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Lack of lymphoid chemokines CCL19 and CCL21 enhances allergic airway inflammation in mice

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Abstract

Lymphoid chemokines CCL19 and CCL21 are crucial for the recruitment of circulating naive T cells into lymph nodes. However, it is not completely known how they contribute to the development of allergic diseases. To determine whether the lack of CCL19 and CCL21 affects allergic airway inflammation, CCL19- and CCL21-deficient [paucity of lymph node T cells (*plt/plt*)] and wild-type (WT) mice were immunized intra-peritoneally and then challenged intra-nasally with chicken ovalbumin (OVA). *Plt/plt* mice developed more severe allergic airway inflammation characterized by increased eosinophils and lymphocytes in bronchoalveolar lavage (BAL) and profound inflammation in peribronchiolar and perivascular regions than did WT mice. $CD4^+$ α_4 integrin$^+$ and $CD4^+$ β_7 integrin$^+$ T cells were significantly increased in the BAL of OVA-immunized and OVA-challenged (OVA/OVA) *plt/plt* mice compared with OVA/OVA WT mice. Moreover, there were higher levels of IL-4 and IL-13 mRNAs and lower levels of IL-2 and IFN-γ mRNAs in inflamed lungs of OVA/OVA *plt/plt* mice compared with OVA/OVA WT mice. *Plt/plt* mice produced higher levels of total and OVA-specific IgE antibody. Thus, our results suggest that lack of lymphoid chemokines CCL19 and CCL21 enhances allergic airway inflammation by modulating the recruitment of $CD4^+$ T cells into the lung, the balance between T_h1 and T_h2 cytokines and the IgE production.

Introduction

Allergic asthma is a chronic airway inflammatory disease caused by an inappropriate immune response to an inhaled antigen and is characterized by airway inflammation, airway hyperreactivity, airway remodeling and antigen-specific IgE production (1). Although many immune cells, including dendritic cells, B cells, T cells, mast cells and eosinophils, are involved, a balance between T_h1 and T_h2 is fundamental to the development of allergic asthma. T_h2 produce IL-4, IL-5 and IL-13, which directly or indirectly contribute to recruitment and activation of principal inflammatory effector cells such as eosinophils and mast cells. In contrast, T_h1 inhibit asthma pathology by producing IFN-γ (2).

Allergic asthma attacks occur when a previously sensitized individual encounters the same or a cross-reactive allergen. Sensitization to an inhaled allergen is primarily initiated in the bronchial lymph nodes (LNs) where the allergen, which is transported by airway mucosal dendritic cells, is presented to naive $CD4^+$ and $CD8^+$ T cells in the context of MHC II and MHC I molecules, respectively (3–6). This results in T cell activation, proliferation, differentiation and eventually allergen-specific memory T cell generation. Thus, the structural integrity of LNs is critical for the priming of naive T cells by inhaled allergens.

Homeostatic migration of lymphocytes from the bloodstream into LNs is one important mechanism for the maintenance of normal LN structure and function. The migration requires multiple sequential events on endothelial venules, including specific recognition of lymphocyte L-selectin by

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vascular peripheral node addressin (PNAd) and the interaction of lymphocyte chemokine receptor CCR7 with its vascular ligands (chemokines CCL19 and CCL21) (7). These two chemokines are predominantly expressed on high endothelial venules and/or stromal cells in T cell zones of LNs and Peyer's patches and on lymphatic vessels (8–10). Mice homozygous for the paucity of lymph node T cells (*plt/plt*) lack CCL19 and CCL21 in LNs and Peyer's patches and have disrupted migration of naive T cells (and partially of B cells) and dendritic cells into LNs (8, 11–14). Although the roles of these chemokines in immune responses have previously been investigated, the findings vary depending on experimental settings. For example, contact hypersensitivity to oxazolone was reduced on day 2 but enhanced on day 6 after sensitization in *plt/plt* mice as compared with wild-type (WT) mice (15). T cells from chicken ovalbumin (OVA)-immunized *plt/plt* mice produced more IL-2 to OVA stimulation *in vitro* (15). *Plt/plt* mice showed a slight reduction in the initiation and maintenance of protective anti-viral memory responses but normal neutralizing anti-viral B cell response and Ig class switching (16). Blockade of CCL21 with neutralizing anti-CCL21 antibody exacerbated acute pulmonary inflammation in mice infected with *Propionibacterium acnes* (17). CCL21 antagonist and anti-CCL21 antibody inhibited chronic graft-versus-host disease and contact hypersensitivity, respectively, in mice (18, 19). However, it is not completely known whether the lack of CCL19 and CCL21 will affect the development of allergic airway inflammation in *plt/plt* mice.

Thus, this study was aimed at investigating the development of allergic airway inflammation in mice lacking lymphoid chemokines CCL19 and CCL21. P*lt/plt* and WT mice were immunized by daily intra-peritoneal injection of OVA for 14 days followed by three consecutive intra-nasal OVA challenges on days 25, 26 and 27. We found that allergic airway inflammation was enhanced in *plt/plt* mice. The enhanced allergic airway inflammation in *plt/plt* mice was characterized by increased infiltrates of lymphocytes and eosinophils in airway lumen, airway and pulmonary vasculatures, increased total and OVA-specific IgE levels and increased T_h2, but decreased T_h1, cytokine mRNAs.

Methods

Mice

Plt/plt mice were obtained from the Laboratory Animal Research Center, University of Tokyo, Tokyo, Japan. Ddy mice that have similar genetic background with *plt/plt* mice were purchased from Japan SLC, Hamamatsu, Shizuoka, Japan, and used as WT controls. Mice were housed under specific pathogen-free conditions in the Animal Facility of Kagoshima University, Kagoshima, Japan. Female *plt/plt* and WT mice at 8 weeks of age were used for experiments. The Institute Animal Care and Use Committee approved all experimental protocols.

Induction of allergic airway inflammation

Allergic airway inflammation was provoked according to the published protocols with some modifications (20–22). Briefly, mice were immunized by daily intra-peritoneal injection of 100 microliter of OVA (100 microgram ml^{-1}, Sigma–Aldrich, St Louis, MO, USA) in PBS from day 1 to day 14. Control mice received the equal volume of PBS. On days 25, 26 and 27, mice were challenged intra-nasally with 50 microliter of OVA (1 mg ml^{-1} in PBS) or PBS alone. Mice were sacrificed under terminal anesthesia 2 days after the last OVA or PBS challenge.

Bronchoalveolar lavage analysis

Airways were lavaged three times with 0.8 ml of PBS via a tracheal cannula. Bronchoalveolar lavage (BAL) fluid from each mouse was centrifuged at 1500 r.p.m. at 4°C for 10 min and re-suspended in PBS for total leukocyte counting. For differential leukocyte counts, we prepared a slide for each BAL sample by cytospin and stained with Wright–Giemsa staining reagents according to the manufacturer's instructions (Muto Chemical Co., Tokyo, Japan). The differential counts of eosinophils, lymphocytes, macrophages and neutrophils were determined blind by counting a total of 200 cells per slide at high-powered fields (×400).

Lung histology

Lungs were inflated with <1 ml of optimal cutting temperature (OCT) compound-embedding medium (Sakura Finetechnical Co. Ltd., Tokyo, Japan):PBS mixture (1:1) and embedded in OCT medium on dry ice. The lungs were cut into 6 micrometer sections and fixed with cold acetone. The sections were stained with hematoxylin and eosin (H&E). Peribronchiolar (medium to small airway) and perivascular inflammation was graded as described previously (23). To evaluate the inflammation in the lung interstitium, the nucleated cells in 10 randomly selected areas, from a 10 × 10 grid at high-powered fields (×400), were counted for each mouse. The interstitial inflammation score was expressed as the ratio of the cell number in each treatment group to that in respective control group (unimmunized and PBS challenged). The ratio that is significantly higher than theoretical value, which is set at 1, indicates interstitial inflammation in lungs.

Flow cytometric analysis

BAL cells were suspended in PBS containing 0.1% fetal bovine serum and 0.01% sodium azide, stained with the indicated mAbs on ice for 30 min and then fixed with 2% formalin–PBS. PE–Cy5–anti-CD4 and PE–anti-CD8 mAbs were obtained from BD Biosciences (San Diego, CA, USA). FITC–anti-CD4 (GK1.5), FITC–anti-L-selectin (MEL-14), FITC–anti-α_4 integrin (PS/2) and FITC–anti-β_7 integrin (FIB504) mAbs were labeled in our laboratories. Data on stained samples were collected on a BD FACScan flow cytometry using CellQuest software (San Jose, CA, USA) and analyzed using WinMDI free software (version 2.8, http://facs.scripps.edu/software.html).

Analysis of cytokine mRNAs in lung tissues

Total RNA was extracted from lungs and semi-quantitative reverse transcription–PCR analysis of cytokine mRNAs was performed as previously described (24, 25). The primers used were IFN-γ, sense TGAACGCTACACACTGCATCTTGG and anti-sense CGACTCCTTTTCCGCTTCCTGAG; IL-2,

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sense TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG and anti-sense GACAGAAGGCTATCCATCTCCTCAGAAAGTCC; IL-4, sense ATGGGTCTCAACCCCCAGCTAGT and anti-sense GCTCTTTAGGCTTTCCAGGAAGTC; IL-13, sense ATGGCG-CTCTGGGTGACTGCAGTCC and anti-sense GAAGGGGCC GTGGCGAAACAGTTGC and GAPDH, sense ACCACAGTC CATGCCATCAC and anti-sense TCCACCACCCTGTTGCT-GTA. PCR cycling conditions were 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, 35 cycles for IL-13 and 1 min at 94°C, 1 min at 60°C and 2 min at 72°C, 35 cycles for other cytokines and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The signal level for each cytokine mRNA was expressed as the ratio relative to GAPDH.

Measurement of serum IgE

Serum total and OVA-specific IgE levels were measured using sandwich ELISA assays. Purified rat anti-mouse IgE mAb (11B11, BD Biosciences) was used as the coating antibody. Biotinylated rat anti-mouse IgE mAb (BVD6-24G2, BD Biosciences) and biotinylated OVA labeled as reported previously (26) were used to detect total and OVA-specific IgE, respectively. The formed IgE and biotinylated anti-IgE mAb complexes or OVA-specific IgE and biotinylated OVA complexes were then detected by streptavidin-conjugated houseradish peroxidase (Sigma–Aldrich) in the presence of hydrogen peroxide and diammonium 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma–Aldrich). Total serum IgE levels were calculated from the standard curve generated from mouse IgE standard (BD Biosciences) and expressed as nanogram per milliliter. OVA-specific IgE levels were calculated from the pooled standard serum generated from OVA-immunized BALB/c mice in our laboratory and were assigned the arbitrary values (units per milliliter) (26).

Statistical analysis

Data are expressed as mean value ± standard deviation. Analysis of variance was used to determine statistical significance among the groups. $P < 0.05$ was considered to be statistically significant.

Results

Leukocytes in the BAL

In allergic asthma, infiltrates of eosinophils and lymphocytes are found in the airway lumen, walls of the airways and the lung interstitium (1). To determine whether *plt/plt* mice develop allergic pulmonary inflammation, we first analyzed BAL cells from unimmunized *plt/plt* and WT mice. There were no differences in the number of total leukocytes and cellular composition in the BAL between *plt/plt* and WT mice even after three intra-nasal OVA challenges (Fig. 1A). The BAL cells were mainly composed of macrophages and lymphocytes. In contrast, total cell numbers were dramatically increased in the BAL of ovalbumin-immunized and ovalbumin-challenged (referred as OVA/OVA thereafter) *plt/plt* and OVA/OVA WT mice (Fig. 1B). However, the total cell number was significantly higher in OVA/OVA *plt/plt* mice than that in OVA/OVA WT mice. Eosinophils, macrophages and lymphocytes were most common cells in the BAL of OVA/OVA *plt/plt* and OVA/OVA WT mice. Moreover, we observed more eosinophils and lymphocytes in the BAL from OVA/OVA *plt/plt* mice than from OVA/OVA WT mice. Conversely, there were fewer macrophages in the BAL from OVA/OVA *plt/plt* mice than from OVA/OVA WT mice. There was no difference in the numbers of neutrophils between OVA/OVA *plt/plt* and OVA/OVA WT mice.

Lung histology

Next, we examined H&E-stained sections of lungs from different groups. We did not find leukocytic infiltrates in the lungs of unimmunized *plt/plt* and WT mice that were challenged

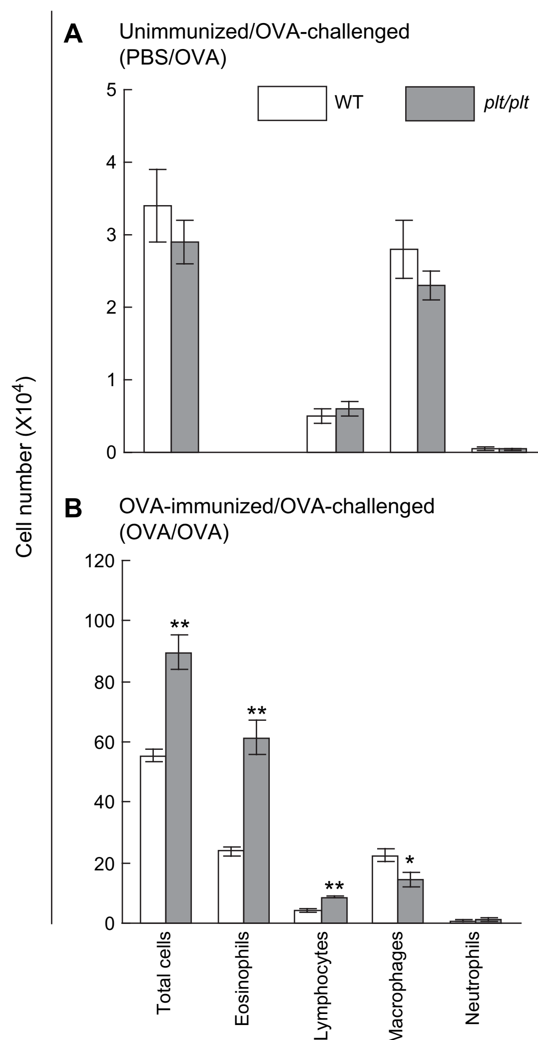

Fig. 1. Leukocytes in the BAL of WT and *plt/plt* mice. BAL cells were collected from OVA/OVA or unimmunized (PBS-treated)/OVA-challenged (PBS/OVA) mice, counted and stained with Wright–Giemsa. (A) There were no differences between PBS/OVA WT and PBS/OVA *plt/plt* mice in the numbers of total leukocytes, lymphocytes, neutrophils or macrophages in the BAL. (B) Eosinophils, lymphocytes and macrophages were significantly increased in the BAL of OVA/OVA *plt/plt* and OVA/OVA WT mice as compared with PBS/OVA. The BAL of OVA/OVA *plt/plt* mice contained more eosinophils and lymphocytes, but fewer macrophages, than the BAL of OVA/OVA WT mice. $*P < 0.05$ and $**P < 0.01$ compared with OVA/OVA WT mice, analysis of variance; $n = 6$ mice in each group.

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with OVA (Fig. 2A and 2B) or PBS (data not shown). Consistent with the findings in the BAL, there were significant peribronchial and perivascular leukocytic infiltrates in the lungs of OVA/OVA *plt/plt* and OVA/OVA WT mice (Fig. 2C and D). The inflammation was more severe in the OVA/OVA *plt/plt* mice, particularly in small airways and vessels than in the OVA/OVA WT mice (Figs 2E, 2F and 3). Similarly, the cell ratios in lung interstitium of OVA/OVA *plt/plt* and OVA/OVA WT mice were significantly higher than theoretical value (set at 1) (Fig. 3), indicating interstitial inflammation in lungs. Relative high cell ratio in lung interstitium of OVA/OVA *plt/plt* mice indicates more severe interstitial inflammation in OVA/OVA *plt/plt* mice as compared with OVA/OVA WT mice.

T cells in the BAL of OVA/OVA mice

Since $CD4^+$ and $CD8^+$ T cells contribute to allergic asthma (3, 27), we stained BAL lymphocytes with anti-CD4 and anti-CD8 mAbs to determine the dominant T cell subset in the BAL of OVA/OVA *plt/plt* and OVA/OVA WT mice. As shown in Fig. 4, the BAL of OVA/OVA *plt/plt* and OVA/OVA WT mice had more $CD4^+$ T cells than $CD8^+$ T cells. However, there were significantly more $CD4^+$ T cells in the BAL of OVA/OVA *plt/plt* mice (61.4 $\pm$ 3.5%) than in the BAL of OVA/OVA WT mice (24.6 $\pm$ 6.0%). There was no difference in the proportion of $CD8^+$ T cells in the BAL between OVA/OVA *plt/plt* and OVA/OVA WT mice. OVA/OVA *plt/plt* mouse BAL had fewer lymphocytes that were not stained with anti-CD4 or anti-CD8 mAb compared with OVA/OVA WT mice.

Adhesion molecules on the BAL T cells of OVA/OVA mice

Recognition of lymphocyte adhesion molecules by their vascular endothelial ligands is crucial for the migration of circulating lymphocytes into lymphoid and inflamed tissues. Lymphocyte L-selectin and endothelial PNAd are important for the recruitment of lymphocytes into LNs, bronchus-associated lymphoid tissues (BALTs) and chronically inflamed airways (28, 29). $\alpha_4\beta_1$ integrin, which binds mainly to vascular cell adhesion molecule-1 (VCAM-1), is important for the migration of $\alpha_4\beta_1$ memory T cells into BALTs (29) and

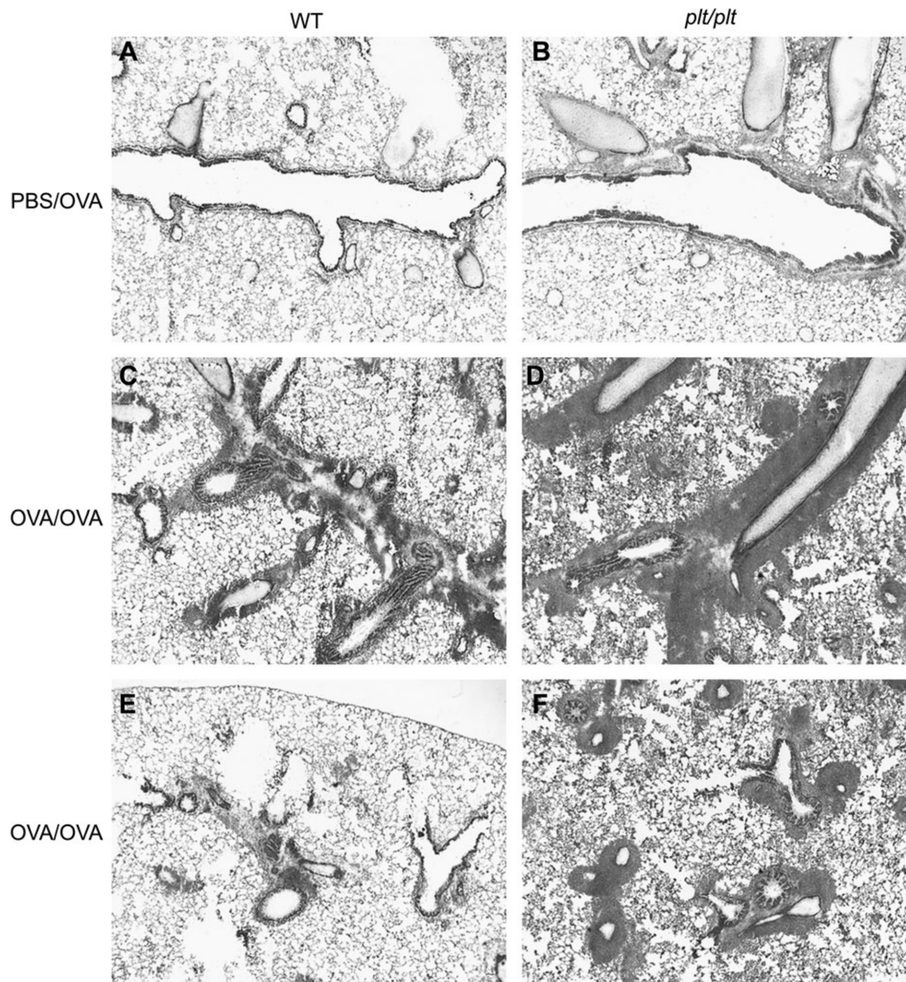

Fig. 2. Lung histology in WT and *plt/plt* mice. Acetone-fixed frozen sections of lungs were stained with H&E. (A and B) There was no obvious inflammation in the lungs of PBS-treated/OVA-challenged (PBS/OVA) WT (A) and PBS/OVA *plt/plt* (B) mice. (C and D) Significant peribronchiolar and perivascular inflammation was found in medium to large sizes of airways and vessels of OVA/OVA WT (C) and OVA/OVA *plt/plt* (D) mice. Specifically, there was more severe inflammation in the small airways and vessels in OVA/OVA *plt/plt* mice (F) than OVA/OVA WT (E) mice.

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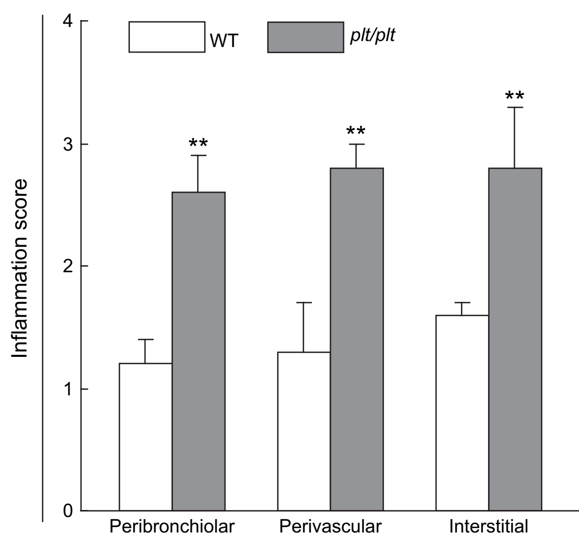

Fig. 3. Quantitative analysis of lung inflammation. Inflammation scores in peribronchiolar, perivascular and interstitial regions were significantly higher in the lungs of OVA/OVA *plt/plt* mice than those of OVA/OVA WT mice. $**P < 0.01$ compared with OVA/OVA WT mice, analysis of variance; $n = 6$ mice in each group.

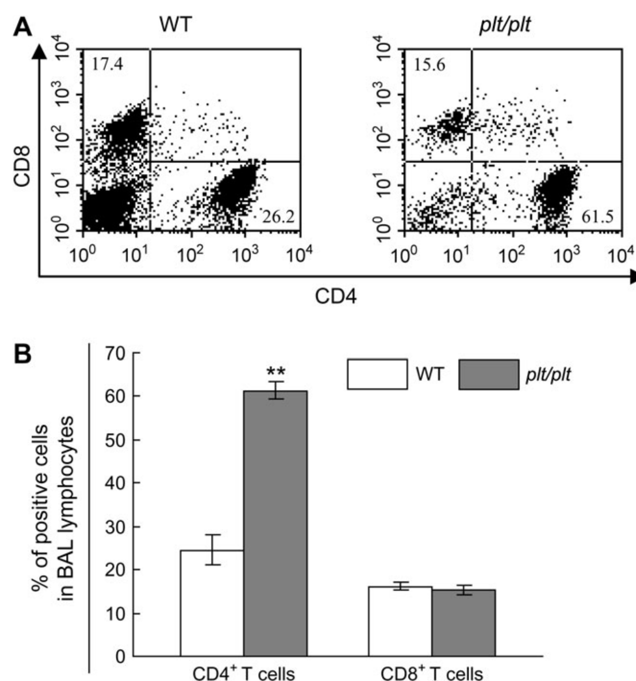

Fig. 4. T cells in the BAL of OVA/OVA mice. BAL cells were prepared from OVA-immunized mice 2 days after the last OVA challenge and stained with anti-CD4 and anti-CD8 mAbs. (A) Representative flow cytometric plots for CD4 and CD8 staining on gated BAL lymphocytes from one WT mouse (left) and one *plt/plt* mouse (right). (B) The BAL of OVA/OVA *plt/plt* mice contained more $CD4^+$ T cells than did the BAL of OVA/OVA WT mice. $**P < 0.01$ compared with OVA/OVA WT mice, analysis of variance; $n = 6$ mice in each group.

inflamed lung (30). $\alpha_E\beta_7$ integrin, which binds to epithelial E-cadherin, plays a major role in recruitment of and retention of intra-epithelial lymphocytes (31).

To determine if the predominance of $CD4^+$ T cells in the BAL of OVA/OVA *plt/plt* mice may result from the preferential recruitment of these cells from blood vessels into lungs, we analyzed the expression of L-selectin, α_4 integrin and β_7 integrin on BAL lymphocytes. As shown in Fig. 5(A and B), BAL lymphocytes recovered from OVA/OVA *plt/plt* mice contained more L-selectin$^-$, α_4 integrin$^+$ and β_7 integrin$^+$ $CD4^+$ T cells than those from OVA/OVA WT mice. More than 80% of the $CD4^+$ T cells in the BAL of OVA/OVA *plt/plt* (84.9 ± 2.5%) and OVA/OVA WT (85.7 ± 3.3%) mice were L-selectin$^-$ (Fig. 5A). More $CD4^+$ T cells in the BAL of OVA/OVA *plt/plt* mice expressed α_4 integrin (84.1 ± 1.9%) and β_7 integrin (56.2 ± 4.4%) than those in the BAL of OVA/OVA WT mice (71.8 ± 3.7% for α_4 integrin$^+$ and 28.2 ± 3.0% for β_7 integrin$^+$) (Fig. 5A). Moreover, BAL $CD4^+$ T cells from OVA/OVA *plt/plt* mice expressed higher levels of α_4 integrin and β_7 integrin than those in OVA/OVA WT mice (Fig. 5C). In contrast, the proportions of BAL $CD8^+$ T cells that expressed L-selectin, α_4 integrin and β_7 integrin were almost identical in OVA/OVA *plt/plt* and OVA/OVA WT mice (Fig. 6A and B). BAL $CD8^+$ T cells from OVA/OVA *plt/plt* mice expressed higher levels of L-selectin than did those from OVA/OVA WT mice (Fig. 6C). There was no difference, however, in the expression levels of α_4 integrin and β_7 integrin on the BAL $CD8^+$ T cells between OVA/OVA *plt/plt* and OVA/OVA WT mice (Fig. 6C).

T_h1 and T_h2 cytokine mRNAs in the lungs of OVA/OVA mice

T_h2 cytokine production is a hallmark of allergic asthma (1, 2). More specifically, high levels of IL-4, IL-5 and IL-13 and low levels of IL-2 and IFN-γ are associated with a T_h2-directed response to an allergen in the lung (1, 2). To determine whether the severe airway inflammation in *plt/plt* mice is associated with an imbalance between T_h1 and T_h2 cytokines, we analyzed T_h1 (IL-2 and IFN-γ) and T_h2 (IL-4 and IL-13) cytokine mRNAs in the lungs of *plt/plt* and WT mice. Very low levels of IL-2 and IFN-γ mRNAs were detected in the lungs of *plt/plt* and WT mice after OVA-immunized/PBS-challenged (or unimmunized/OVA-challenged), whereas IL-4 and IL-13 mRNAs were undetectable (data not shown). In contrast, OVA challenge significantly enhanced the expression of T_h1 and T_h2 cytokine mRNAs in the lungs of OVA-immunized *plt/plt* and WT mice (Fig. 7). However, lungs from OVA/OVA *plt/plt* mice expressed significantly higher levels of IL-4 and IL-13 mRNAs and lower levels of IL-2 and IFN-γ mRNAs than did lungs from OVA/OVA WT mice.

IgE production

IgE is important in the development of allergic asthma (1, 2). To determine whether *plt/plt* mice have a high IgE response to allergen immunization, we measured total and OVA-specific IgE levels in the sera of *plt/plt* and WT mice that were immunized with OVA or unimmunized. Although unimmunized *plt/plt* mice had slightly higher total IgE levels than unimmunized WT mice (Fig. 8A), OVA-specific IgE was undetectable in the sera from unimmunized *plt/plt* and WT mice (Fig. 8B). OVA immunization significantly increased total and OVA-specific IgE levels in *plt/plt* and WT mice. However, total and antigen-specific IgE levels were significantly

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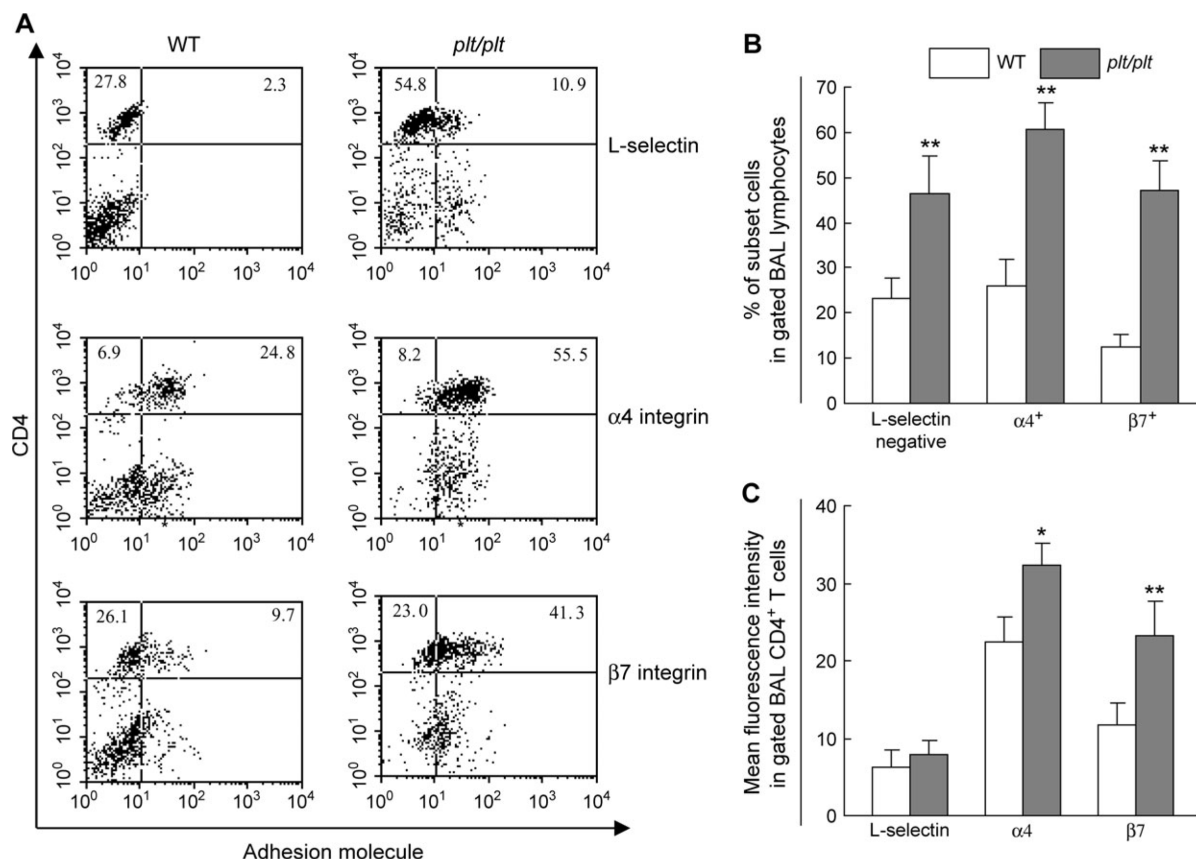

Fig. 5. The expression of adhesion molecules on BAL CD4$^+$ T cells from OVA/OVA mice. BAL cells were prepared from OVA-immunized mice 2 days after the last OVA challenge and stained with mAbs against CD4 and lymphocyte adhesion molecules. (A) Representative flow cytometric plots for the staining with anti-CD4 mAb and anti-L-selectin, anti-α_4 integrin or anti-β_7 integrin mAb from one WT mouse (left panels) and one *plt/plt* mouse (right panels). (B) The proportions of CD4$^+$ L-selectin$^-$, CD4$^+$ α_4 integrin$^+$ and CD4$^+$ β_7 integrin$^+$ in the BAL lymphocytes of OVA/OVA *plt/plt* mice were significantly higher than those in OVA/OVA WT mice. (C) The expression levels (mean fluorescence intensity) of α_4 integrin and β_7 integrin on BAL CD4$^+$ T cells of OVA/OVA *plt/plt* mice were significantly higher than those of OVA/OVA WT mice. **P* < 0.05 and ***P* < 0.01 compared with OVA/OVA WT mice, analysis of variance; *n* = 6 mice in each group.

higher in OVA-immunized *plt/plt* mice than those in OVA-immunized WT mice.

Discussion

We observed the enhanced allergic airway inflammation in *plt/plt* mice, which lack the lymphoid chemokines CCL19 and CCL21, as compared with WT mice. Specially, the OVA/OVA *plt/plt* mice had significantly more eosinophils and lymphocytes in the BAL (Fig. 1) and more severe peribronchiolar, perivascular and interstitial infiltrates in the lungs than did OVA/OVA WT mice (Figs 2 and 3). The BAL of OVA/OVA *plt/plt* mice contained more CD4$^+$ T cells than did OVA/OVA WT mice (Fig. 4). Most BAL CD4$^+$ T cells in either OVA/OVA *plt/plt* or OVA/OVA WT mice were L-selectin$^-$; thus, these cells have a memory/effector phenotype, as previously reported in asthma patients and mouse models of asthma (32–34). Furthermore, BAL CD4$^+$, but not CD8$^+$, T cells in OVA/OVA *plt/plt* mice expressed the higher levels of α_4 integrin and β_7 integrin than those in OVA/OVA WT mice (Figs 5 and 6).

α_4 integrin forms the $\alpha_4\beta_1$ and $\alpha_4\beta_7$ heterodimers with β_1 and β_7 integrins, respectively. $\alpha_4\beta_1$ integrin mainly binds to VCAM-1 and recruits $\alpha_4\beta_1^+$ memory T cells and eosinophils into inflamed lungs, whereas $\alpha_4\beta_7$ integrin binds to mucosal addressin cell adhesion molecule-1 on endothelia and recruits $\alpha_4\beta_7^+$ lymphocytes into inflamed gut (30, 35). $\alpha_E\beta_7$ integrin binds to epithelial E-cadherin and recruits $\alpha_E\beta_7^+$ lymphocytes into epithelial layers of mucosal tissues (31). However, $\alpha_4\beta_7$ integrin is rarely expressed on lung lymphocytes (32, 36, 37). Thus, the relatively high levels of α_4 and β_7 integrins on BAL CD4$^+$ T cells from OVA/OVA *plt/plt* mice suggest that more $\alpha_4\beta_1$ and $\alpha_E\beta_7$ memory CD4$^+$ T cells were recruited into inflamed lungs of *plt/plt* mice.

There is impaired migration of naive T cells from the bloodstream into LNs of *plt/plt* mice and L-selectin-deficient mice. In contrast to *plt/plt* mice, L-selectin-deficient mice develop allergic airway inflammation as well as do WT mice in response to OVA immunization and challenge (38). Thus, CCL19 and CCL21 in LNs and L-selectin on lymphocytes may play distinct roles in the development of allergic airway inflammation.

Binding of CCL19 or CCL21 to CCR7 on naive lymphocytes causes activation of a tyrosine kinase which in turn-leads to conformational changes in β_2 integrin and α_4

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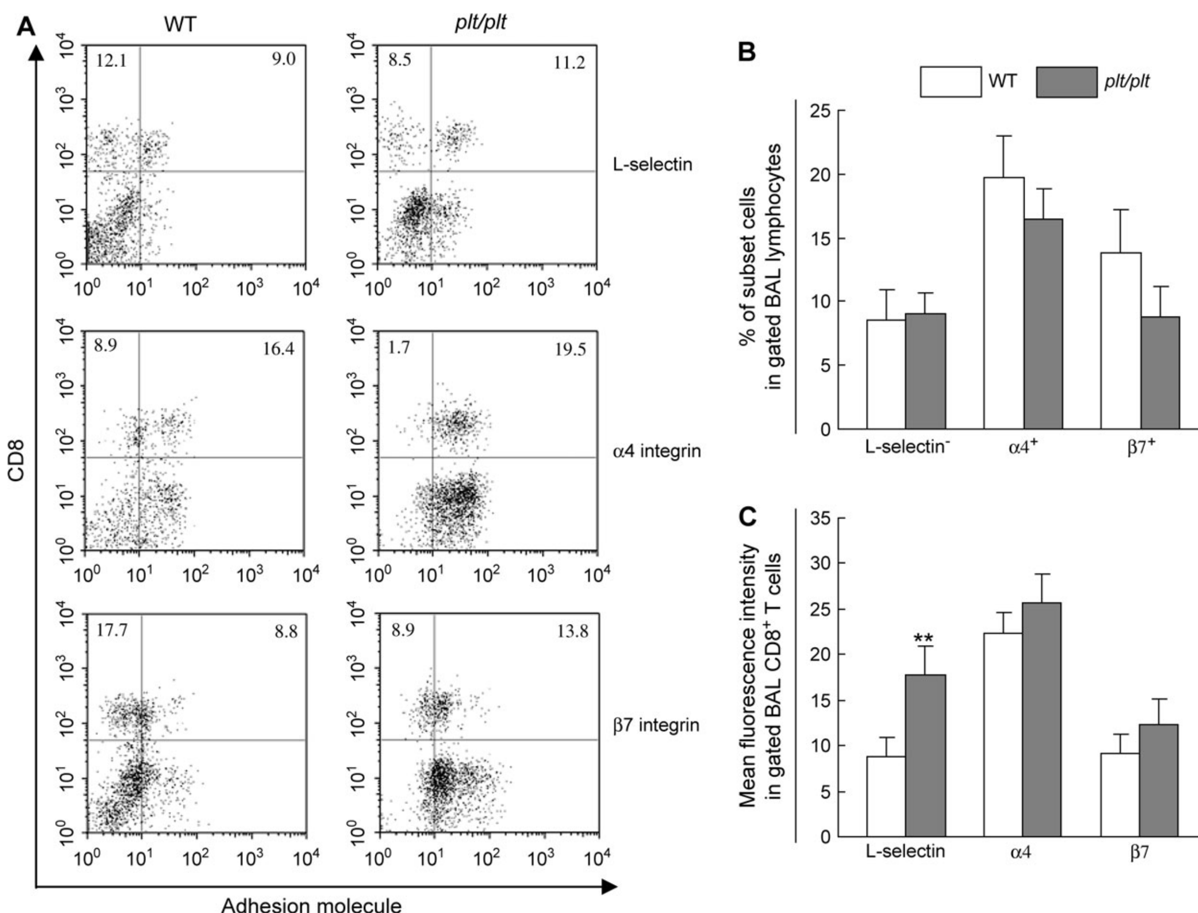

Fig. 6. The expression of adhesion molecules on BAL $CD8^+$ T cells from OVA/OVA mice. BAL cells were prepared from OVA-immunized mice 2 days after the last OVA challenge and stained with mAbs against CD8 and lymphocyte adhesion molecules. (A) Representative flow cytometric plots for staining with anti-CD8 mAb and anti-L-selectin, anti-α_4 integrin or anti-β_7 integrin mAb. (B) The proportions of $CD8^+$ L-selectin$^-$, $CD8^+$ α_4 integrin$^+$ and $CD8^+$ β_7 integrin$^+$ in the BAL lymphocytes were the same in OVA/OVA *plt/plt* and OVA/OVA WT mice. (C) The expression levels (mean fluorescence intensity) of α_4 integrin and β_7 integrin on BAL $CD8^+$ T cells of OVA/OVA *plt/plt* were the same in those of OVA/OVA WT mice. The expression levels of L-selectin in the BAL $CD8^+$ T cells of OVA/OVA *plt/plt* mice were significantly higher than those of OVA/OVA WT mice. $^{**}P < 0.01$ compared with OVA/OVA WT mice, analysis of variance; $n = 6$ mice in each group.

integrin on the lymphocyte surface (39, 40). Thus, $\alpha_L\beta_2$ integrin binds to intercellular cell adhesion molecule (ICAM)-1 and ICAM-2, while $\alpha_4\beta_1$ integrin binds to VCAM-1. In the present study, most $CD4^+$ T cells in the BAL of OVA/OVA *plt/plt* mice had an activated/memory phenotype (Fig. 5). Therefore, recruitment of α_4 integrin^{high} $CD4^+$ T cells into the BAL of OVA/OVA *plt/plt* mice does not depend on CCR7 signaling-induced integrin activation.

We found increased T_h2 cytokine (IL-4 and IL-13) mRNAs and decreased T_h1 cytokine (IL-2 and IFN-γ) mRNAs in the lungs of OVA/OVA *plt/plt* mice compared with OVA/OVA WT mice (Fig. 7). Our findings are consistent with the differential roles of T_h1 and T_h2 cytokines in T_h2-driven allergic asthma (2). For example, IL-4 and IL-13 are essential for the polarization and expansion of T_h2 and for the production of downstream cytokines such as eotaxin which is crucial for the full development of eosinophilia (41–43). IL-4 can up-regulate the expression of endothelial ICAM-1 and VCAM-1, which play critical roles in recruiting eosinophils and $\alpha_4\beta_1{}^+$ memory T cells into inflamed lungs (30, 43, 44). In contrast, IFN-γ produced by T_h1 inhibits eosinophil recruitment and mucus production (45, 46). Local expression of IFN-γ inhibits airway hyperresponsiveness and antigen-specific IgE secretion (47). Thus, the increased T_h2 and decreased T_h1 cytokines in the lungs of OVA/OVA *plt/plt* mice favor the development of enhanced allergic airway inflammation in *plt/plt* mice.

We showed that *plt/plt* mice produce more total and OVA-specific IgE than WT mice in response to OVA immunization (Fig. 8). These results are consistent with the enhanced T_h2 cytokine production in OVA/OVA *plt/plt* mice (Fig. 7) and the well-known regulatory roles of IL-4 and IL-13 in IgE synthesis (48). Elevated levels of total IgE have also been found in CCR7-deficient mice (49). IgE contributes to allergic asthma mainly by modulating the effects of mast cells (50–52). Binding of IgE to its high-affinity receptor, FcepsilonRI, on mast cells increases receptor levels and thus effector mediator release in response to specific antigens. In addition, the binding of specific antigen to FcepsilonRI-expressing cells increases the recruitment of $CD4^+$ T cells into the airway in trinitrophenyl-specific IgE transgenic mice (53). Thus, high

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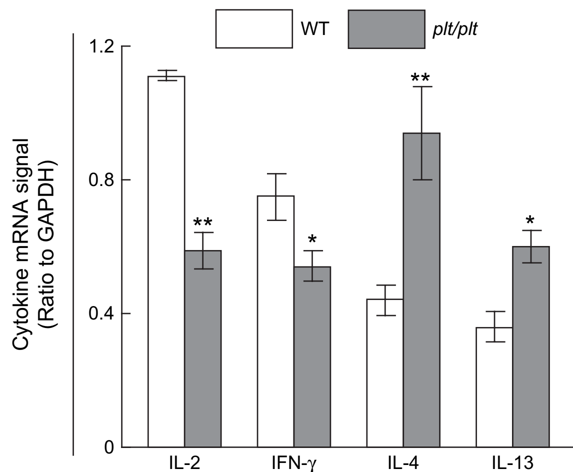

Fig. 7. Cytokine mRNAs in the inflamed lungs of WT and *plt/plt* mice. Total RNA was isolated from lungs of OVA/OVA *plt/plt* and OVA/OVA WT mice and used for semi-quantitative reverse transcription–PCR analysis. Lungs from OVA/OVA *plt/plt* mice expressed higher levels of IL-4 and IL-13 mRNAs and lower levels of IL-2 and IFN-γ mRNAs than those in OVA/OVA WT mice. $*P < 0.05$ and $**P < 0.01$ compared with OVA/OVA WT mice, analysis of variance; $n = 6$ mice in each group.

IgE levels in OVA/OVA *plt/plt* mice may enhance allergic airway inflammation by promoting the mast cell function and the recruitment of CD4$^+$ T cells into airways.

$\alpha_4\beta_7$ integrin recruits mast cell progenitors into small intestine, whereas α_4 integrin and VCAM-1 recruit mast cell progenitors into inflamed lung (54, 55). To address whether enhanced airway inflammation in *plt/plt* mice is associated with the recruitment of mast cells into the lungs, we stained mast cells on the longitudinal left lung frozen sections of OVA/OVA *plt/plt* and OVA/OVA WT mice using acidic toluidine blue. Mast cells per lung section were comparable in OVA/OVA *plt/plt* (2.0 ± 2.0) and OVA/OVA WT (2.2 ± 3.3) mice (n = 6 mice in each group). Thus, enhanced allergic airway inflammation in *plt/plt* mice does not accompany increased recruitment of mast cells into the lungs.

Yamashita *et al.* (56) recently reported the development of allergic airway inflammation in *plt/plt* mice. They focused on the disease's resolution phase and observed the enhanced airway inflammation in OVA-immunized *plt/plt* mice on days 21, 28 and 42 after several aerosol OVA challenges. However, they did not examine airway inflammation on day 1–3 after OVA challenge, the time points at which airway inflammation reach the peak in most published mouse models of asthma. We and Grinnan *et al.* (57) showed the increased numbers of eosinophils and CD4$^+$ T cells in the BAL of OVA-immunized *plt/plt* mice 24 or 48 h after the last OVA challenge. Thus, our and Grinnan's (57) studies compliment the data from Yamashita *et al.* (56), further suggesting that allergic airway inflammation is enhanced in *plt/plt* mice. However, neither Yamashita *et al.* (56) nor Grinnan *et al.* (57) found significant differences in total and OVA-specific IgE levels between OVA-immunized *plt/plt* and OVA-immunized WT mice. This is contrast to our findings that OVA immunization induced more IgE production in *plt/plt* mice than WT mice. The different IgE response in these studies may be due to differences in immunization protocols and sampling time points.

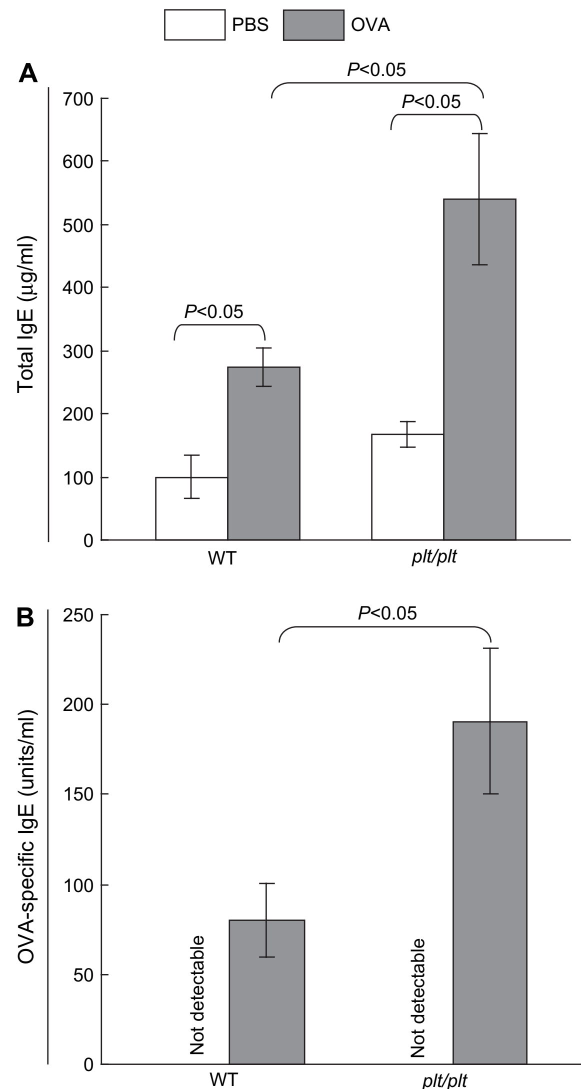

Fig. 8. Serum IgE levels in WT and *plt/plt* mice. Sera were prepared from OVA-immunized (filled bars) or unimmunized (PBS-treated, unfilled bars) WT and *plt/plt* mice. The levels of total and OVA-specific IgE were measured using sandwich ELISA assays. (A) OVA immunization significantly increased total IgE levels in WT and *plt/plt* mice compared with corresponding unimmunized group. However, serum total IgE levels in OVA-immunized *plt/plt* mice were significantly higher than those in OVA-immunized WT mice. (B) OVA-specific IgE antibody was not detected in unimmunized WT and *plt/plt* mice. OVA immunization significantly induced OVA-specific IgE in WT and *plt/plt* mice. The OVA-specific IgE response was much stronger in *plt/plt* mice than WT mice. n = 12 mice in each group.

Finally, CCR7, the receptor for CCL19 and CCL21, is expressed on B cells, naive T cells, central memory T cells and dendritic cells. Campbell *et al.* (33) showed that nearly half of CD4$^+$ or CD8$^+$ T cells recovered from the BAL of asthma patients expressed CCR7. Conversely, Syed *et al.* (58) reported down-regulation of CCR7 on peripheral blood CD4$^+$ T cells isolated from asthma patients. Bromley *et al.* (59) recently reported that most CD4$^+$CD44^{high} T cells in the BAL and lung of OVA/OVA mice did not express CCR7. In the present study, we did not examine CCR7 on the BAL

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T cells of OVA/OVA *plt/plt* and OVA/OVA WT mice. However, Grinnan *et al.* (57) found more CCR7-expressing $CD4^+$ and $CD8^+$ T cells in the lungs of OVA/OVA *plt/plt* mice than those in OVA/OVA WT mice. Given the important role of CCR7 in lymphocyte exit from peripheral tissues (59, 60) and the reduced expression of CCL21 on lymphatic endothelia in *plt/plt* mice (8, 11, 61), increased $CD4^+$ T cells in the BAL and lung of OVA/OVA *plt/plt* mice observed by us and Grinnan *et al.* (57) may in part result from reduced lymphocyte exit from inflamed lung via afferent lymphatic vessels.

Based on our findings and those in two previous studies, (56, 57) we propose that enhanced allergic airway inflammation in *plt/plt* mice is mainly caused by increased recruitment of lymphocytes and eosinophils into the lungs, increased T_h2 and decreased T_h1 cytokines and high IgE production by B cells. Moreover, the reduced CCL21 on lymphatic vessels in *plt/plt* mice may delay or impair lymphocyte exit from inflamed lung which in turn augments airway inflammation. CCL19 and CCL21 are absent in stromal cells and high endothelial cells but not dendritic cells in *plt/plt* mice (9). The number of dendritic cells was almost equal in the inflamed lung of OVA/OVA *plt/plt* and OVA/OVA WT mice (57). Therefore, it is unlikely that lung dendritic cells make major contributions to enhanced allergic airway inflammation in *plt/plt* mice.

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Abbreviations

BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
H&E	hematoxylin and eosin
ICAM	intercellular cell adhesion molecule
LN	lymph node
OCT	optimal cutting temperature
OVA	ovalbumin
OVA/OVA	ovalbumin-immunized and ovalbumin-challenged
plt/plt	paucity of lymph node T cell
PNAd	peripheral node addressin
VCAM-1	vascular cell adhesion molecule-1
WT	wild type

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