

## ORIGINAL ARTICLE

## Osteopontin is involved in migration of eosinophils in asthma

A. Takahashi<sup>\*1</sup>, M. Kurokawa<sup>†1</sup>, S. Konno<sup>\*</sup>, K. Ito<sup>‡</sup>, S. Kon<sup>‡</sup>, S. Ashino<sup>§</sup>, T. Nishimura<sup>§</sup>, T. Uede<sup>‡</sup>, N. Hizawa<sup>¶</sup>, S-K Huang<sup>†</sup> and M. Nishimura<sup>\*</sup><sup>\*</sup>First Department of Medicine, Hokkaido University School of Medicine, Sapporo, Hokkaido, Japan, <sup>†</sup>Johns Hopkins Asthma and Allergy Center, Baltimore, MD, USA,<sup>‡</sup>Division of Molecular Immunology, Institute for Genetic Medicine, Hokkaido University, Hokkaido, Japan, <sup>§</sup>Division of Immunoregulation, Institute for GeneticMedicine, Hokkaido University, Hokkaido, Japan and <sup>¶</sup>Division of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, JapanClinical &  
Experimental  
Allergy

## Summary

**Background** Osteopontin (OPN) is an extracellular matrix protein with a wide range of functions, and is involved in various inflammatory diseases. However, the role of OPN in eosinophilic airway inflammation is unclear.**Objective** To elucidate the role of OPN in eosinophilic airway inflammation.**Methods** OPN protein levels in induced sputum from asthmatic patients and healthy controls were measured. Eosinophil migration assays were performed in the presence or absence of OPN, a blocking antibody (Ab) recognizing its integrin-binding domain (2K1) and an anti-integrin  $\alpha 4$  Ab (P1H4). In the mouse asthma model, the levels of eosinophilia were examined in bronchoalveolar lavage fluids (BALFs) from ovalbumin (OVA)-sensitized and -challenged mice with or without administration of an Ab (M5) corresponding to human 2K1.**Results** Levels of OPN in induced sputum were significantly higher in asthmatic patients when compared with healthy controls. In addition, levels of OPN were correlated with the percentage of sputum eosinophils. OPN induced significant migration of human eosinophils and this effect was inhibited by 2K1 and P1H4. M5 significantly attenuated OVA-induced eosinophilia in BALFs.**Conclusion** These results indicate that OPN plays a role in the migration of eosinophils into the airways and may be involved in the pathogenesis of asthma.**Keywords** asthma, eosinophil, migration, osteopontin*Submitted 15 May 2008; revised 17 December 2008; accepted 27 January 2009*

## Correspondence:

Satoshi Konno, First Department of Medicine, School of Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-Ku, Sapporo 060-8638, Japan.

E-mail: satkonno@med.hokudai.ac.jp

## Introduction

Osteopontin [(OPN), also known as early T lymphocyte activation-1 (Eta-1)] is an extracellular matrix protein with a wide range of functions. It contains arginine-glycine-aspartate (RGD) and SVVYGLR (SLAYGLR in mouse OPN) domains that bind various integrins and facilitate adhesion and migration of inflammatory cells such as macrophages [1], monocytes [2], lymphocytes [3] and neutrophils [4]. Studies using OPN-deficient mouse models and neutralizing antibodies (Abs) have demonstrated the importance of OPN in the development of several inflammatory diseases, particularly those involving T-helper type 1 (Th1)-associated immune responses [2, 5–7]. In humans, high levels of OPN have been observed

in several Th1-associated diseases, such as sarcoidosis [8, 9], tuberculosis [9], rheumatoid arthritis [10], Crohn's disease [11] and multiple sclerosis (MS) [12]. However, the role of OPN in inflammatory responses is complex due to its complicated structure and the existence of several functional motifs [13]. Contradictory reports have been published regarding the contribution of OPN in several inflammatory conditions [7, 14–16] and dual roles of this molecule in a single disease have been also reported [17–19].

Asthma, which is characterized by eosinophilic airway inflammation, exhibits several potential mechanisms for pathogenesis, such as enhancement of the Th2 immune response, migration of eosinophils into the airway, airway mucus production and airway remodelling. OPN is reportedly involved in Th1-associated immune responses [2, 5–12], migration of inflammatory cells [1–4] and fibrosis [20]. Interestingly, a recent study by Xanthou et al. [17]

<sup>1</sup>Contributed equally to this work.

suggested that OPN has an inhibitory effect on OVA-induced allergic airway inflammation in studies using anti-OPN Ab and OPN-deficient mice. However, the direct effects of OPN on pulmonary eosinophils remain unclear.

In the present study, in order to elucidate the role of OPN in the development of eosinophilic airway inflammation, we examined the expression of OPN in human asthmatic subjects and in an OVA-immunized murine asthma model. The effects of blocking Abs recognizing human and mouse integrin-binding domains on the recruitment and migration of eosinophils were also evaluated. Our results provided an indication of yet another aspect of OPN's role in the development of eosinophilic airway inflammation.

## Materials and methods

### Measurement of osteopontin levels in induced sputum

Characteristics of healthy controls and asthmatic patients are shown in Table 1. Sputum samples were obtained from patients in Hokkaido University Hospital who were diagnosed as having asthma, and from hospital staff and students who had no history of asthma and no chest or nasal symptoms. The institutional review board of the School of Medicine, Hokkaido University, approved the study and all of the patients and healthy control subjects agreed with the purpose of our research and provided informed consent. We collected sputum as reported previously [21]. Briefly, sputum was induced by inhalation of 3 mL of 4.5% saline with a nebulizer, after pretreatment with inhaled salbutamol (200 µg). Subjects were instructed to rinse their mouths with water to avoid contamination with saliva. Induced sputum was placed in a 50 mL

**Table 1.** Characteristics of healthy controls and asthmatic patients for measuring OPN levels in induced sputum

|                                                 | Healthy controls<br>( <i>n</i> = 19) | Asthmatic patients<br>( <i>n</i> = 25) |
|-------------------------------------------------|--------------------------------------|----------------------------------------|
| Sex (male/female)                               | 13/6                                 | 14/11                                  |
| Age (years)* <sup>#</sup>                       | 30 (23–73)                           | 62 (35–74)                             |
| Smoking (never/ex/current)                      | 11/6/2                               | 13/11/1                                |
| %FEV <sub>1</sub> (%)*                          | –                                    | 85.1 (25.4–118.8)                      |
| Asthma severity [Step (1/2/3/4)] <sup>†</sup>   | –                                    | 1/1/6/17                               |
| Asthma therapy                                  |                                      |                                        |
| Inhaled corticosteroid                          | –                                    | 20                                     |
| Oral corticosteroid                             | –                                    | 7                                      |
| Eosinophils (%) <sup>*</sup>                    | 0.8 (0–3.2)                          | 25.6 (2.0–94.4)                        |
| Inflammatory cells/total cells (%) <sup>‡</sup> | 52.3 ± 5.46                          | 56.6 ± 4.71                            |

\*Median (range),

<sup>†</sup>GINA 2005,

<sup>‡</sup>Mean ± SEM,

<sup>#</sup>*P* < 0.05.

FEV<sub>1</sub>, forced expiratory volume in 1 s; OPN, osteopontin; SEM, standard error of mean.

polystyrene tube, and a two-fold volume of 0.1% dithiothreitol (DTT)/phosphate-buffered saline (PBS) was added, after which tubes were shaken in a water bath for 30 min. After centrifuging for 10 min, the supernatant was collected and a smear was prepared and stained with Diff-Quik (International Reagent Corp., Green Cross, Osaka, Japan). OPN levels in the supernatant were measured with an ELISA kit (R&D Systems, Minneapolis, MN, USA). Any values for OPN which fell below the detection level (0.011 ng/mL) were assigned the value of 0.011 ng/mL to use for statistical analysis.

### Human lung immunohistochemistry

Human lung sections were obtained from six patients with (*n* = 3) or without (*n* = 3) asthma undergoing primary lung cancer surgery, and non-cancerous lesions were used for staining. All of the patients were non-smokers. The institutional review board of the School of Medicine, Hokkaido University, approved the study, and all of the patients agreed with the purpose of our research and provided their written informed consent. Lung sections were incubated with non-specific staining blocking reagent (Dako, Carpinteria, CA, USA) for 10 min, followed by overnight incubation at 4 °C with a primary monoclonal Ab (10A16) (IBL Japan, Gumma, Japan) at a concentration of 0.5 µg/mL in a humidified chamber. The immunoreaction product was detected with the Catalyzed Signal Amplification (CSA) system (Dako). Sections were counterstained with haematoxylin. The primary Ab was replaced with normal mouse IgG (IBL Japan) for negative controls.

### Human anti-osteopontin antibodies and anti-integrin α4 antibody

Monoclonal Abs raised against two defined internal sequences of human OPN were used for the *in vitro* study. 2K1 was raised against VDTYDGRGDSVYGLRS [13]. 5A1, which recognizes IPVKQADSGSSEKQ [13] within the amino-terminal half of OPN, was used as a control Ab. A monoclonal anti-human integrin α4 Ab (P1H4) was purchased from Chemicon (Pittsburgh, PA, USA).

### Preparation of human eosinophils

Human eosinophils were isolated from healthy controls and asthmatic patients by negative selection using anti-CD16-bound micromagnetic beads (Miltenyi BioTech, Bergisch-Gladbach, Germany). After purification, the mean eosinophil purity was consistently > 98%, as determined by Diff-Quik staining.

### In vitro migration assay

Each well of 24-well plate was filled with various concentration of Glutathione *S*-transferase (GST)–OPN protein

[13] or GST alone in 800  $\mu$ L of PBS. Then, Millicell Culture Plate insert (pore size 5  $\mu$ m, Millipore, Billerica, MA, USA) was placed into well for 1 h at 37 °C, thus allowing the lower side of the insert being coated with OPN proteins. Then each insert was re-placed into new 24-well, filled with 800  $\mu$ L of PBS with 0.5% BSA and incubated at room temperature for 1 h for blocking. After blocking, each insert was placed into 24-well, filled with 800  $\mu$ L of TIL medium with 1% fetal bovine serum (FBS) and then  $2 \times 10^5$  cells in 400  $\mu$ L of serum-free TIL medium was applied to each insert. In a series of blocking experiments using 2K1 or 5A1, each insert was placed into well, filled with PBS with 20  $\mu$ g/mL of Ab, and was re-placed into well in TIL medium/FBS with 20  $\mu$ g/mL of Ab before cells were applied to each insert. In an experiment using P1H4, P1H4 (20  $\mu$ g/mL) was added to cell suspension (20  $\mu$ g/mL) and cells were incubated at 37 °C for 20 min before final incubation. Plate was finally incubated for 4 h at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. After culture, upper side of insert was scrubbed with cotton swab. Thus, non-migrated cell was scraped off. Cells were fixed in 300  $\mu$ L of methanol for 20 min at -80 °C and stained with Giemsa's stain solution for 10 min at room temperature. Excess dye was washed with water. Filter was removed from insert and mounted on Slide Glass. Migrated cells were counted in 10 fields through light microscopy at a magnification of  $\times 200$ . These experiments were performed using a total of six donors (three healthy controls and three asthmatic patients).

To compare the migratory effect of OPN on human eosinophils to that of eotaxin, which is a well known eosinophil chemoattractant, a migration assay was also conducted using 5  $\mu$ m polyvinylpyrrolidone-free polycarbonate membranes (Nucleopore) in Boyden chambers (NeuroProbe Inc., Gaithersburg, MD, USA) as previously described [22, 23] with slight modification. Human recombinant eotaxin (R&D Systems) or recombinant OPN (R&D Systems) were diluted in PBS at a concentration of 10 nM and placed in the lower chambers (30  $\mu$ L). The cells were suspended in HBSS containing 0.1% BSA at  $2 \times 10^6$ /mL, and 50  $\mu$ L of the suspension were placed into the upper chamber. The chamber was incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. After culture, the membrane was stained with Diff-Quick (International Reagent Corp.), and the upper side of the membrane was scrubbed with a cotton swab. Migrated cells were counted in 10 fields using light microscopy at a magnification of  $\times 400$ . These experiments were performed using a total of three donors (two healthy controls and one asthmatic patient).

#### *Mice and immunization*

All mouse experiments were approved by the Ethics Committee on Animal Research of Hokkaido University.

Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were sensitized with PBS or 20  $\mu$ g of ovalbumin (OVA; grade V; Sigma, St Louis, MO, USA) and 4.0 mg of aluminium hydroxide (Pierce, Rockford, IL, USA) by intraperitoneal injection on days 0 and 7. From days 21 to 23 after initial sensitization, mice were challenged intranasally with PBS or 20  $\mu$ g of OVA. Twenty-four hours after the final challenge (day 24), bronchoalveolar lavage (BAL) was performed, using three 0.5 mL doses of PBS and lung samples were collected. Total cell counts were performed and cytopsin preparations of BALF fluid (BALF) were stained with Diff-Quick and differentials were performed based on morphology and staining characteristics.

#### *Mouse anti-osteopontin antibody*

A purified IgG fraction of rabbit sera immunized with a synthetic peptide (VDVPNGRGDSLAYGLRS) referred to as M5 [2] was used in this study. In a series of blocking experiments, mice were injected M5 (50 or 100  $\mu$ g/200  $\mu$ L/mouse) intraperitoneally (i.p.) or control Ab (Normal Rabbit IgG, R&D Systems) 60 min before intranasal OVA administration on days 21–23. Endotoxin levels in the M5 and control Abs were <0.1 ng/mL.

#### *Measurement of cytokine levels in bronchoalveolar lavage Fluid*

Concentrations of OPN and several cytokines (IL-4, IL-5, IL-13, IFN- $\gamma$ ) in BALF were measured by ELISA (IBL Japan for OPN; R&D Systems for IL-13; Biosource International, Camarillo, CA, USA for IL-4 and IL-5; Bender Medsystems, Vienna, Austria for IFN- $\gamma$ ). The sensitivity of these assays was 1 ng/mL for OPN, 4 pg/mL for IL-4, 15.6 pg/mL for IL-5, 1.5 pg/mL for IL-13 and 5.28 pg/mL for IFN- $\gamma$ .

#### *Ribonucleic acid isolation and reverse transcription-polymerase chain reaction*

Mouse lung tissues were homogenized in ISOGEN (Nippon Gene, Tokyo, Japan) and chloroform was added. After centrifugation, the supernatant was extracted and mixed with isopropanol, and RNA was isolated by precipitation. Total RNAs (250 ng) were reverse-transcribed to cDNAs using an oligo dT primer, followed by quantitative detection of OPN transcripts using TaqMan probes and primers (ID: Mn01611440, Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7700 sequence detector (Applied Biosystems) as follows: initial step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The mRNA expression levels were normalized against the levels of a housekeeping gene, r18S.

### Mouse lung immunohistochemistry

Lung sections were incubated with non-specific staining blocking reagent (Dako) for 10 min, followed by overnight incubation at 4 °C with a primary monoclonal Ab (AF808) (R&D Systems) at a concentration of 1 µg/mL in a humidified chamber. The immunoreaction product was detected with the CSA System (Dako). Sections were counterstained with haematoxylin. Primary Ab was replaced by normal goat IgG (R&D Systems) for negative controls.

### Statistical analysis

Statistical analyses were performed using SYSTAT software (Version 11, Systat Inc., San Jose, CA, USA). Differences between groups were assessed by the Kruskal–Wallis test followed by Bonferroni's test for multiple comparisons or Mann–Whitney *U*-test. Correlations between levels of OPN and percentage of eosinophils in induced sputum were assessed by the Spearman's rank correlation coefficient. A *P*-value of <0.05 was considered to be significant.

## Results

### Increase in osteopontin protein level in induced sputum from asthmatic patients

In order to investigate the expression of OPN in the airways of asthmatic patients, we measured the levels of OPN in induced sputum from asthmatic patients and healthy control subjects for comparison. OPN levels in induced sputum from asthmatic patients were significantly higher when compared with healthy controls ( $P < 0.005$ , Fig. 1a). Correlation analysis showed a significant positive correlation between OPN levels and percentage of eosinophils ( $r = 0.530$ ,  $P < 0.01$ , Fig. 1b). However,

there was no correlation with severity of asthma or with treatment status (data not shown). Moreover, positive immunostaining for OPN was seen in the infiltrating inflammatory cells, including macrophages, lymphocytes, plasma cells and eosinophils, around the airways in asthmatic patients, while in the control subjects, few OPN-positive inflammatory cells were noted (Fig. 2). Although OPN staining was confirmed in airway epithelial cells and endothelial cells, there were no differences between asthmatic airways and non-asthmatic airways. Therefore, up-regulation of OPN in the lungs might be, at least in part, as a result of recruitment of OPN-expressing inflammatory cells around airways.

### Osteopontin-induced migration of human eosinophils, and inhibition by anti-osteopontin antibody (2K1) and anti-integrin $\alpha 4$ antibody (P1H4) *in vitro*

As OPN is involved in migration of inflammatory cells such as monocytes [2, 13] and neutrophils [24] by ligation with RGD and SVVYGLR (SLAYGLR in mouse OPN) domains, we postulated that OPN is also involved in migration of eosinophils through the ligation with these domains. The effect of OPN on human eosinophils was thus evaluated *in vitro*. Membrane-coated OPN induced significant migration of human eosinophils ( $P < 0.05$ , Fig. 3a,  $n = 3$ ; eosinophils from healthy controls). A similar result was observed when eosinophils from the three asthmatic subjects were used (data not shown). In addition, this migratory effect was blocked by 2K1, which recognizes the integrin-binding domain of OPN and by an anti-integrin  $\alpha 4$  Ab, P1H4 ( $P < 0.05$ ) (Fig. 3a). However, 5A1, which recognizes the N-terminal end of OPN, did not inhibit the OPN-induced migration of eosinophils (Fig. 3a). To compare the effect of OPN on eosinophil migration with that of eotaxin, either OPN or eotaxin (10 nM each) was dissolved in the lower chamber, and the migratory effect was evaluated. As shown in Fig. 3b, dissolved OPN

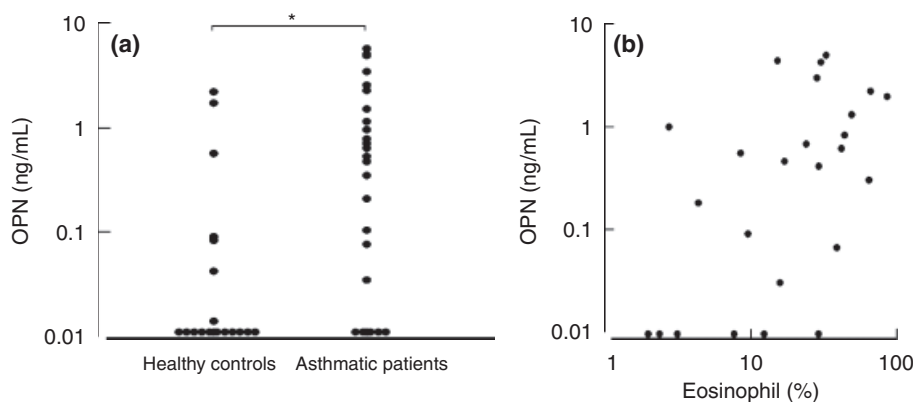


Fig. 1. (a) Osteopontin (OPN) protein levels in supernatant of induced sputum from healthy controls and asthmatic patients. OPN levels were measured by ELISA. \* $P < 0.005$ . (b) Correlation between percentage of eosinophils and OPN levels in induced sputum from asthmatic patients. There was a significant positive correlation between OPN levels and percentage of eosinophils ( $r = 0.530$ ,  $P < 0.01$ ).

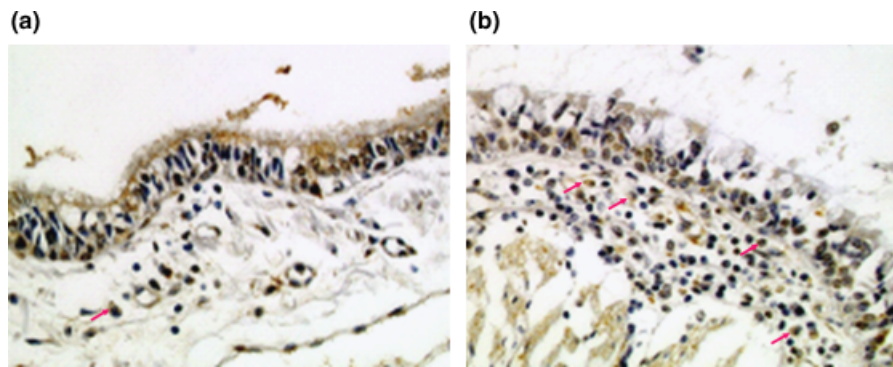


Fig. 2. Immunohistochemical staining for osteopontin (OPN) in a non-asthmatic 55-year-old female (a) and a 49-year-old female with severe persistent asthma (Step 4) (b). Representative sections from three non-asthmatic subjects and three asthmatic subjects are shown ( $\times 400$ ). The pink arrow shows OPN-positive inflammatory cells around the airway.

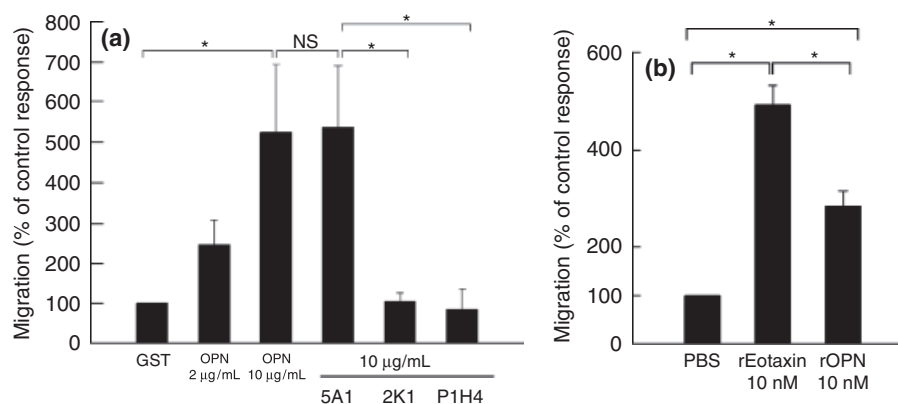


Fig. 3. Eosinophil migration assay was performed *in vitro*. Cells migrating to the lower surface were counted in 10 fields through light microscopy in each subject. Data were expressed as the mean of the average of migration index [relative value (% of control), compared with Glutathione S-transferase (GST) or phosphate-buffered saline (PBS) alone] in each subject. Each bar represents the means  $\pm$  standard error of mean of three different donors. (a) The effect of membrane-coated osteopontin (OPN) and several antibodies on the migration of eosinophils. The actual number of migrating cells in response to 10  $\mu$ g of OPN is  $13.3 \pm 11.1$ .  $*P < 0.05$ . (b) Comparisons of the migratory effect of OPN with that of eotaxin (10 nM of each recombinant protein was dissolved in the lower chamber). The actual number of migrating cells in response to 10 nM of eotaxin is  $54.6 \pm 13.8$ .  $*P < 0.05$ .

significantly induced cell migration, though this effect was less than that of the same molar concentration of eotaxin.

#### *Increase in osteopontin protein and messenger ribonucleic acid expression in a murine model of asthma*

In order to investigate the expression pattern of OPN in the lungs of a murine asthma model, we measured the levels of OPN in BALF and OPN mRNA expression at 24 h after the final OVA challenge. In our model, only mice in the OVA-sensitized and -challenged group (OVA/OVA group) showed an increase in total cells and eosinophils (data not shown). Levels of OPN in BALF were significantly elevated only in the OVA/OVA group ( $P < 0.05$ , Fig. 4a). OPN mRNA was also up-regulated in the OVA/OVA group ( $P < 0.05$ , Fig. 4b). This increase in OPN levels was associated with the increased number of inflammatory cells, as judged by significant staining of the OPN-

expressing cells in the lungs of mice challenged with OVA (Fig. 5). Although OPN was also stained in airway epithelial cells, there was no difference in expression levels in the OVA/OVA group as compared with the PBS/PBS group (Fig. 5).

#### *Anti-osteopontin antibody (M5)-attenuated eosinophilic airway inflammation in murine asthma models*

In an effort to evaluate the effects of 2K1 on inhibition of eosinophilic airway inflammation *in vivo*, we injected anti-OPN Ab (M5) i.p., which corresponds to human 2K1, before OVA challenge. Treatment with M5 significantly decreased total cell number, the number of eosinophils and macrophages in BALF as compared with treatment with control Ab ( $P < 0.05$ , Fig. 6). Levels of Th2 cytokines (IL-4, IL-5, IL-13) and IFN- $\gamma$  in BALF did not differ between the two groups (data not shown).

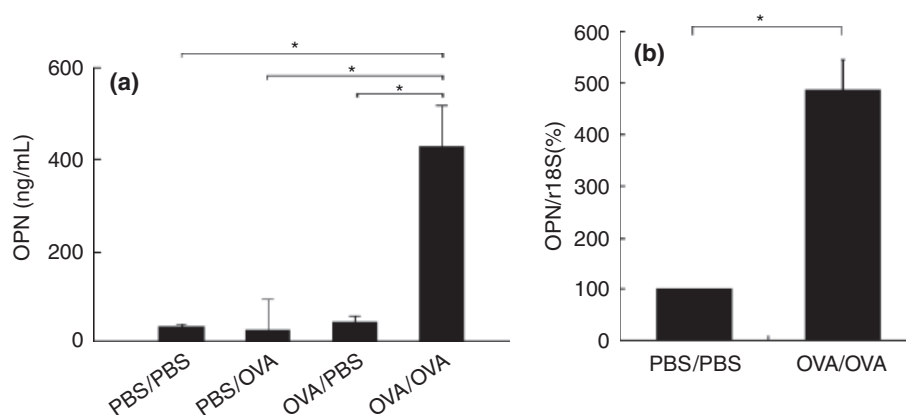


Fig. 4. (a) Osteopontin (OPN) levels in mouse bronchoalveolar lavage fluid (BALF). Twenty-four hours after the final challenge (on day 24), bronchoalveolar lavage was performed and OPN levels in BALF were measured by ELISA. Data are expressed as means  $\pm$  standard error of mean (SEM). \* $P$  < 0.05 ( $n$  = 3–4 in each group). (b) OPN mRNA levels in mouse lung tissue. The OPN/r18S ratio in ovalbumin (OVA)/OVA mice is compared with that in phosphate-buffered saline (PBS)/PBS mice. Data are expressed as means  $\pm$  SEM. \* $P$  < 0.05 ( $n$  = 3–4 in each group).

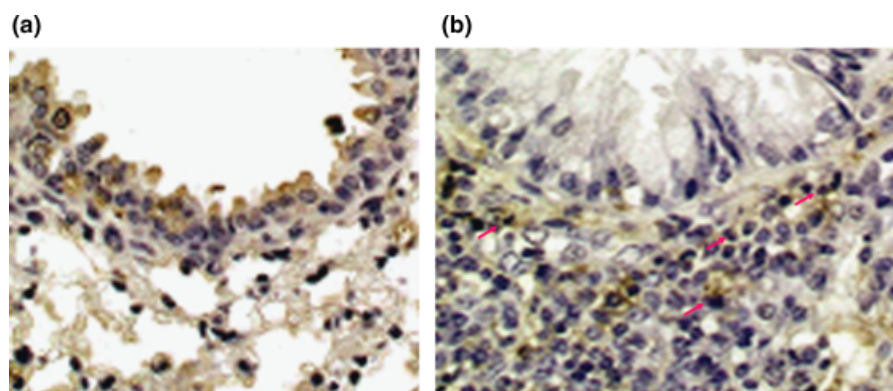


Fig. 5. Immunohistochemical staining of osteopontin (OPN) in mouse lung from the phosphate-buffered saline (PBS)/PBS group (a) and the ovalbumin (OVA)/OVA group (b). Representative sections are shown ( $\times 400$ ). Pink arrow shows OPN positive inflammatory cells around the airway.

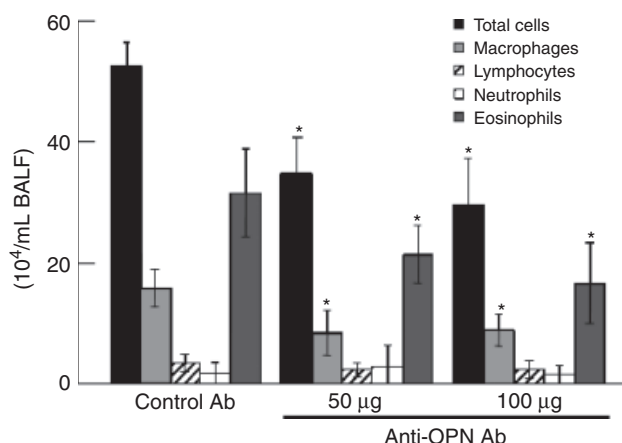


Fig. 6. Differential cell counts in mouse bronchoalveolar lavage fluid (BALF). One hour before each challenge (on days 21, 22 and 23), anti-osteopontin (OPN) antibody (Ab; M5) (50 or 100  $\mu\text{g}$ ) or control Ab was injected intraperitoneally. Data are expressed as means  $\pm$  standard error of mean. \* $P$  < 0.05 vs. control group ( $n$  = 7–8 in each group).

## Discussion

In the present study, we identified a significant association between OPN and asthma in both human subjects and a murine model. OPN is expressed in the airways of asthmatic patients, with the levels of OPN protein being positively correlated with the percentage of eosinophils in induced sputum. OPN significantly induces migration of human eosinophils *in vitro*, and this effect is inhibited by anti-OPN Ab (2K1), which recognizes the RGD and SVVYGLR domains on OPN, and by anti-integrin  $\alpha 4$  Ab (P1H4). Anti-OPN Ab (M5), which corresponds to human 2K1, attenuates OVA-induced eosinophilia in mouse BALFs. These results indicate that OPN plays a role in the pathogenesis of asthma, and this role is at least partially mediated by the migration of eosinophils into the airways.

OPN has been implicated in a number of physiological and pathological events and has been shown to play a role in the migration of inflammatory cells, such as monocytes

[2, 13], lymphocytes [3] and neutrophils [4, 24]. The effects of OPN were mediated through the interaction with several types of integrin on the cell surface of inflammatory cells [2, 24, 25]. In this study, we demonstrated that OPN is also involved in the migration of eosinophils and that this effect was inhibited by 2K1 and P1H4, suggesting that OPN-induced migration of eosinophils was mediated via interaction between the RGD and SVVYGLR domains on OPN and  $\alpha 4$  integrin expressed on the surface of eosinophils. In an animal model, the inhibitory effects of anti- $\alpha 4$  integrin Ab on the development of allergic airway inflammation have been reported [26, 27], and this effect is mediated by the inhibition of migration of eosinophils into the airway [27]. Accordingly, the inhibitory effects of anti- $\alpha 4$  integrin Ab on the development of allergic airway inflammation may partially be mediated through the inhibition of OPN-induced migration of eosinophils.

It is noted, however, that our results appear to be at variance with a recent study by Xanthou et al. [17], which showed that administration of anti-OPN Abs before OVA challenge enhanced allergic airway inflammation, suggesting that OPN has an inhibitory effect on eosinophilic inflammation. The reason for these discrepant results is currently unclear, but it is likely due, in part, to the use of different anti-OPN Abs recognizing different functional domains of the OPN molecule. OPN is known to have a diverse function mediated through an integrin-binding domain consisting of an RGD and SVVYGLR (SLAYGLR in mouse OPN) sequence [2, 25, 28], and via ligation with the N- and C-terminal half of the protein [13]. In fact, recent studies by Koguchi and colleagues demonstrated that the effects of OPN on IL-12 and IFN- $\gamma$  induction in human peripheral blood mononuclear cells can be blocked by a C-terminal region-specific Ab, 10A16 but not by an integrin-binding domain-specific Ab, 2K1 [9, 29], indicating that ligation between the C-terminal half of OPN and an unidentified receptor(s) is involved in the induction of Th1 cytokines, while blockade of integrin binding is ineffective in regulating the cytokine response.

In the present study, the anti-OPN Ab used (M5) recognizes the RGD and SLAYGLR domains of OPN, and, interestingly, administration of M5 did not affect Th2 cytokine levels, suggesting that the effects of M5 on the decreased eosinophilia in BALF are independent of Th2 immune suppression. As a corollary, several studies have shown an inhibitory effect of M5 on mouse models of inflammatory diseases [2, 24, 28] and on the OPN-induced migration and adhesion of several inflammatory cell types [2, 24]. In contrast, in the study by Xanthou and colleagues, an anti-OPN Ab, AF-808, was used: however, at present, its epitope specificity is currently unknown. One possibility is that AF-808 Abs may be functionally similar to 10A16 Abs in blocking OPN-mediated Th1 cytokine generation, resulting in a polarized Th2 response and consequently elevated levels of chemokine-mediated

eosinophilia. It would be of interest to directly investigate whether AF-808 Abs are able to regulate eosinophil migration, but this is beyond the scope of the current study.

OPN has also been implicated in the production of fibrosis in damaged tissue [30]. Recent findings by Kohan et al. [31] demonstrated that OPN is up-regulated in a murine model of allergen-induced airway remodelling and that expression of OPN is correlated with structural changes, suggesting that OPN may be involved in the development of airway remodelling in asthma. Taken together with the report by Xanthou et al. [17] and our results, on OPN's functions, it is likely that OPN may play a multi-functional role in asthma, including enhancement of the Th2 immune response, migration of eosinophils into the airway and airway remodelling.

The effects of a chimeric Ab, C2K1 (in which the variable region of 2K1 was fused with the human IgG1 constant region) on a cynomolgus monkey model of rheumatoid arthritis were recently reported [32]. In addition, phase I clinical trials of C2K1 (ASK8007) for patients with rheumatoid arthritis are currently underway. Given the inhibitory effects of 2K1 on OPN-induced migration of human eosinophils, and of M5 on eosinophilia in BALFs in murine model, 2K1 might represent a novel therapeutic target for amelioration of eosinophilic airway inflammation, which is one of the important features in asthma.

In conclusion, our data indicate that OPN is involved in the migration of eosinophils into the airway through ligation between the RGD and SVVYGLR (SLAYGLR in mouse OPN) domains and  $\alpha 4$  integrin on the surface of eosinophils, and that OPN plays a role in the development of asthma. Given its complex structure and the existence of several functional motifs, as well as previous reports that contradict our results, the role of OPN in asthma appears to be complex. Further studies are needed to clarify the involvement of OPN in various aspects of asthma.

## Acknowledgements

The authors would like to thank Dr Hiroshi Nishihara and Dr Mishie Tanino for advice on pathological evaluation. The authors would like to thank Dr Akira Isada, Dr Takeshi Hattori and Dr Yukiko Maeda for helpful discussions. Shau-Ku Huang was supported, in part, by NIH grants (AI052468 and AI073610).

## References

- 1 Weber GF, Zawaideh S, Hikita S, Kumar VA, Cantor H, Ashkar S. Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. *J Leukoc Biol* 2002; 72:752–61.

- 2 Yamamoto N, Sakai F, Kon S *et al*. Essential role of the cryptic epitope SLAYGLR within osteopontin in a murine model of rheumatoid arthritis. *J Clin Invest* 2003; **112**:181–8.
- 3 Higuchi Y, Tamura Y, Uchida T, Matsuura K, Hijiya N, Yamamoto S. The roles of soluble osteopontin using osteopontin-transgenic mice in vivo: proliferation of CD4+ T lymphocytes and the enhancement of cell-mediated immune responses. *Pathobiology* 2004; **71**:1–11.
- 4 Koh A, da Silva AP, Bansal AK *et al*. Role of osteopontin in neutrophil function. *Immunology* 2007; **122**:466–75.
- 5 Ishii T, Ohshima S, Ishida T *et al*. Osteopontin as a positive regulator in the osteoclastogenesis of arthritis. *Biochem Biophys Res Commun* 2004; **316**:809–15.
- 6 O'Regan AW, Hayden JM, Body S *et al*. Abnormal pulmonary granuloma formation in osteopontin-deficient mice. *Am J Respir Crit Care Med* 2001; **164**:2243–7.
- 7 Chabas D, Baranzini SE, Mitchell D *et al*. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science* 2001; **294**:1731–5.
- 8 O'Regan AW, Chupp GL, Lowry JA, Goetschkes M, Mulligan N, Berman JS. Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties in vitro. *J Immunol* 1991; **162**:1024–31.
- 9 Koguchi Y, Kawakami K, Uezu K *et al*. High plasma osteopontin level and its relationship with interleukin-12-mediated type 1 T helper cell response in tuberculosis. *Am J Respir Crit Care Med* 2003; **167**:1355–9.
- 10 Xu G, Nie H, Li N *et al*. Role of osteopontin in amplification and perpetuation of rheumatoid synovitis. *J Clin Invest* 2005; **115**:1060–7.
- 11 Sato T, Nakai T, Tamura N *et al*. Osteopontin/Eta-1 upregulated in Crohn's disease regulates the Th1 immune response. *Gut* 2005; **54**:1254–62.
- 12 Comabella M, Pericot I, Goertsches R *et al*. Plasma osteopontin levels in multiple sclerosis. *J Neuroimmunol* 2005; **158**:231–9.
- 13 Kon S, Yokosaki Y, Maeda M *et al*. Mapping of functional epitopes of osteopontin by monoclonal antibodies raised against defined internal sequences. *J Cell Biochem* 2002; **84**:420–32.
- 14 Blom T, Franzén A, Heinegård D, Holmdahl R. Comment on "The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease". *Science* 2003; **299**:1845.
- 15 Ashkar S, Weber GF, Panoutsakopoulou V *et al*. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 2000; **287**:860–4.
- 16 Abel B, Freigang S, Bachmann MF, Boschert U, Kopf M. Osteopontin is not required for the development of Th1 responses and viral immunity. *J Immunol* 2005; **175**:6006–13.
- 17 Xanthou G, Alissafi T, Semitekolou M *et al*. Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nat Med* 2007; **13**:570–8.
- 18 Crawford HC, Matrisian LM, Liaw L. Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo. *Cancer Res* 1998; **58**:5206–15.
- 19 Sodek J, Chen J, Nagata T *et al*. Regulation of osteopontin expression in osteoblasts. *Ann N Y Acad Sci* 1995; **760**:223–41.
- 20 Pardo A, Gibson K, Cisneros J *et al*. Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Med* 2005; **2**:891–903.
- 21 Yamaguchi E, Nishihira J, Shimizu T *et al*. Macrophage migration inhibitory factor (MIF) in bronchial asthma. *Clin Exp Allergy* 2000; **30**:1244–9.
- 22 Adachi T, Hanaka S, Masuda T, Yoshihara H, Nagase H, Ohta K. Transduction of phosphatase and tensin homolog deleted on chromosome 10 into eosinophils attenuates survival, chemotaxis, and airway inflammation. *J Immunol* 2007; **179**:8105–11.
- 23 Royer JF, Schratl P, Lorenz S *et al*. A novel antagonist of CRTH2 blocks eosinophil release from bone marrow, chemotaxis and respiratory burst. *Allergy* 2007; **62**:1401–9.
- 24 Diao H, Kon S, Iwabuchi K *et al*. Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases. *Immunity* 2004; **21**:539–50.
- 25 Senger DR, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L. Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *Mol Biol Cell* 1994; **5**:565–74.
- 26 Henderson WR Jr, Chi EY, Albert RK *et al*. Blockade of CD49d (alpha4 integrin) on intrapulmonary but not circulating leukocytes inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. *J Clin Invest* 1997; **100**:3083–92.
- 27 Schneider T, Issekutz TB, Issekutz AC. The role of alpha4 (CD49d) and beta2 (CD18) integrins in eosinophil and neutrophil migration to allergic lung inflammation in the Brown Norway rat. *Am J Respir Cell Mol Biol* 1999; **20**:448–57.
- 28 Kitamura M, Iwabuchi K, Kitaichi N *et al*. Osteopontin aggravates experimental autoimmune uveoretinitis in mice. *J Immunol* 2007; **178**:6567–72.
- 29 Koguchi Y, Kawakami K, Kon S *et al*. Penicillium marneffeii causes osteopontin-mediated production of interleukin-12 by peripheral blood mononuclear cells. *Infect Immun* 2002; **70**:1042–8.
- 30 Mori R, Shaw TJ, Martin P. Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring. *J Exp Med* 2008; **21**:43–51.
- 31 Kohan M, Bader R, Puxeddu I, Levi-Schaffer F, Breuer R, Berkman N. Enhanced osteopontin expression in a murine model of allergen-induced airway remodelling. *Clin Exp Allergy* 2007; **37**:1444–54.
- 32 Yamamoto N, Nakashima T, Torikai M *et al*. Successful treatment of collagen-induced arthritis in non-human primates by chimeric anti-osteopontin antibody. *Int Immunopharmacol* 2007; **7**:1460–70.