptive transfer of spleen cells depleted of a specific phenotype would suppress bronchial eosinophilia. CD4+ cells, CD8+ cells, or APCs (CD11c+, CD19+, and CD11b+ cells) were depleted from whole spleen cells using magnetic beads and adoptively transferred i.v. into naive BALB/c mice, after which the BALB/c mice were immunized with OVA/alum and bronchial eosinophilia was induced. Transfer of the spleen cells depleted of APC failed to suppress the bronchial eosinophilia, whereas the depletion of CD4+ or CD8+ cells partially abrogated the suppression. The depletion of CD4+ cells abrogated active suppression more effectively than did the depletion of CD8+...
These results suggested that not only CD4+ cells but also APC were important for the active suppression in this transfer system of oral tolerance. Next, to determine the major subset of APC required for the transfer of tolerance, CD11c+ cells, CD19+ cells, or CD11b+ cells were removed and adoptively transferred into naive BALB/c mice. The depletion of CD11c+ cells completely blocked the transfer of tolerance and CD11b is expressed on macrophages and myeloid DCs, it was suggested that DCs, especially myeloid DCs, are important for active suppression in this transfer system of tolerance.

To confirm that splenic CD11c+ DCs in OVA-fed mice have a regulatory function that can be transferred in this model, we positively selected CD4+ T cells or CD11c+ DCs from the spleen of OVA-fed mice using magnetic beads and adoptively transferred them by i.v. injection into naive BALB/c mice just before the first
immunization with OVA in aluminum hydroxide. As shown in Fig. 5A, the percentage of eosinophils in the BALF of mice injected with CD4\(^+\) or CD11c\(^+\) cells was significantly lower than that in control mice, which shows that not only CD4\(^+\)T cells alone but also CD11c\(^+\)DCs alone were able to transfer the suppression of eosinophilia. In this experiment, we also checked airway hyperreactivity, peribronchial inflammation, and mucus production and the level of serum OVA-specific Ig. Airway hyperreactivity was assessed by methacholine-induced airflow obstruction. Data are expressed as the mean PC\(_{200}\)Mch (milligrams per milliliter). D, Gene expression of cytokine and chemokine in the whole lung. The total RNA was extracted from the whole lung and the gene expression of IL-13, eotaxin, IFN-\(\gamma\), IL-10, and TGF-\(\beta\) were assessed by RT-PCR using specific primers. E, IL-13 in the BALF. Protein level of IL-13 in the BALF of each group was assayed by ELISA. F, The percentage of eosinophils in BALF, the level of serum total IgE, and airway hyperresponsiveness of mice that were fed 30 mg of OVA between the first and the second i.p. immunization (Intermediate-i.p.) were checked. The mice were immunized as described for Fig. 1. Statistically significant data (*) are indicated.

FIGURE 2. Influence of the timing of oral OVA administration upon the suppression of experimental asthma. Mice were fed 30 mg of OVA only before the first i.p. immunization (Pre-i.p.), between the first and the second i.p. immunization in some experiments (E: Intermediate-i.p.), or only after the second i.p. immunization (Post-i.p.) every other day for a total of five feedings. The mice were immunized as described for Fig. 1. The control group was fed water and was immunized with OVA in aluminum hydroxide (OVA/Non-fed) or saline (Saline/Non-fed). On day 21, airway hyperresponsiveness was measured. After the measurement of airway hyperresponsiveness, BALF, blood samples, and the whole lung were obtained. The whole lung was removed for the study of the gene expression of cytokines and chemokines. A, The percentage of eosinophils in BALF. B, The level of serum total IgE was assayed by ELISA. C, Airway hyperresponsiveness was assessed by methacholine-induced airflow obstruction. Data are expressed as the mean PC\(_{200}\)Mch (milligrams per milliliter). D, Gene expression of cytokine and chemokine in the whole lung. The total RNA was extracted from the whole lung and the gene expression of IL-13, eotaxin, IFN-\(\gamma\), IL-10, and TGF-\(\beta\) were assessed by RT-PCR using specific primers. E, IL-13 in the BALF. Protein level of IL-13 in the BALF of each group was assayed by ELISA. F, The percentage of eosinophils in BALF, the level of serum total IgE, and airway hyperresponsiveness of mice that were fed 30 mg of OVA between the first and the second i.p. immunization (Intermediate-i.p.) were checked. The mice were immunized as described for Fig. 1. Statistically significant data (*) are indicated.
level of IgG2a was not changed significantly by the transfer (Fig. 5C). In contrast, whole spleen cells or CD4⁺ T cells suppressed both IgG1 and IgG2a, although the suppression was not statistically significant. These results suggested that the mechanism of suppression by the transfer of CD11c⁺ DCs was different from that by the transfer of CD4⁺ cells.

**Ag-loading in vivo is important for conferring a regulatory function onto DCs**

To examine whether Ag-loading by DCs in vivo is important for the transfer of this inhibitory function, the effect of in vivo and in vitro Ag-loading were compared. We prepared splenic DCs purified from OVA-fed and nonfed mice, and DCs from naive mice pulsed with OVA in vitro. These three types of splenic DCs were transferred into naive mice just before the first immunization with OVA in aluminum hydroxide. As shown in Fig. 6, the eosinophilia of the mice injected with OVA-pulsed splenic DCs was worsened, whereas that of the mice injected with splenic DCs from OVA-fed mice was improved. This result indicates that Ag-loading in vivo is very important in allowing DCs to have a regulatory function.

**Discussion**

We demonstrated in this study that the oral administration of Ag successfully induced tolerance in the murine model of asthma. We then evaluated whether the feeding protocol had an influence on the induction of tolerance in this model. Moreover, the present findings suggest the possibility that “tolerogenic DCs” induced by Ag feeding play a major role in the active suppression of oral tolerance. First, we demonstrated that the oral administration of Ag prevented not only bronchial eosinophilia but also airway hyperresponsiveness. We then evaluated whether the feeding dose exerted an influence on the induction of tolerance in this model. Feeding dose is critical for the induction of oral tolerance (22) and it is very important in clinical applications to estimate the most effective conditions for the induction of oral tolerance. Recent studies of oral tolerance in an OVA-induced asthma model have examined only high-dose OVA administration (9 –12). In other disease models, the dose and the delivery route of the Ag were found to exert effects on the induction of tolerance (23–25). However, there have been few reports addressing whether feeding dose affects the induction of oral tolerance in a murine model of asthma (14). We showed in this study that both low- and high-dose feeding prevented not only bronchial eosinophilia but also airway hyperresponsiveness. Our data indicate that high-dose feeding is more effective than low-dose feeding in this model (Fig. 1).
FIGURE 5. Prevention of experimental asthma by the transfer of CD4⁺ cells and CD11c⁺ spleen cells from high-dose OVA-fed mice. Naive BALB/c mice were immunized as described for Fig. 1. A total of 10⁷ CD4⁺ cells or 10⁶ CD11c⁺ spleen cells of OVA-fed mice were adoptively transferred by i.v. injection into naive BALB/c mice just before the first immunization with OVA in aluminum hydroxide (Fed/CD4⁺ or Fed/CD11c⁺ transfer). The control mice were injected with whole spleen cells of water-fed mice (Non-fed/Whole transfer) or OVA-fed (Fed/Whole transfer) mice. At 24 h after the final inhalation (day 21), airway hyperresponsiveness was measured. After the measurement of airway hyperresponsiveness, BALF and blood samples were obtained and the whole lung was removed for histological examination. A, The percentage of eosinophils in BALF. The cell differentials in the BALF were identified by morphologic criteria. B, Airway hyperresponsiveness was assessed by methacholine-induced airflow obstruction. Airway hyperresponsiveness was expressed as PC₂₀₀Mch. C, The levels of serum OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a were assayed by ELISA. D, Histological examination of the lung tissue. Histological findings were evaluated and scored into four grades based on the distribution and intensity of inflammation as described in Materials and Methods. Representative histological findings in lungs were stained with H&E or PAS. Magnification ×100 with H&E stain (a), ×200 with H&E (e), and ×200 with PAS (i) of lung tissue from Non-fed/Whole transfer mice. Magnification ×100 with H&E stain (b), ×200 with H&E (f), and ×200 with PAS (j) of lung tissue from Fed/Whole transfer mice. Magnification ×100 with H&E stain (c), ×200 with H&E (g), and ×200 with PAS (k) of lung tissue from Fed/CD4⁺ transfer mice. Magnification ×100 H&E stain (d), ×200 with H&E (h), and ×200 with PAS (l) of lung tissue from Fed/CD11c⁺ transfer mice. Mucus production is stained red with PAS. Statistically significant data (*) are indicated.
For clinical applications, it is important to determine whether Ag feeding exerts a suppressive effect on immune responses that are already in progress. To evaluate whether the timing of the feeding affected the induction of tolerance, we tested the animals to determine whether OVA feeding after an i.p. immunization would also prevent the disease. Our data indicated that the development of bronchial eosinophilia and airway hyperresponsiveness was not suppressed by high-dose OVA feeding just before the Ag challenge, but OVA feeding after the first immunization suppressed the development of the disease (Fig. 2). A recent report demonstrated that OVA feeding after the intranasal OVA challenge did not suppress bronchial eosinophilia, but OVA feeding after the second immunization successfully suppressed bronchial eosinophilia (12). The discrepancy between those results and ours might be due to the use of two different immunization protocols. However, it is of note that both datasets suggest the possibility of a clinical application for sensitized patients.

Oral tolerance is mediated by more than one mechanism, and the major proposed mechanisms are as follows: 1) induction of regulatory T cells secreting immunoregulatory cytokines such as TGF-β (22) and IL-10 (26); 2) immune deviation to a Th1-type response by IFN-γ, which has been shown to inhibit the Th2-type response in vitro (27, 28) and in vivo (29 –31); and 3) anergy or deletion of Ag-specific T cells (6, 18). To examine whether the suppression of bronchial eosinophilia and airway hyperresponsiveness is consistent with local cytokine and chemokine patterns in the lung, we also analyzed the gene expression of major cytokines and chemokines in whole lung tissue using RT-PCR. Th2 cytokines such as IL-4, IL-5, and IL-13 and chemokines such as eotaxin were regarded as essential mediators for the development of bronchial eosinophilia and/or airway hyperresponsiveness (32–35). Although the levels of IL-13 and eotaxin in the lung were remarkably decreased in OVA-fed mice before the first immunization (Fig. 2, D and E), the level of IL-4 or IL-5 were not suppressed as much as IL-13 (data not shown). Some reports have suggested that IL-13 is more important key regulator in the experimental airway hyperreactivity model than are IL-4 or IL-5 (36–38).

Our findings suggest that the inhibition of airway hyperreactivity and eosinophilia by direct feeding of Ag was partially due to the suppression of IL-13 and eotaxin. The levels of TGF-β, IL-10, and IFN-γ in the lung did not increase in tolerized mice, suggesting that these cytokines were not involved in the mechanism of oral tolerance in this model. Our data are in agreement with the findings of recent reports showing that treatment with anti-TGF-β Abs had no significant effect on the inhibition of tracheal eosinophilia (11, 12); however, the present data contradict the findings of another previous report (9) in this regard. This discrepancy could be due to the use of different immunization protocols or to the use of transgenic vs nontransgenic mice. Conversely, a recent report demonstrated that both IL-10 and TGF-β gene expression of CD14+ macrophages in BALF from Ag-fed dogs was up-regulated in a dog model of asthma, which was assessed by RT-PCR (13). However, the levels of IL-10 and TGF-β in BALF from tolerized mice did not increase in the present series of experiments, as determined by ELISA (data not shown).

To determine whether bronchial eosinophilia in this model was actively suppressed by the cell transfer, we adoptively transferred the spleen cells of high-dose OVA-fed mice and examined whether bronchial eosinophilia was suppressed. Our data show that bronchial eosinophilia in this model was actively suppressed by whole spleen cell transfer from fed mice (Fig. 3A), even when the transfer took place during the immunization period (Fig. 3B), suggesting the possibility of a clinical application for the treatment of sensitized patients. To explore the regulatory cells in this transfer system of oral tolerance, we conducted the adoptive transfer of spleen cells depleted of specific subpopulations. Our results show that the removal of CD4+ cells partially blocked active suppression (Fig. 4A), whereas the depletion of CD19+ cells did not affect the transfer of tolerance (Fig. 4B). Active suppression was entirely blocked by the depletion of whole APCs or CD11b+ cells, and the depletion of CD11c+ cells made the bronchial eosinophilia worse than that in nontolerized mice (Fig. 4). One previous report demonstrated that the depletion of CD4+ cells, but not that of CD8+ cells, blocked the active suppression of pulmonary eosinophilia (9). We demonstrated in this study that the depletion of CD8+ cells led only to a partial reversal of active suppression. In contrast, the depletion of CD11c+ DCs blocked active suppression more effectively than did the depletion of CD4+ cells. We therefore followed-up with a trial in which we purified only CD11c+ cells from the spleen of OVA-fed mice and adoptively transferred them. By the transfer of CD11c+ DCs, eosinophilia in BALF, airway hyperreactivity, peribronchial inflammation, and OVA-specific IgE productions were all suppressed, indicating that splenic CD11c+ DCs of OVA-fed mice functioned as “regulatory cells” in the suppression of disease in this model.

Several recent studies have proposed the existence of tolerogenic or regulatory DCs (19, 39–44). Especially in the murine model of airway hyperreactivity, Akbari et al. (19, 43) have reported that the tolerance to Ag can be adoptively transferred by the pulmonary DCs exposed to respiratory Ag, which secrete IL-10. Alpan et al. (42, 44) have shown that DCs from Ag-fed animals are educated to induce tolerance to OVA in the OVA-induced murine model of asthma (Fig. 5). Mouse splenic DCs are categorized into the subpopulations of CD8−CD11b−CD4+ DCs, CD8+CD11b+CD4− DCs, and CD8−CD11b+CD4− DCs (45). The results of the present study suggest that CD11b+ DCs were more important than CD8− DCs because the depletion of CD11b+ cells partially abrogated the transfer of suppression (Fig. 4B). The mechanism by which these DCs suppress airway hyperreactivity and eosinophilia in this model remains to be elucidated. Regarding the site at which these regulatory DCs are generated, it is possible that immature DCs encounter the Ags in mucosal lymphoid tissues and acquire the regulatory function. We have data showing that DCs in the dome of Peyer’s patches move quickly after the capture of Ag and interact with Ag-specific T cells in the T cell

**FIGURE 6.** Comparison of the effects of in vivo and in vitro Ag loading upon the transfer of oral tolerance. Naive BALB/c mice were immunized as described in Fig. 1. A total of 10^6 CD11c+ spleen cells of water-fed mice (Non-fed Sp-DC transfer), 10^6 CD11c+ spleen cells of OVA-fed mice (Fed Sp-DC transfer), or 10^6 CD11c+ spleen cells of naive BALB/c mice that were pulsed with OVA in vitro (OVA-pulsed Sp-DC transfer) were adoptively transferred by i.v. injection into naive BALB/c mice just before the first immunization with OVA in aluminum hydroxide. Data are expressed as the mean percentage of eosinophils in BALF on day 21.
area of Peyer’s patches (K. Nagatani, K. Sagawa, and Y. Komagata, unpublished observations). It is possible that DCS that loaded mucosal Ags in the gut acquire tolerogenic function and move to systemic lymphoid organs, including the spleen.

We show that the splenic tolerogenic DCS induced by oral Ag play a major role in the active suppression of the experimental murine model of asthma. These findings may provide a new strategy for the management of allergic diseases such as bronchial asthma and for autoimmune diseases. Future studies are needed to investigate how such tolerogenic DCS function in the induction of oral tolerance.

Acknowledgments

We thank M. Katakawa, E. Ogawa, K. Kuroaki, I. Makino, and H. Hotate for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References