Original Article

Contribution of CD4+ or CD8+ T cell subsets in the induction of asthma in C57BL/6 mice

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ABSTRACT

Background: Respiratory viral infections are known to be a risk factor for the exacerbation of asthma. In a model of asthma with influenza A virus infection, dendritic cells play an important role during antigen sensitization and antigen challenge. However, the role of T cells in the asthma–influenza A model was unclear. The aim of the present study was to assess the roles of T cells during antigen sensitization and antigen challenge in the asthmatic model.

Methods: C57BL/6 mice were infected with influenza A virus and were sensitized with inhaled antigen during the acute phase of the virus infection. Anti-CD4 or anti-CD8 monoclonal antibodies were administered during antigen sensitization or antigen challenge to evaluate the role of CD4+ or CD8+ T cells in this murine model of asthma. The onset of asthma was determined by eosinophil recruitment into the lung.

Results: During antigen sensitization, depletion of either CD4+ or CD8+ T cells resulted in an absence of eosinophil infiltration into the lung after antigen challenge. However, during antigen challenge, depletion of CD4+ T cells did cause such an absence of eosinophil infiltration.

Conclusions: During antigen sensitization, both CD4+ and CD8+ T cells were required in C57BL/6 mice for exacerbation of asthma. During antigen challenge, CD4+ T cells were important for the onset of asthma, whereas CD8+ T cells do not affect eosinophil recruitment into the lung.

Key words: asthma, C57BL/6 mice, CD4+ T cell, CD8+ T cell, influenza virus.

INTRODUCTION

Allergy is a disorder of the immune system. In healthy individuals, the immune system is poised between maximizing protection from unwelcome invasion or internal imbalance and minimizing harmful overreaction to external or internal events. However, in an allergic disorder, this delicate balance is disturbed, with consequent adverse effects on morbidity and mortality. Asthma is a common allergic disease that is accompanied by eosinophil infiltration into the tissues of the airways. Reversible dyspnea, airway hyperresponsiveness and pulmonary inflammation are the main features of this disease.1,2 The development of asthma depends on the immune response and its mechanism is highly complex. To study asthma, many murine models have been developed. Recent studies indicate that Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, play important roles in the onset of asthma.3-7 Interleukin-4 and IL-5 are essential in antigen-induced eosinophil infiltration into inflammation sites.3,4 Interleukin-4 induces vascular cell adhesion molecule (VCAM)-1 expression in endothelial cells and VCAM-1 selectively binds to very late antigen (VLA)-4 on eosinophils and recruits them to the site of inflammation.5 Interleukin-5 induces the differentiation of eosinophils and prolongs their survival.6,7 In contrast, the Th1 cytokines interferon (IFN)-γ and IL-12 inhibit eosinophil infiltration and IgE and IgG1 secretion, while enhancing IgG2a secretion.
in vivo. Thus, the cross-talk in the cytokine network regulates asthma exacerbation.

In clinical studies, respiratory viral infections have been shown to increase the sensitization and exacerbation of asthma. Johnston et al. have shown that upper respiratory viral infections were strongly associated in time with hospital admissions for asthma in children and adults. Bronchitis due to respiratory syncytial virus (RSV) infections during infancy was considered to be an important risk factor for antigen sensitization, leading to the development of asthma in children with an inherited susceptibility to allergic diseases. Viral infections elicit strong CD8+ T cell responses and these CD8+ T cells are predominantly cytotoxic and IFN-γ-secreting cells. Interestingly, a recent study has demonstrated that a local environment containing IL-4 can transform virus-specific CD8+ cytotoxic T cells into non-cytotoxic IL-5-producing cells, both in vitro and in vivo. Suzuki et al. have developed a murine asthma model with a combination of influenza A virus infection and ovalbumin (OVA)-sensitization in BALB/c mice. They demonstrated that the inhalation of low-dose OVA during the acute phase of an influenza A virus infection accelerated OVA-specific IgE production and intensified airway hyperresponsiveness after antigen challenge. We have demonstrated that dendritic cells (DC) play a pivotal role during antigen sensitization and challenge in the same asthmatic murine model. After an influenza A viral infection, DC accumulated on the bronchial epithelium and disappeared within 1 week. During this period, if the mice received consecutive aerosolized OVA sensitization, DC appeared on the bronchial epithelium for more than 2 months. If the mice were challenged with inhaled OVA within this period, eosinophils infiltrated into the airways. However, the role of CD4+ and CD8+ T cells in this murine asthmatic model has not been examined. In the present study, we determined the requirements for T cell subsets during both antigen sensitization and asthma onset in the murine model with influenza A virus infection.

METHODS

Animals

Virus-free male C57BL/6 mice between 6 and 8 weeks of age were obtained from Japan SLC (Shizuoka, Japan). Animals were fed with OVA-free diets and water ad libitum and were kept under specific pathogen-free conditions in a laminar flow container. All experimental animals used in the present study were kept under conditions approved by the Institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

Virus

The mouse-adapted strain of Influenza A/PR/8/34 (H1N1) virus was prepared as described previously. We used a diluted virus suspension with a titer of $1.5 \times 10^2$ PFU/mL, which was determined to be a sublethal dose. Animals were inoculated intranasally with 50 μL virus solution under diethylether anesthesia.

Airway antigen sensitization and challenge

Animals were sensitized by the inhalation of aerosolized 0.05% OVA (Sigma, St Louis, MO, USA) with 0.5% alum adjuvant using a DeVilbiss 646 nebulizer (Somerset, PA, USA). Animals in a Plexiglas chamber (23.5 L capacity) were exposed to aerosols of OVA for 30 min each day over a 5 day period (from day 3 to day 7 after virus infection). For the inhalation challenge, the aerosol (2% OVA in phosphate-buffered saline (PBS)) was administered by a DeVilbiss 646 nebulizer for 30 min each day over a 5 day period (from day 28 to day 32 after virus infection).

Experimental protocol

Mice were inoculated intranasally with influenza A virus (day 0) and sensitized with inhaled OVA + alum (days 3–7). They were then challenged with inhaled OVA 4 weeks after the viral inoculation (days 28–32). On day 34, mice were killed and blood, bronchoalveolar lavage fluid (BALF) and spleens were collected for further experimentation. Some mice were killed for the collection of BALF and spleens after sensitization (on day 9). The following experimental groups ($n = 5–8$ each) were used: (i) T cell depletion during sensitization (CD4+ T or CD8+ T cell-depleted group), in which the animals were injected intraperitoneally with anti-CD4 (GK1.5) or anti-CD8 (53.6.72) monoclonal antibody (mAb) on days 1, 3, 5 and 7; (ii) T cell depletion during challenge (CD4+ T or CD8+ T cell-depleted group), in which mice were injected intraperitoneally with anti-CD4 or anti-CD8 mAb on days 26, 28, 30 and 32.
Bronchoalveolar lavage fluid

Animals were lavaged with 0.8 mL ice-cold PBS under anesthesia with pentobarbital sodium (40 mg/kg body-weight). Lavage was repeated four times and the recovered fluid (BALF) was centrifuged immediately and resuspended in 500 µL PBS. The number of cells was counted and the cells were then smeared onto a glass slide and stained with Diff Quick (International Reagent, Kobe, Japan) for cell differentiation. The differential cell count was made by counting at least 200 cells under a light microscope. For T cell subset differentiation, cells in BALF were immunostained with phycoerythrin (PE)-conjugated anti-CD4 mAb and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 mAb (BD PharMingen, San Jones, CA, USA) and were analyzed by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA, USA).

Measurement of immunoglobulins

On day 34, blood was collected, and the serum was obtained by centrifugation and stored at –80°C until analysis. Ovalbumin-specific IgG1 and IgG2a titers were determined by enzyme-linked immunosorbent assay (ELISA). Plates were coated with 20 µg/well OVA (Sigma). After blocking with 1% bovine serum albumin (BSA), the diluted serum dilutions were incubated and detection of OVA-specific IgG1 and IgG2a was performed with isotype-specific secondary antibodies (biotin-conjugated rabbit anti-mouse IgG1 or IgG2a; Cortex Biochem, San Leandro, CA, USA) and avidin-peroxidase conjugate (Sigma). These plates were developed using a tetramethylbenzidine (TMB)-1 component microwell substrate (KPL, Gaithersburg, MD, USA) and the optical densities of the enzymatic reactions were measured at 450 nm. Immunoglobulin titers were determined using control sera and expressed in arbitrary units (a.u.). The control sera were taken from mice injected intraperitoneally three times with 0.5 mg OVA absorbed in alum (0.2 mg/mL). The OVA-specific antibody titers used as internal controls were determined as 6.4 × 10^4 a.u./mL for IgG1 and 6.4 × 10^3 a.u./mL for IgG2a.

The OVA-specific IgE titer was measured by fluorometric reverse ELISA, as described previously. Briefly, plates were coated with 1 µg/mL anti-mouse IgE (Yamasa Shoyu, Chiba, Japan) in the presence of BSA in 0.05 mol/L carbonate-bicarbonate buffer (pH 9.5) for 3 h. After the wells were washed with PBS containing 0.05% Tween 20, mouse serum was added to duplicate wells and incubated overnight at room temperature; then, after a second washing, 0.5 µg/mL biotinylated OVA was added to each well and the mixture was incubated for 3 h at room temperature. The wells were washed a third time, and β-D-galactosidase-conjugated streptavidin (Roche Diagnostics GmbH, Mannheim, Germany) was added and incubated for 1 h at room temperature. After a further washing, 0.1 mmol/L 4-methylumbelliferyl (MU)-β-D-galactoside was added to each well and incubated for 2 h at 37°C. The reaction was stopped with 0.1 mol/L glycine–NaOH (pH 10.3) and the fluorescence intensity was read from a microplate fluorescence reader. Ovalbumin-specific antibody titers were determined as 1.3 × 10^4 a.u./mL for IgE. Antibody titers were expressed by reference to the internal control.

Cell culture

Spleen cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Biological Industries, Haemek, Israel), 100 U/mL penicillin (Banyu Pharmaceutical, Tokyo, Japan), 100 µg/mL streptomycin (Meiji Seika, Tokyo, Japan) and 2 mmol/L L-glutamine (WAKO Chemicals, Tokyo, Japan) for 6 h at 37°C in an incubator with 5% CO₂ and 85% humidity. These cells were stimulated simultaneously with 50 ng/mL phorbol 12 myristate 13 acetate (PMA), 500 ng/mL calcium ionophore and 2 µmol/L monensin (Sigma).

Immunofluorescence staining for intracellular cytokine

Stimulated spleen cells were incubated with FITC-conjugated anti-mouse CD8 mAb and PC-5 conjugated anti-mouse CD4 mAb (BD PharMingen) for 30 min on ice. After being washed twice with PBS containing 1% BSA, these cells were fixed with cold 2% paraformaldehyde in PBS for 20 min on ice. After two additional washes with PBS, cells were made permeable with PBS containing 0.1% saponin and PE-conjugated anti-mouse IFN-γ or anti-mouse IL-4 mAb (BD PharMingen) was added at a concentration of 1 µg/mL for 30 min on ice. Cells were washed twice in PBS containing 0.1% saponin and once in PBS, then resuspended in approximately 500 µL PBS.

Flow cytometry

Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) equipped with 15 mA argon ion
laser. Twenty thousand cells for BALF or 10,000 cells for splenocytes were acquired into the list mode and data were analyzed with CELLQuest software (Becton Dickinson). Analysis gates were set on lymphocytes according to forward- and side-scatter properties. For cytokine-producing splenic T cells, results were expressed as percentages of the cytokine-producing cells in the relevant CD4⁺ or CD8⁺ cell population.

**Immunohistochemical analysis**

The expression of CD11c and MHC class II molecules (I-A<sup>b</sup>) in the lung was examined 2–3 days after final OVA sensitization or OVA challenge. Lungs were removed after fixation with intratracheal instillation of an optimum cutting temperature (OCT) compound (Bayer-Pharma, Zürich, Switzerland) in an equivalent volume of PBS. Lungs were embedded in OCT compound, frozen in liquid nitrogen and stored at −80 °C until use. Frozen tissues were cut into 8–10 µm sections using a cryostat. Tissue sections were then mounted on silanized slides, air-dried and fixed in cold acetone for 20 min. These slides were incubated with blocking serum (ZYM Histo-stain SP kit; Zymed Laboratory, San Francisco, CA, USA) at room temperature for 10 min and were incubated with primary antibody, anti-mouse CD11c mAb (BD PharMingen) or anti-mouse I-A<sup>b</sup> mAb (BD PharMingen) at room temperature for 30 min. After washing, slides were incubated with FITC-conjugated anti-rat IgG (BD PharMingen) at room temperature for 30 min. After final washes, slides were mounted with FA mounting fluid (DIFCO Laboratories, Detroit, MI, USA). Non-specific rat IgG was used as a negative control for primary antibodies.

**Statistical analysis**

Values are presented as the mean ± SEM. Statistical significance of differences between experimental groups was examined using the two-tailed Student’s t-test.

**RESULTS**

**Influenza virus infection affects antigen sensitization and recruits eosinophils into the lung after antigen challenge**

We have reported that, in BALB/c mice sensitized with inhaled antigen during the acute phase of influenza virus infection, eosinophil recruitment into the lung, antigen-specific IgE production and airway hypersensitivity were induced after antigen challenge. We first examined the effects of influenza virus infection on the antigen sensitization in C57BL/6 mice. Influenza virus infection successfully enhanced the effect of inhaled antigen sensitization and recruited increased numbers of macrophages, lymphocytes, neutrophils and eosinophils after antigen challenge (Table 1). However, inhaled antigen sensitization without virus infection or virus infection alone without antigen sensitization did not recruit eosinophils.

<table>
<thead>
<tr>
<th>Infection/sensitization</th>
<th>Macrophages (×10⁻³)</th>
<th>Lymphocytes (×10⁻³)</th>
<th>Neutrophils (×10⁻³)</th>
<th>Eosinophils (×10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–/–</td>
<td>29.1 ± 0.4</td>
<td>0.5 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+/–</td>
<td>43.0 ± 3.0</td>
<td>3.8 ± 3.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>–/+</td>
<td>42.8 ± 3.8</td>
<td>4.4 ± 3.9</td>
<td>0.1 ± 0.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+/-</td>
<td>100.8 ± 22.7</td>
<td>27.9 ± 22.7</td>
<td>3.7 ± 1.2</td>
<td>4.5 ± 4.0</td>
</tr>
</tbody>
</table>

Each cell population in bronchoalveolar lavage fluid was assessed 2 days after the final antigen challenge.

**Table 2** Antibody titers for inhaled antigen sensitization during the acute phase of influenza virus infection and production of antigen-specific IgE after antigen challenge

<table>
<thead>
<tr>
<th>Infection/sensitization</th>
<th>OVA-IgG1 (a.u./mL)</th>
<th>OVA-IgG2a (a.u./mL)</th>
<th>OVA-IgE (a.u./mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–/–</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+/–</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>–/+</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>+/-</td>
<td>594.5 ± 66.3</td>
<td>102.4 ± 14.4</td>
<td>325.7 ± 77.1</td>
</tr>
</tbody>
</table>

The serum antibody titer of each group was assessed 2 days after final antigen challenge.

OVA, ovalbumin.
into the lung after the antigen challenge. The total cell number in BALF of the influenza virus-infected group and the antigen-sensitized group was significantly higher than that of the other three groups ($P < 0.01$).

**Influenza virus infection and the following inhaled antigen sensitization induced antigen-specific antibody responses**

To confirm the antigen-specific immune responses induced by influenza virus infection and the following sensitization with low-dose inhaled antigen, serum OVA-specific immunoglobulin titers were measured. Ovalbumin-specific IgE was detected in the serum from influenza virus-infected and OVA-sensitized mice after antigen challenge (Table 2). Ovalbumin-specific IgG1 and IgG2a were also detected in the sera from the virus-infected and antigen-sensitized groups, whereas no antigen-specific antibodies (IgE, IgG1 or IgG2a) were detected in the serum from the other groups (Table 2).

**T Cell subsets in BALF after antigen sensitization or challenge**

Next, we examined the frequency of CD4$^+$ T or CD8$^+$ T cells in BALF after antigen sensitization or challenge. Bronchoalveolar lavage fluid was collected on day 9 (after sensitization) and day 34 (after challenge) and the frequency of CD4$^+$ or CD8$^+$ T cells was examined by flow cytometry and cell number and percentage of lymphocytes by Diff Quick staining. In C57BL/6 mice, the frequency of CD4$^+$ T cells was approximately one-quarter of that of CD8$^+$ T cells after antigen sensitization ($5.3 \pm 1.5 \text{ vs } 22.4 \pm 11.1\%)$. Two days after the last antigen challenge, the frequency of CD4$^+$ T cells in BALF was approximately twice that of CD8$^+$ T cells in BALF ($25.7 \pm 13.9 \text{ vs } 13.0 \pm 7.4\%)$. The relative cell number of CD4$^+$ or CD8$^+$ T cells is indicated in Table 3.

**Frequency of cytokine-producing splenic T cells after antigen sensitization and challenge**

To determine the cytokine production of splenic T cells after antigen sensitization or challenge, the splenocytes were stimulated for 6 h in the presence of PMA and the calcium ionophore and the intracellular cytokines IL-4 and IFN-γ in CD4$^+$ or CD8$^+$ T cells were examined by flow cytometry. The frequency of IL-4-producing CD8$^+$ T cells was higher than that of CD4$^+$ T cells after antigen sensitization ($2.87 \text{ vs } 0.74\%$; Table 4). In contrast, the frequency of IL-4-producing CD4$^+$ T cells was much higher than CD8$^+$ T cells after antigen challenge ($7.63 \text{ vs } 0.23\%$; Table 4). Approximately 20% of IFN-γ-producing CD8$^+$ T cells was observed after antigen challenge, whereas less than 5% of IFN-γ producing CD4$^+$ T cells was seen at the same time (Table 4).

**Effects of anti-CD4 or anti-CD8 mAb treatment during antigen sensitization or challenge on eosinophil infiltration into BALF after antigen challenge**

To determine the role of CD4$^+$ T or CD8$^+$ T cells in the provocation of asthma in C57BL/6 mice by intraperitoneal administration of anti-CD4 or CD8 mAb, CD4$^+$ T

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**Table 3** Number of T cells in bronchoalveolar lavage fluid after antigen sensitization and challenge

<table>
<thead>
<tr>
<th></th>
<th>After sensitization</th>
<th>After challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4$^+$ T cells ($\times 10^{-3}$)</td>
<td>7.0 ± 2.0</td>
<td>7.6 ± 4.1</td>
</tr>
<tr>
<td>CD8$^+$ T cells ($\times 10^{-3}$)</td>
<td>30.0 ± 14.7</td>
<td>3.8 ± 2.2</td>
</tr>
</tbody>
</table>

**Table 4** Frequency of interleukin-4- or interferon-γ-producing CD4$^+$ or CD8$^+$ T cells in the spleen after antigen sensitization and challenge

<table>
<thead>
<tr>
<th></th>
<th>After sensitization</th>
<th>After challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4$^+$ T cells ($\times 10^{-3}$)</td>
<td>IL-4</td>
<td>0.8 ± 0.7</td>
</tr>
<tr>
<td>CD8$^+$ T cells ($\times 10^{-3}$)</td>
<td>IFN-γ</td>
<td>3.9 ± 1.8</td>
</tr>
</tbody>
</table>

The frequency indicates the number of interleukin (IL)-4- or interferon (IFN)-γ-producing cells among CD4$^+$ or CD8$^+$ T cells.
or CD8+ T cells were depleted selectively during antigen sensitization or challenge. First, the contribution of CD4+ or CD8+ T cells during antigen sensitization was determined. Mice were treated with anti-CD4 or anti-CD8 mAb during antigen sensitization, and eosinophil recruitment into the bronchial lumen after antigen challenge was assessed for onset of asthma. CD4+ T cell depletion during sensitization resulted in the recruitment of very few eosinophils, eosinophils were detected in only one mouse of eight, and CD8+ T cell depletion resulted in the recruitment of no eosinophils into the BALF (Table 5).

Next, to determine the contribution of CD4+ or CD8+ T cells at the time of onset of asthma, eosinophil recruitment was examined after selective T cell depletion during antigen challenge. C57BL/6 mice treated with anti-CD4 mAb showed a decreased number of eosinophils in BALF compared with non-depleted mice (Table 6). However, depletion of CD8+ T cells did not affect the number of eosinophils recruited into the bronchial lumen (Table 6).

**Longevity of DC under the bronchial epithelium was associated with the presence of CD4+ T and CD8+ T Cells**

Immunohistochemical staining of both major histocompatibility complex (MHC) class II molecules and CD11c was undertaken in order to determine whether DC were present, because the long-term presence of DC in the subepithelial region of a bronchium or bronchiole was essential for the onset of asthma after antigen challenge in this mouse model. In the virus-infected and antigen-sensitized mice, DC were present in the subepithelial region at 10 days after the infection, whereas DC disappeared from the subepithelial region at the same time point in the virus-infected mice, but not in the antigen-sensitized mice. In C57BL/6 mice treated with anti-CD4 mAb or anti-CD8 mAb during OVA sensitization, DC were observed in the subepithelial region until 10 days after the virus infection and then disappeared (data not shown).

**DISCUSSION**

A murine asthma model with OVA sensitization during influenza A virus infection has been developed in BALB/c mice.15,16 This model has shown that influenza A virus infection induces enhanced responsiveness to sensitization with inhaled OVA and airway responsiveness to methacholine in mice. In the present study, we demonstrated that C57BL/6 mice were successfully sensitized with inhaled antigen during the acute phase of influenza A virus infection and eosinophil infiltration into the lung after antigen challenge was observed, as in BALB/c mice. This result was supported by a recent report that C57BL/6 mice showed more severe local pulmonary inflammation in asthma.18 In both BALB/c and C57BL/6 murine models, DC were observed on the bronchial epithelium during the acute phase of virus infection and disappeared within 1 week. In mice administered an antigen during this period, DC were

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**Table 5**  Effect of administration of anti-CD4 or anti-CD8 monoclonal antibody during sensitization

<table>
<thead>
<tr>
<th>mAb administered</th>
<th>Macrophages ($\times 10^3$)</th>
<th>Lymphocytes ($\times 10^3$)</th>
<th>Neutrophils ($\times 10^3$)</th>
<th>Eosinophils ($\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>64.4 ± 2.9</td>
<td>55.6 ± 6.1</td>
<td>0.6 ± 0.3</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Anti-CD4 mAb</td>
<td>49.9 ± 3.9</td>
<td>5.4 ± 3.4</td>
<td>1.8 ± 1.7</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Anti-CD8 mAb</td>
<td>42.6 ± 4.3</td>
<td>9.3 ± 4.1</td>
<td>0.6 ± 0.5</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*The number of each type of cell in bronchoalveolar lavage fluid was assessed 2 days after final antigen challenge.*

PBS, phosphate-buffered saline; mAB, monoclonal antibody.

**Table 6**  Effect of administration of anti-CD4 or anti-CD8 monoclonal antibody during challenge

<table>
<thead>
<tr>
<th>mAb administered</th>
<th>Macrophages ($\times 10^3$)</th>
<th>Lymphocytes ($\times 10^3$)</th>
<th>Neutrophils ($\times 10^3$)</th>
<th>Eosinophils ($\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>128.8 ± 6.2</td>
<td>5.1 ± 2.6</td>
<td>1.2 ± 1.0</td>
<td>2.3 ± 3.7</td>
</tr>
<tr>
<td>Anti-CD4 mAb</td>
<td>63.4 ± 2.6</td>
<td>3.9 ± 2.1</td>
<td>0.7 ± 0.7</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>Anti-CD8 mAb</td>
<td>115.0 ± 10.2</td>
<td>9.8 ± 5.6</td>
<td>0.7 ± 1.6</td>
<td>5.0 ± 5.6</td>
</tr>
</tbody>
</table>

*The number of each type of cell in bronchoalveolar lavage fluid was assessed 2 days after final antigen challenge.*

PBS, phosphate-buffered saline; mAB, monoclonal antibody.
found on bronchial epithelium for 2 months and the mice were successfully sensitized and showed asthmatic responses after antigen challenge. These DC were thought to play an important role during antigen sensitization and challenge.

In C57BL/6 mice, CD8+ T cells constituted a major portion of the cell population in BALF after virus infection and antigen sensitization. The frequency of IL-4-producing CD8+ T cells in splenocytes is higher than in CD4+ T cells after antigen sensitization. From these results, CD8+ T cells were considered to be essential during antigen sensitization. However, the depletion study with anti-CD4 or anti-CD8 mAb showed that both CD4+ and CD8+ T cells were required during antigen sensitization. In mice treated with anti-CD4 or anti-CD8 mAb during antigen sensitization, DC on the bronchial epithelium disappeared within 10 days and eosinophil infiltration was not observed after antigen challenge. Anti-CD4 or anti-CD8 mAb treatment may affect the thymocytes to deplete double-positive T cells and decrease the number of the other T cell subset in the periphery. However, our preliminary study showed anti-CD4 or anti-CD8 mAb did not affect the number of the other T cell population at 9 days after treatment (data not shown). In addition, a previous study has shown that depletion of CD4+ or CD8+ T cells by the administration of specific mAb did not affect the number of the other T cell subset for more than 1 month. So, we considered that anti-CD4 or -CD8 treatment did not affect the number of the other T cell subset in the periphery. Therefore, these results suggest that CD8+ T cells play a pivotal role during antigen sensitization in C57BL/6 mice and that CD4+ T cells may help to induce antigen-specific CD8+ T cell activation during sensitization. This hypothesis is supported by previous reports showing that a reduction or progressive loss of memory cytotoxic T lymphocytes (CTL) occurs in the absence of helper T cells. In another role of CD4+ T and CD8+ T cells, the prolonged presence of DC on the bronchial epithelium was due to an interaction between both CD4+ T and CD8+ T cells and DC during antigen sensitization. Further investigation is required to define the roles of the interaction between the T cells and the DC. In a previous study in BALB/c mice, CD4+ T cells were required for airway antigen sensitization during the acute phase of influenza A virus infection (T Takamasu et al., unpubl. obs.). Schwarze et al. reported that CD8+ T cells are essential in airway hyperresponsiveness after RSV infection. These findings suggest that different immune cell populations are required for the onset of asthma in different mouse strains or in different viral infections.

In this asthmatic mouse model, influenza A virus infection enhances airway sensitization of suboptimal concentrations of OVA. Wohlleben et al. have shown that influenza A virus infection inhibits the efficient recruitment of Th2 cells and eosinophils into the airway. They explained that influenza A virus infection induced the Th1 immune response and inhibited the Th2 immune response. However, we have demonstrated that antigen sensitization during the acute phase of influenza A virus infection successfully induces eosinophil recruitment into the airway after antigen challenge. We have also suggested that the timing of antigen sensitization is important because antigen sensitization during the recovery phase of influenza A virus infection induced the Th1 immune response and IFN-γ production after antigen challenge. It is possible that differences in the timing of antigen sensitization between our model and that of Wohlleben et al. were responsible for the different immune responses after antigen challenge. Another possible explanation is that the amount of influenza A virus virion used for the asthma model is likely to induce different immune responses. Eisenbarth et al. have reported that low levels of lipopolysaccharide (LPS) led to a Th2 immune response against inhaled antigen, whereas high levels of LPS caused induction of a Th1 immune response against the same antigen. This suggests that a small amount of pathogen-associated molecular patterns (PAMP) induces a Th2 immune response, but larger amounts induce a Th1 immune response in the airway. Thus, the amount of influenza A virus used for the asthma model is important in determining which immune response, Th1 or Th2, is induced against an inhaled antigen.

After antigen challenge, CD4+ T cells made up the majority of the cell population in BALF in C57BL/6 mice. The frequency of IL-4-producing CD4+ T cells increases greatly among splenocytes after antigen challenge compared with after antigen sensitization, whereas the frequency of IL-4-producing CD8+ T cells is decreased. Furthermore, CD4+ T cell depletion in C57BL/6 mice during antigen challenge showed decreased eosinophil recruitment into BALF. These results indicate that CD4+ T cells in C57BL/6 mice mainly contribute to the onset of asthma after antigen challenge. CD8+ T cell depletion did not decrease eosinophil recruitment in C57BL/6 mice. Intracellular cytokine staining shows that IFN-γ-producing CD8+ T cells were present in approximately 20% of splenic CD8+ T cells after antigen challenge.
Interferon-γ acts on T cells to inhibit type 2 cytokine secretion. Suzuki et al. have reported that IFN-γ secretion by CD8+ T cells inhibits allergen-induced airway eosinophilia.24 CD8+ T cell depletion during antigen challenge is not likely to result in a decrease of type 2 cytokine production. Herrick et al.27 reported that diisocyanate-induced asthma occurred in BALB/c mice, but not in C57BL/6 mice. C57BL/6 mice are thought to be a model for Th1 dominant animals. However, in the present study, influenza A virus infection enhances airway antigen sensitization and exacerbates asthma in C57BL/6 mice.

This suggests that airway virus infection is a risk factor for the exacerbation of asthma in non-atopic individuals.

REFERENCES


18 Morokata T, Ishikawa J, Ida K, Yamada T. C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. Immunology 1999; 98: 345–51.


20 von Herrath MG, Yokoyama M, Dockter J, Oldstone MB, Whitton JL. CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. J. Virol. 1996; 70: 1072–9.


