学位論文（博士）

Histological characteristics of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 in asthmatic murine model during A(H1N1)pdm09 infection.

（A(H1N1)pdm09 に感染した喘息モデルマウスにおける MMP-9 と TIMP-1 の病理的特徴）

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令和 4 年 11 月

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1. 要旨

Pandemic influenza virus A(H1N1)pdm09 infection occurred in healthy children and young adults, but asthmatic patients presented more rapid progression of respiratory distress and plastic bronchitis. To investigate the pathogenesis of worsening respiratory symptoms after A(H1N1)pdm09 infection, we focused on matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1). MMP-9 and TIMP-1 levels in bronchoalveolar lavage fluid and serum from mice with and without asthma were evaluated after A(H1N1)pdm09 or seasonal A(H1N1) infection. MMP-9 levels were more elevated in Asthma/A(H1N1)pdm09-infected mice than in non-Asthma/A(H1N1)pdm09-infected mice on both 3 and 7 days post-infection. Immunohistochemical findings in this pneumonia model showed that MMP-9 and TIMP-1 positive cells were observed in blood vessels and bronchus of lung tissue in severe pathological findings of pneumonia with asthma. Microscopically, shedding cells and secretions were conspicuous in the trachea on days 3 and 7 post-infection, in the A(H1N1)pdm09-infected mice with asthma. Our results suggest that MMP-9 and TIMP-1 expressions are related to severe pneumonia in the A(H1N1)pdm09 infection with asthma, leading to cause epithelial cell shedding.

2. 研究背景

A global pandemic of influenza with virus A(H1N1)pdm09 began in Mexico in 2009[1]. Approximately 80% of deaths due to A(H1N1)pdm09 infection occurred in individuals <65 years of age[2]. Many severe and fatal cases of A(H1N1)pdm09 infection occurred in healthy children and young adults[3–5], presenting with severe pneumonia, acute respiratory distress syndrome, and plastic bronchitis[6–8]. Some reports have suggested that bronchial asthma was one of the most common underlying medical conditions in patients hospitalized with A(H1N1)pdm09[9,10]. We previously reported that unlike seasonal influenza A H1N1 infection, A(H1N1)pdm09 infection in a murine model of asthma induces severe pulmonary inflammation on 7 days after infection[11–14]. Furthermore, in asthmatic mice with A(H1N1)pdm09 infection, interleukin-6 and tumor necrosis factor-α levels peak earlier on 3 d post-infection and with higher values compared to those in asthmatic mice with seasonal influenza (A/Puerto Rico/8/34, A/PR/8/34) infection[13]. Moreover, virus titers in bronchoalveolar lavage (BAL) fluid after influenza infection showed the highest levels in A(H1N1)pdm09-infected asthmatic mice on 3 and 7 d post-infection, and immunohistochemical finding stained with influenza A nucleoprotein (InfA-NP) revealed that InfA-NP was high expressed at site of pneumonia in A(H1N1)pdm09 infection[13]. Also, the airways in A(H1N1)pdm09-infected asthmatic mice showed greater hyperresponsiveness compared to those of A/PR/8/34 infection[14].

3. 目的

Matrix metalloproteinases (MMPs) are proteinases that belong to a large family of zinc-containing enzymes responsible for degrading extracellular matrix components, such as collagen and proteoglycans, during tissue remodeling[15–17]. MMP-9 is mainly produced by macrophages and neutrophils, but also epithelial cells, mast cells, fibroblasts, and smooth myocytes[18]. Increased MMP-9 levels have been observed in blood, sputum, and BAL fluid of patients experiencing asthma exacerbation[19]. Furthermore, tissue inhibitor of metalloproteinases-1 (TIMP-1) levels and MMP-9/TIMP-1 ratio are elevated in BAL fluid of asthmatic patients and immunohistochemical analysis has revealed that MMP-9 and TIMP-1 are expressed in airway tissue[20]. MMP-9 has also been reported to affect the severity of pneumonia in mice infected with seasonal influenza A(H1N1) in which the active form of MMP-9 is upregulated[21]. Moreover, in the mechanism of the severity of influenza infection, inflammatory cytokines induced the expression of MMP-9, which leads to increase the vascular permeability and multiple organ failure[22]. However, there have been no studies regarding the role of MMP-9 and TIMP-1 in the pathophysiology of lung inflammation in asthmatic patients with A(H1N1)pdm09 infection.

In this study, we investigated whether MMP-9 was involved in more severe lung inflammation using a mouse model of bronchial asthma in combination with A(H1N1)pdm09 infection. We also compared sequential changes of MMP-9 and TIMP-1 expression in intra-tracheal and intra-vascular tissue of mice from the bronchial asthma model that were infected with either A(H1N1)pdm09 or A/PR/8/34 influenza virus.

4. 方法

(1) 対象

Sensitization of mice and allergen challenge

BALB/c male and female mice 6 to 8 weeks of age were obtained from Chiyoda Kaihatsu Co., Ltd. (Tokyo, Japan). To establish the bronchial asthma model, the mice were sensitized and challenged with grade II ovalbumin (OVA; Sigma-Aldrich., St. Louis, MO, USA), as previously described[11–14]. The study was approved by the All Institutional Animal Care and Use Committee of Yamaguchi University (No. 29-S01) and all methods were conducted in accordance with the approved guidelines.

4. 方法

（2）方法

Viruses, infection of mice and preparation of samples

Mouse-adapted A(H1N1)pdm09 (strain A/Narita/1/09) and influenza A H1N1 (strain A/Puerto Rico/8/34) viruses were provided by the National Institute of Infectious Disease (Tokyo, Japan). On day 31 after initiation of the bronchial asthma model, 1.0×10^5 plaque forming units (pfu) of influenza virus in a volume of 20 μL was administered intranasally to mice. Mock-infected mice received 20 μL of vehicle. The mice were then euthanized on 2, 3, or 7 d post-infection and BAL, lung tissue, and blood samples were collected. The BAL fluid was collected, centrifuged, and stored as previously described[13]. The blood samples were collected from the orbital venous plexus. The clotted blood samples were centrifuged at 13,500 rpm for 5 min at 4℃ twice, and the collected serum was stored at −80℃ until analysis for MMP-9 and TIMP-1 levels.

Measurement of MMP-9 and TIMP-1 levels

MMP-9 and TIMP-1 levels in BAL fluid and serum samples were quantified using an enzyme-linked immunosorbent assay (ELISA). A Mouse Total MMP-9 ELISA Kit (R&D Systems, Minneapolis, MN, USA) and Mouse Total TIMP-1 ELISA Kit (R&D Systems) were used according to the manufacturer's instructions. For the analysis of 3 d post-infection, Non-Asthma/A(H1N1)pdm09 mice (n=9), Asthma/A(H1N1)pdm09 mice (n=8), Non-Asthma/A/PR/8/34 mice (n=6), Asthma/A/PR/8/34 mice (n=4) were used and for 7 d post-infection, Non-Asthma/A(H1N1)pdm09 mice (n=6), Asthma/A(H1N1)pdm09 mice (n=6), Non-Asthma/A/PR/8/34 mice (n=7), Asthma/A/PR/8/34 mice (n=8) were used. To quantify MMP-9 and TIMP-1 levels in BAL fluid, the samples were diluted 20- and 10-fold, respectively. Measuring MMP-9 and TIMP-1 levels of serum samples were diluted 50- and 10-fold, respectively. The detection limits of MMP-9 and TIMP-1 were 0.014 ng/mL and 3.5 pg/mL, respectively.

Western blot analysis

Before harvesting lung tissue, mice were exsanguinated to exclude proteins in the blood, and after the harvest the lung tissue was stored at −80℃ until analysis for western blot. Lung tissues were homogenized in lysis buffer containing protease inhibitors and protein concentrations were determined using standard assay method (Bio-Rad laboratories Inc., Hercules, CA, USA). Equal amount (30 μg) of total protein were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). The membranes for MMP-9 and TIMP-1 were blocked with blocking solution (5% skimmed milk with 0.1% Tween 20 dissolved in Tris-buffered saline (pH 7.5)), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was blocked with 5% bovine serum albumin (BSA). The membrane was incubated with the first antibody for MMP-9 (Abcam, Cambridge, UK; cat #ab38898, dilution 1:1000), which was diluted in blocking solution, and TIMP-1 (Abcam, cat #ab179580, dilution 1:1000) was diluted in Can get Signal® immunoreaction enhancer solution (Toyobo, Osaka, Japan), and GAPDH (Abcam, cat #ab9485, dilution 1:1250) was diluted in 5% BSA. The membranes were incubated with the peroxidase-conjugated second antibody diluted in blocking solution, immunoreaction enhancer

solution, or 5% BSA, respectively, as used in the dilution of the first antibodies and visualized with the ECL-Western blotting detection system (Amersham Biosciences, Aylesbury, UK) according to the manufacturer's protocol. All the images were captured by ChemiDoc XRS Plus with Image Lab software (Bio-Rad laboratories Inc.).

Histological and immunohistochemical examination

Lung tissues were fixed in 10% buffered formalin for 24 h at room temperature and embedded in paraffin. Serial sections were cut 3-μm thick and stained with hematoxylin and eosin (H&E; Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Eosinophils and neutrophils were observed at 400–1000 magnification. Eosinophils and neutrophils were distinguished by the nuclear segmentation and eosin staining. Formalin-fixed serial sections of mouse lung tissue with three highly inflamed specimens from each group was also immunologically stained using rabbit polyclonal antibodies against MMP-9 (Abcam, cat #ab38898, dilution 1:1000), occludin (Abcam, cat #ab222691, dilution 1:500), MUC1 (Abcam, cat #ab109185, dilution 1:1000), and MUC5AC (Bioss, Massachusetts, US; cat #bs7166R, dilution 1:500), and goat polyclonal antibody against TIMP-1 (R&D Systems; cat #AF980, 1:1000). For MMP-9, occludin, MUC1, MUC5AC, and TIMP-1 antigen retrieval was performed by heat-induced epitope retrieval using 10 mM sodium citrate (pH 6.0) as buffer. Endogenous peroxidase activity was blocked with methanol solution containing 0.3% hydrogen peroxide. The tissue sections were incubated with the primary antibodies for 1 h. The tissues were then washed and incubated with the appropriate secondary antibody (Histofine, Nichirei Bioscience inc., Tokyo, Japan). 3,3'-diaminobenzidine (DAB) was used as the chromogenic substrate. We used terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay apoptosis detection kit (R&D systems, cat# 4810-30-K) for the analysis of apoptosis. The procedure was carried out according to the manufacturer's instruction and methyl green was used for counterstaining. Additionally, immunostaining of InfA-NP was performed at the National Institute of Infectious Diseases as previously described[13]. We used the lung tissue which was embedded in paraffin in previous study[13], and repeated the findings of InfA-NP at the pneumonia section in this manuscript. All images of the lung sections were captured and analyzed using an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan) and cellSence software version 1.7.1 (Olympus).

4. 方法

（3）解析

Statistical analysis

The difference between groups were analyzed using the Mann-Whitney *U* test. P-values less than 0.05 were considered statistically significant. All analyses and calculations were performed using JMP Pro 14 software (SAS Institute Inc., Cary, NC, USA).

5. 結果

MMP-9 and TIMP-1 levels in BAL fluid, serum, and lung tissue

We compared MMP-9 and TIMP-1 levels in BAL fluid samples collected from non-asthmatic and asthmatic mice infected with A(H1N1)pdm09 or A/PR/8/34. MMP-9 levels were more elevated in Asthma/A(H1N1)pdm09-infected mice compared to that in Non-Asthma/A(H1N1)pdm09-infected mice on both 3 and 7 d post-infection ($p < 0.05$). However, there were no significant differences in MMP-9 levels between Asthma/A(H1N1)pdm09-infected and Asthma/A/PR/8/34-infected mice on either 3 or 7 d post-infection. On 7 d post-infection, MMP-9 levels were higher in Non-Asthma/A/PR/8/34-infected mice compared to that in Asthma/A/PR/8/34-infected mice. TIMP-1 levels among the four groups on either 3 or 7 d post-infection showed no significant difference (Supp. Fig. 1A–D). We then compared MMP-9 and TIMP-1 levels in serum samples collected from the four groups. Non-Asthma/A/PR/8/34-infected mice was elevated in MMP-9 levels among four groups on 3 d post-infection. Although, there were no significant differences in TIMP-1 levels among the four groups on 3 or 7 d post-infection. We also performed western blotting to evaluate the expression of MMP-9 and TIMP-1 proteins in lung tissue (Supp. Fig. 1E). MMP-9 was more strongly expressed in Asthma/A(H1N1)pdm09-infected mice than in Asthma/ A/PR/34-infected mice on 7 d post-infection, although MMP-9 expression was the most prominent in Non-Asthma/A/PR/34-infected mice on 3 and 7 d post-infection. TIMP-1 was strongly expressed only in Non-Asthma/A(H1N1)pdm09 on 3 d post-infection. However, strong expression of TIMP-1 was observed in all groups on 7 d post-infection.

Histopathological finding in the pneumonia, lung blood vessels, and bronchi

Next, we evaluated the pathological characteristics of lungs from non-asthmatic and asthmatic mice infected with A(H1N1)pdm09 or with A/PR/8/34 for the possibility of inflammation spreading hematogenously as the cause of severe pneumonia. A(H1N1)pdm09 infection, but not A/PR/8/34 infection, induces severe pulmonary inflammation in the murine model of asthma on 7 d after infection[11–14]. The pathological findings revealed remarkable inflammatory cell infiltration and abscess formation with reduced area of air space in the alveola of Asthma/A(H1N1)pdm09-infected mice ($p < 0.05$; Fig. 1A, 1B).

MMP-9 was strongly expressed in areas of intense pneumonia in Asthma/A(H1N1)pdm09-infected mice compared to that in Non-Asthma/A(H1N1)pdm09-infected mice on 3 d post-infection (Fig. 2A). The number of MMP-9-positive cells were increased the most in Asthma/A(H1N1)pdm09-infected mice among the four groups, both on 3 and 7 d post-infection ($p < 0.05$; Fig. 2B). TIMP-1 expression was also strongly expressed in concurrence with the MMP-9 expression in Asthma/A(H1N1)pdm09-infected mice (Fig. 2A).

We compared the expressions of MMP-9 and TIMP-1 in the blood vessels and bronchi of asthmatic mice infected with A(H1N1)pdm09. On 3 d post-infection, the congregate of MMP-9-positive cells were observed around the blood vessels (Fig. 3A). The number of MMP-9-positive cells was elevated in Asthma/A(H1N1)pdm09-infected mice compared to that of the other three groups (Fig. 3B). On 7 d post-infection, MMP-9-positive cells in Asthma/A(H1N1)pdm09-infected mice were decreased compared to that on 3 d post-infection. There was no difference between the Asthma/A(H1N1)pdm09 and Asthma/A/PR/8/34 groups. Although there was no significant difference

among the four groups regarding TIMP-1 expression (Fig. 3C), it was consistently expressed in concurrence with the changes in MMP-9 expression (Fig. 3A).

Epithelial cells in the bronchi also were positively stained for MMP-9 (Fig. 4A). TIMP-1 expression was consistent with the expression of MMP-9 (Fig. 4A). Shedding cells and secretions were present in the trachea on 3 and 7 d post-infection, particularly in Asthma/A(H1N1)pdm09-infected mice (Fig. 4A). In accordance with this finding, MMP-9- and TIMP-1-positive cells were each observed in greater numbers in bronchi on 7 d post-infection (Fig. 4B). As MMP-9 is secreted from not only epithelial cells, but also neutrophils and eosinophils[18], we examined the blood cells in the bronchi (Fig. 4C). The greater numbers of neutrophil and eosinophil were detected in the Asthma/A(H1N1)pdm09-infected mice than those in the other groups. Additionally, MMP-9-positive neutrophils and eosinophils were more found in Asthma/A(H1N1)pdm09-infected mice (Fig. 4D).

Comparison of tight junction and mucin with immunostaining

For further investigation on shedding cells and secretions particularly observed in the bronchi of Asthma/A(H1N1)pdm09-infected mice, we compared the occludin which is essential to maintain the tight junctions of tracheal epithelial cells. The expression of occludin was significantly reduced in the tracheal epithelial cells of Asthma/A(H1N1)pdm09-infected mice compared to those of the other three on 3 d post-infection (Fig. 5A, Supp. Fig. 2A). Additionally, in the examination of apoptosis detecting with TUNEL assay, apoptosis was detected only in the Asthma/A(H1N1)pdm09-infected mice on 7 d post-infection, stained at the site of pneumonia (Fig. 5B). Moreover, MUC1 and MUC5AC expressions were evaluated by immunostaining to examine the components of the secretions. MUC1 was strongly expressed in the tracheal secretions only in the Asthma/A(H1N1)pdm09-infected mice and epithelial cells of the trachea were stained in each group (Fig. 5C, Supp. Fig. 2B). However, MUC5AC was slightly expressed in the epithelial cells of the trachea in each group (Fig. 5C, Supp. Fig. 2C).

Comparison of influenza A antigen, MMP-9, and TIMP-1 immunostaining

We reported that InfA-NP immunostaining for influenza antigen was positive in A(H1N1)pdm09-infected mice, but not in A/PR/8/34-infected mice[13]. Therefore, we compared the site of InfA-NP presence in Asthma/A(H1N1)pdm09-infected mice with MMP-9 and TIMP-1 expression on 7 d post-infection (Fig. 6). InfA-NP-positive cells were found at the site of pneumonia, InfA-NP-positive bronchial epithelial cells were also observed. However, immunostaining of perivascular and intravascular blood cells for InfA-NP were negative. MMP-9 and TIMP-1 expressions did not coincide perfectly with the existence of InfA-NPs, but their existences in the area of pneumonia and in the bronchus were in close proximity.

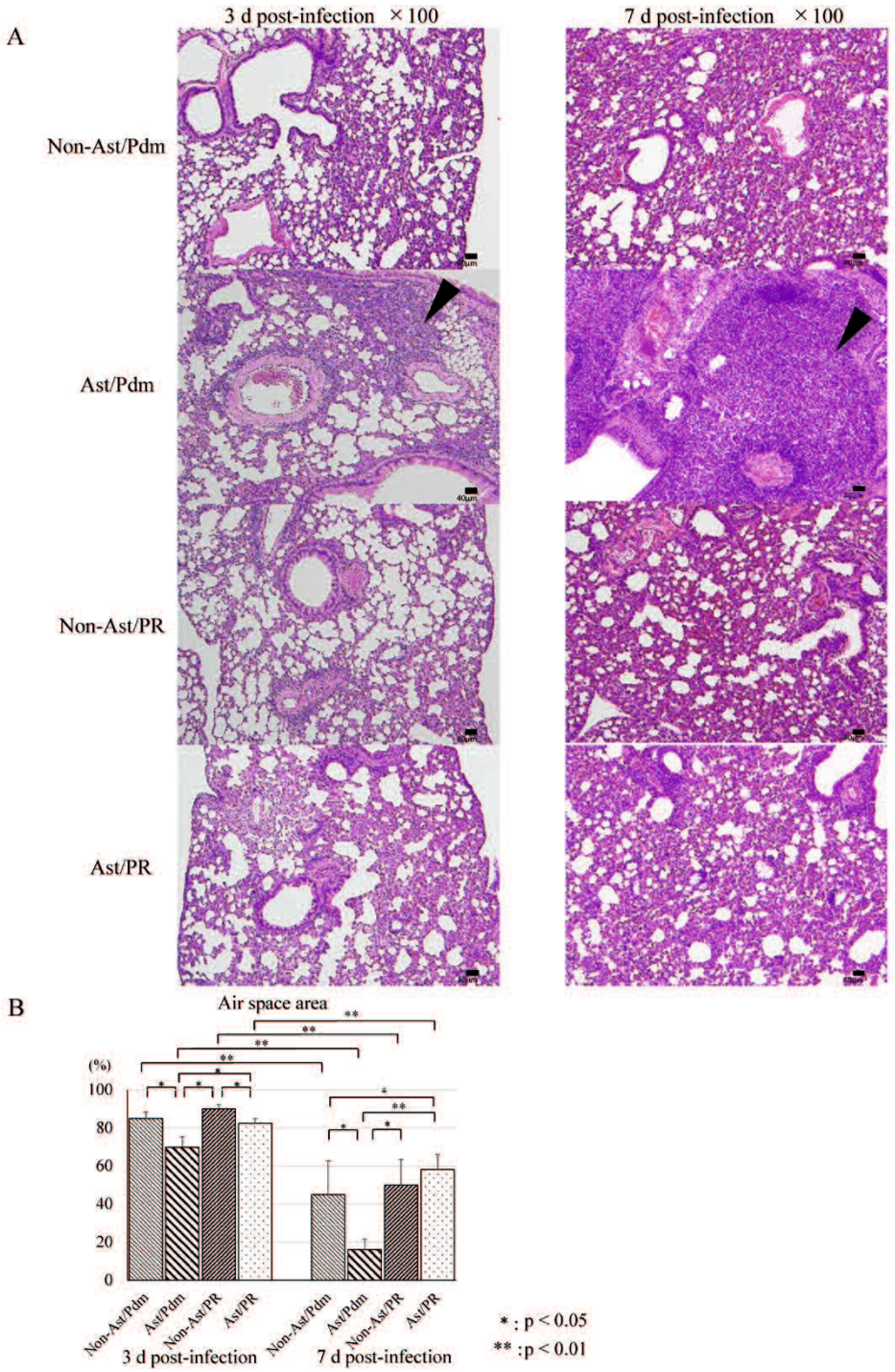


Figure 1. (A) Histopathological findings in mice after A(H1N1)pdm09 and seasonal A(H1N1) influenza (A/PR/8/34) infection. Hematoxylin and eosin (H&E)-stained lung tissue on 3 and 7 d post-infection at the site of pneumonia. Representative findings are shown. Arrow head are pointing to an area of severe pneumonia. **(B)** Comparison of air space area of lungs in influenza-infected mice. The areas of air space in pneumonia per microscopic field were measured and the average of a total of eight fields in each group are graphed. Representative findings were chosen for measurement. *p < 0.05, **p < 0.01. Non-Ast/Pdm: Non-Asthma/A(H1N1)pdm09-infected mice, Ast/Pdm: Asthma/A(H1N1)pdm09-infected mice, Non-Ast/PR: Non-Asthma/A/PR/8/34-infected mice, Ast/PR: Asthma/A/PR/8/34-infected mice. Error bars are indicating standard deviation.

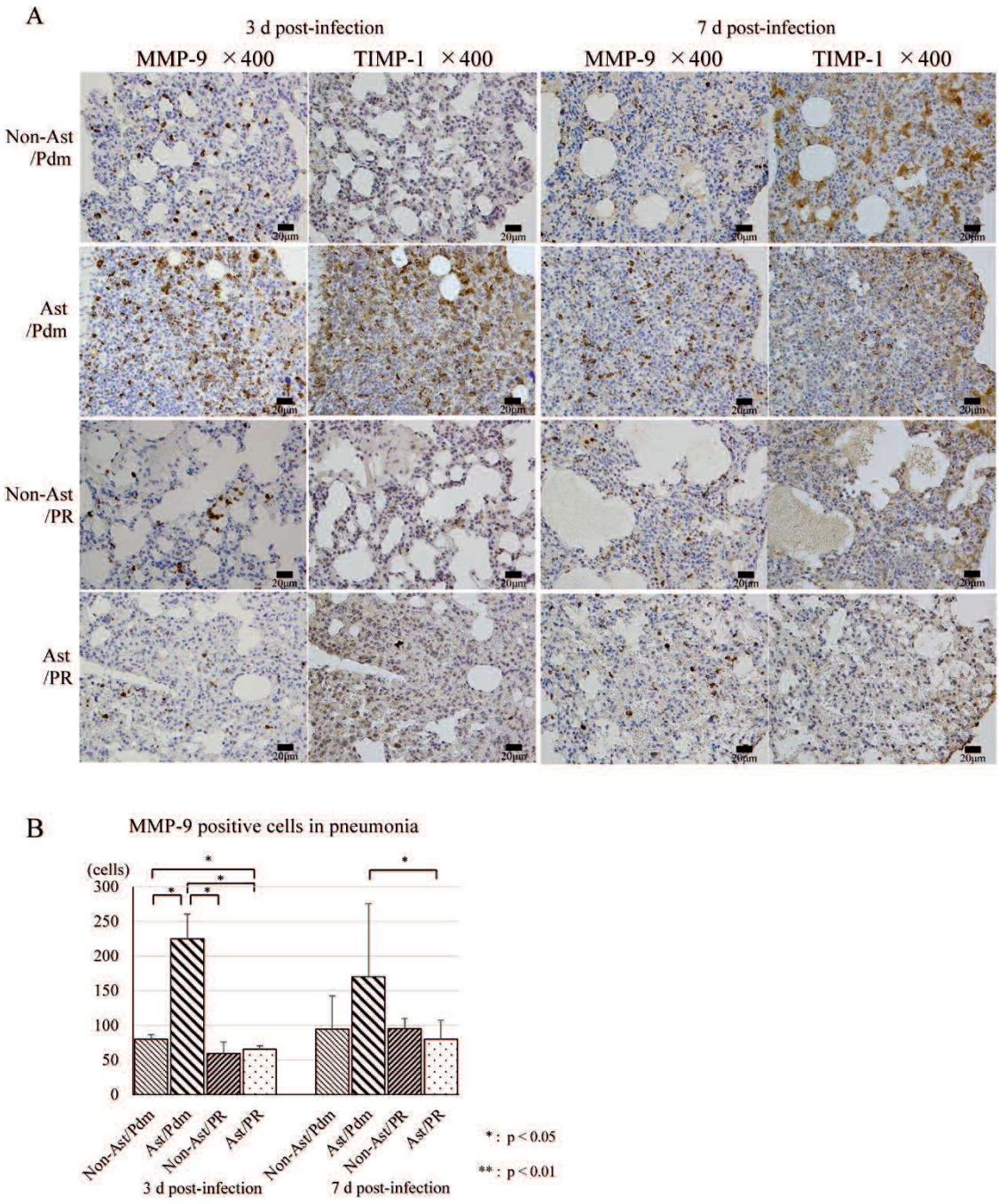


Figure 2. (A) Immunohistochemical analysis of MMP-9 and TIMP-1 expression at the site of pneumonia. Micrographs of immunohistochemical staining of mouse lung tissue on 3 and 7 d post-infection. Similar results were obtained for six independent mice from each group. Representative findings are shown. **(B)** MMP-9-positive cells in the area of pneumonia were counted on 3 and 7 d post-infection. Eight sites of typical findings were counted and the averages graphed. *p < 0.05, **p < 0.01. Error bars are indicating standard deviation. Counts of TIMP-1-positive cells were not performed due to the large number of areas in the intercellular space that were positive for staining.

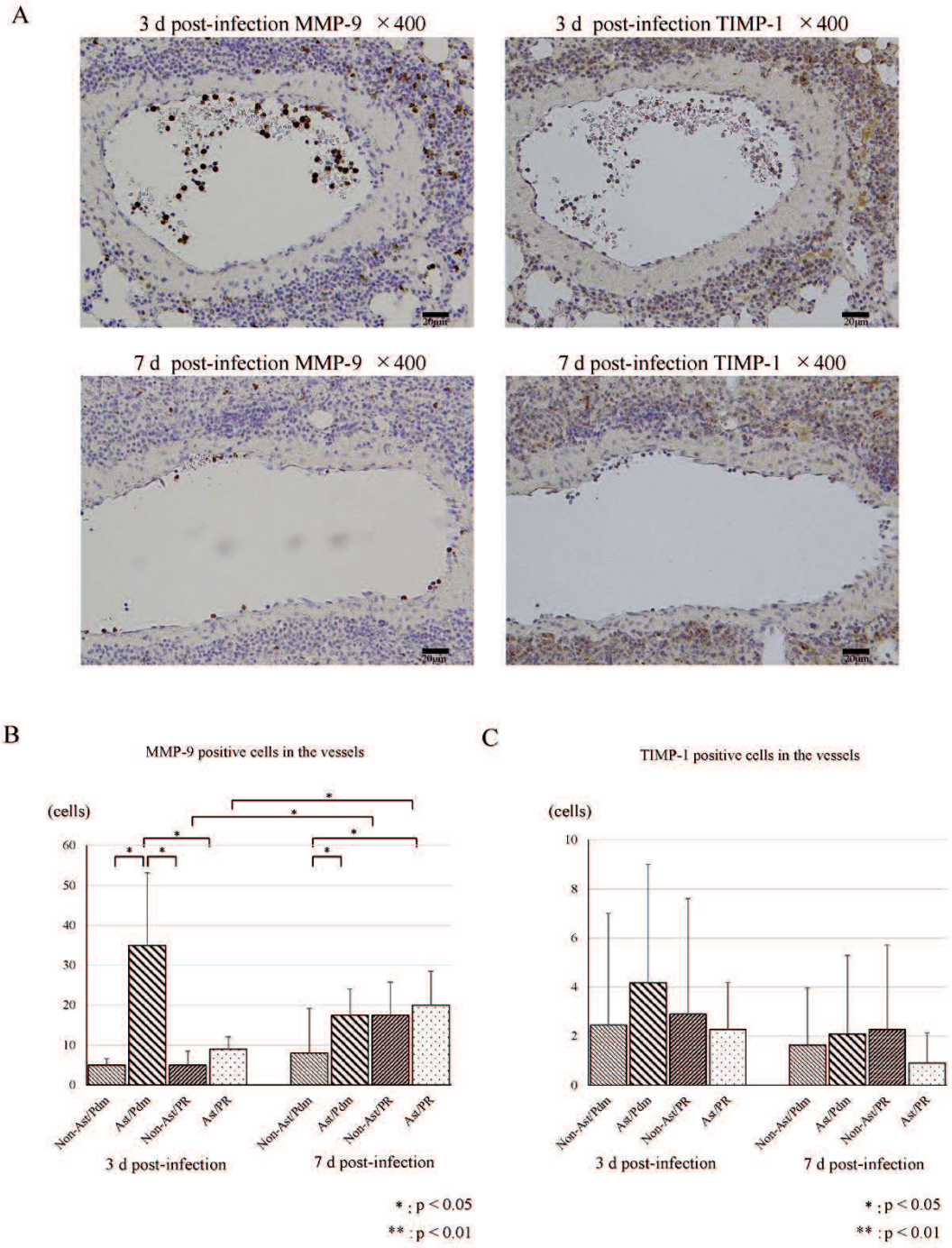


Figure 3. (A) Immunohistochemical analysis of MMP-9 and TIMP-1 in blood vessels of mouse lung tissue on 3 and 7 d post-infection. Similar results were obtained for six independent mice from each group. Representative findings are shown. **(B)** MMP-9-positive cells in the blood vessels of lung tissue were counted on 3 and 7 d post-infection. Eight sites of typical findings were counted and graphed. *p < 0.05, **p < 0.01. **(C)** TIMP-1-positive cells in the blood vessels of lung tissue were counted on 3 and 7 d post-infection. Eight sites of typical findings were counted and the averages graphed. *p < 0.05, **p < 0.01. Error bars are indicating standard deviation.

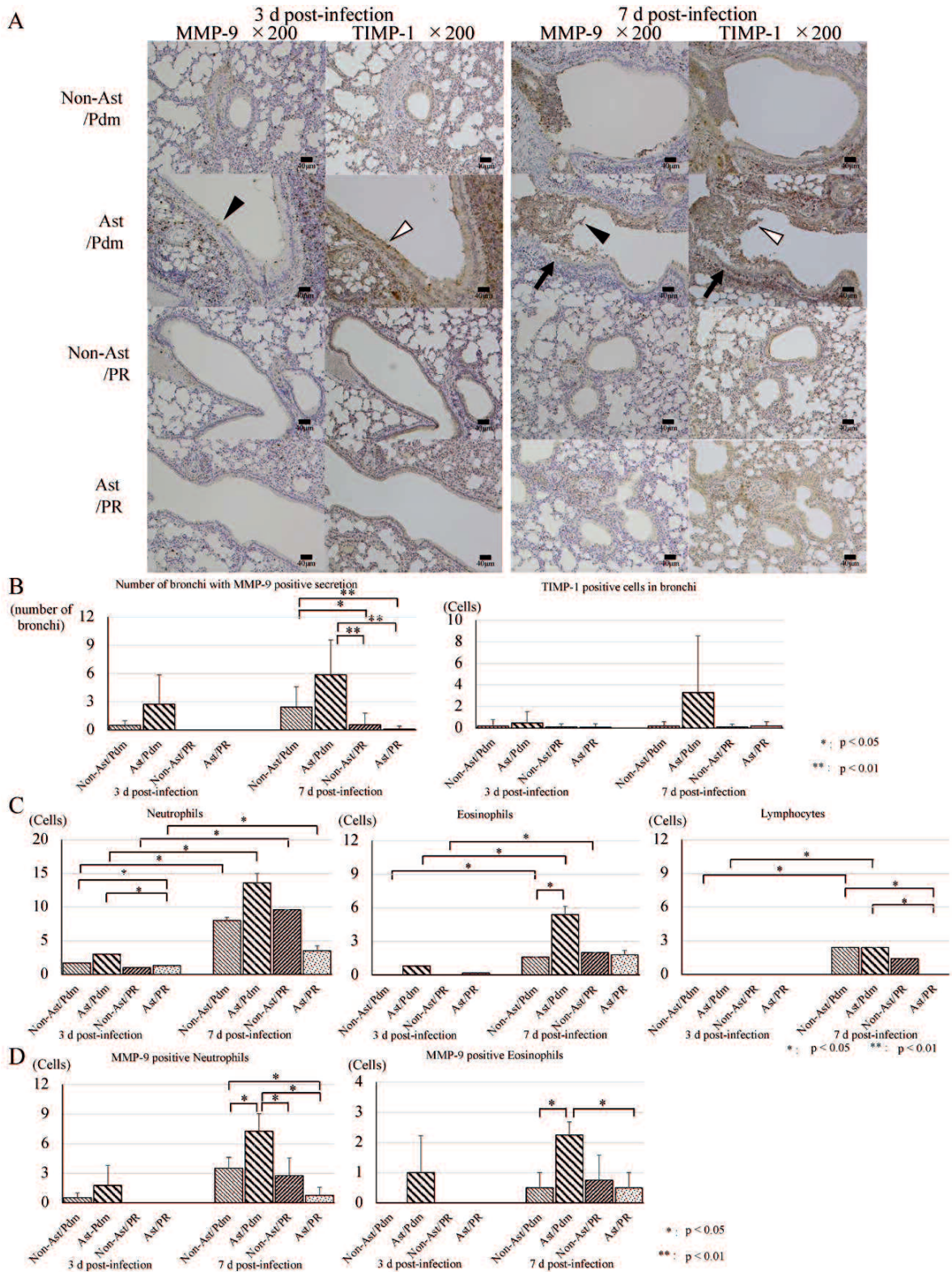


Figure 4. (A) Immunohistochemical analysis of MMP-9 and TIMP-1 expression in the bronchi of mouse lung tissue on 3 and 7 d post-infection. Also, we compared the secretion in the bronchi of mouse lung tissue on 3 and 7 d post-infection. Similar results were obtained for six independent mice from each group. Representative findings are shown. Black arrow heads indicate MMP-9-positive cells and white arrow heads indicate TIMP-1 expression at locations where the MMP-9 and TIMP-1 expression match. Black arrows indicate the epithelial cell shedding. **(B)** MMP-9-positive cells in the bronchi

with secretion were counted on 3 and 7 d post-infection. As the number of bronchi with secretion was small on 3 d post-infection, they were all counted. On 7 d post-infection, all or up to eight bronchi with secretion counted. The averages were graphed. *p < 0.05, **p < 0.01. TIMP-1-positive cells in the bronchi with secretion were counted on 3 and 7 d post-infection. As the number TIMP-1-positive cells in bronchi was small on 3 d post-infection, they were all counted. On 7 d post-infection, all or up to eight were counted. The averages were graphed. *p < 0.05, **p < 0.01. **(C)** Blood cell counts of neutrophils, eosinophils, and lymphocytes in bronchi were counted by lung tissue stained with haematoxylin and eosin. The visible nuclei were counted in eight representative sites and the averages graphed. *p < 0.05, **p < 0.01. Error bars are indicating standard deviation. **(D)** MMP-9 positive neutrophils and eosinophils in bronchi were counted. The visible nuclei were counted in eight representative sites and the averages graphed. *p < 0.05, **p < 0.01. Error bars indicate standard deviation.

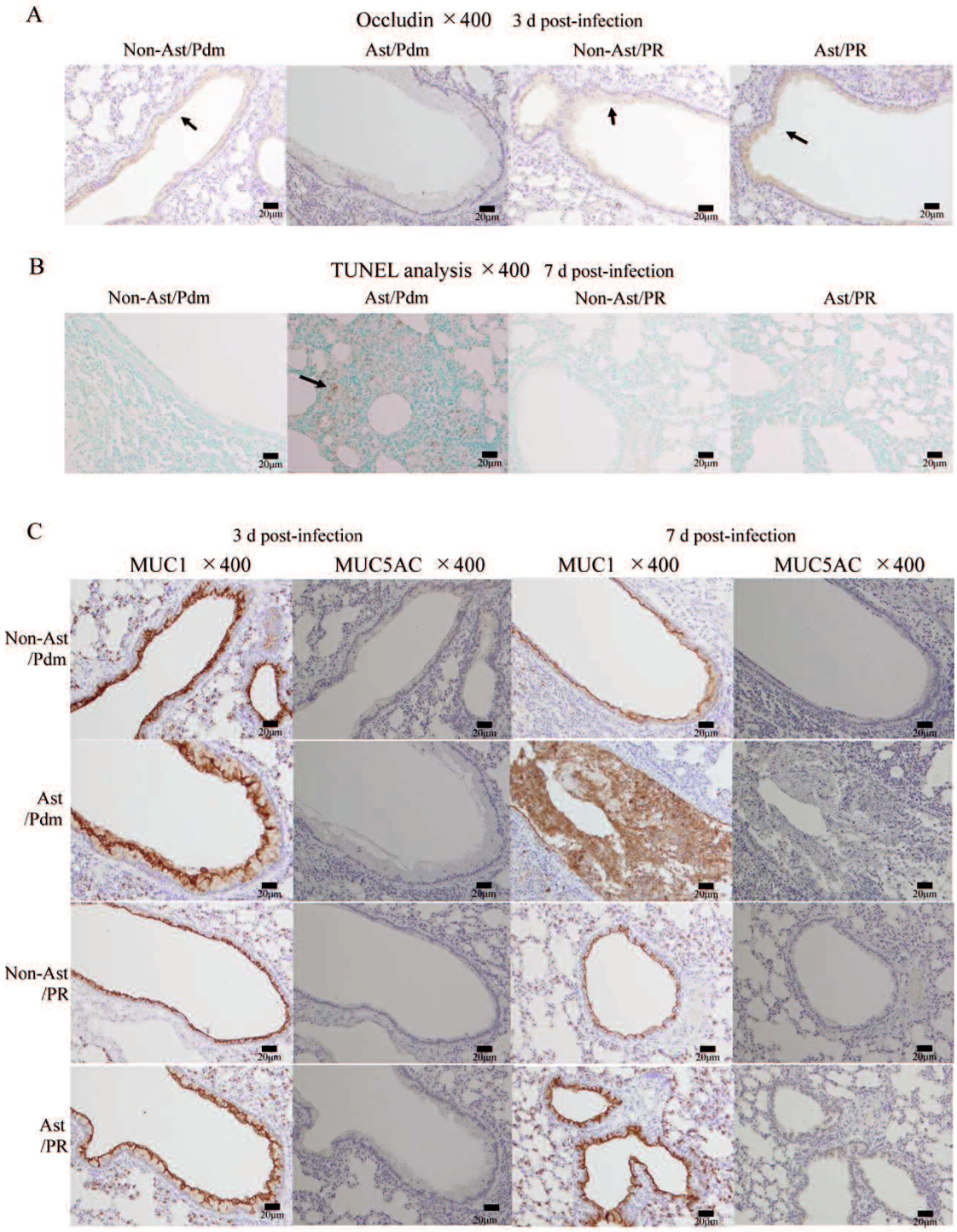


Figure 5. (A) Immunohistochemical analysis of occludin expression in the bronchi of mouse lung tissue on 3 d post-infection. Similar results were obtained for independent mice from each group. Representative findings are shown. Black arrow indicates the occludin-positive cells. Three samples were stained in each group. **(B)** TUNEL assay analysis at the site of pneumonia and bronchi on 7 d post-infection. Black arrow indicates the apoptosis of lung cells in the site of pneumonia. Three samples were stained in each group. **(C)** Immunohistochemical analysis of MUC1 and MUC5AC expression in bronchi on 3 and 7 d post-infection. Three samples were stained in each group. Representative findings are shown.

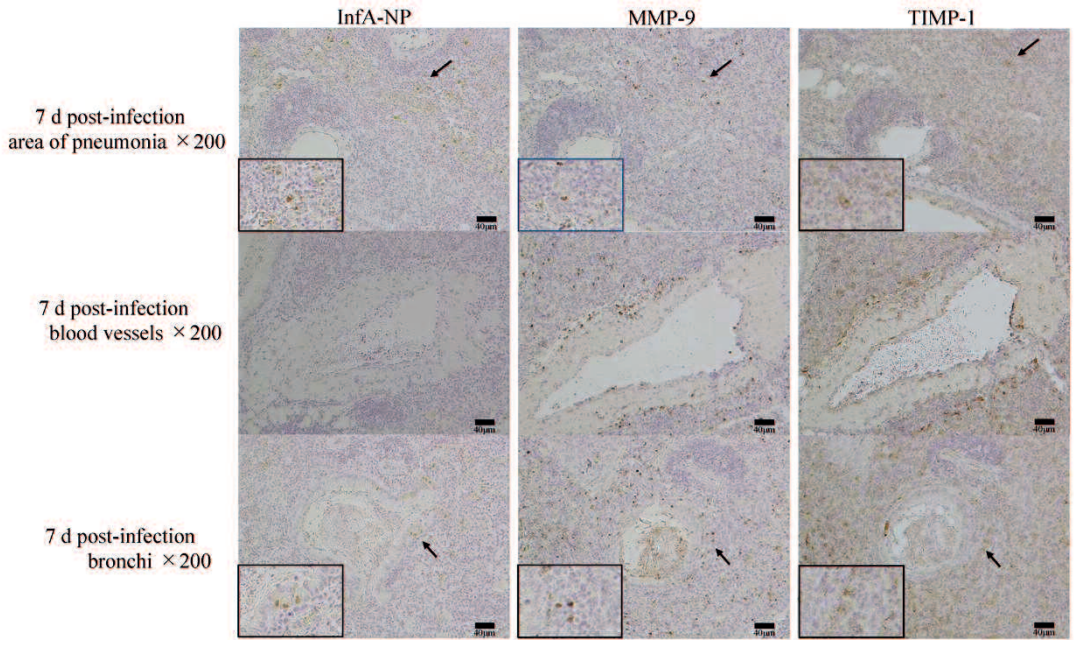
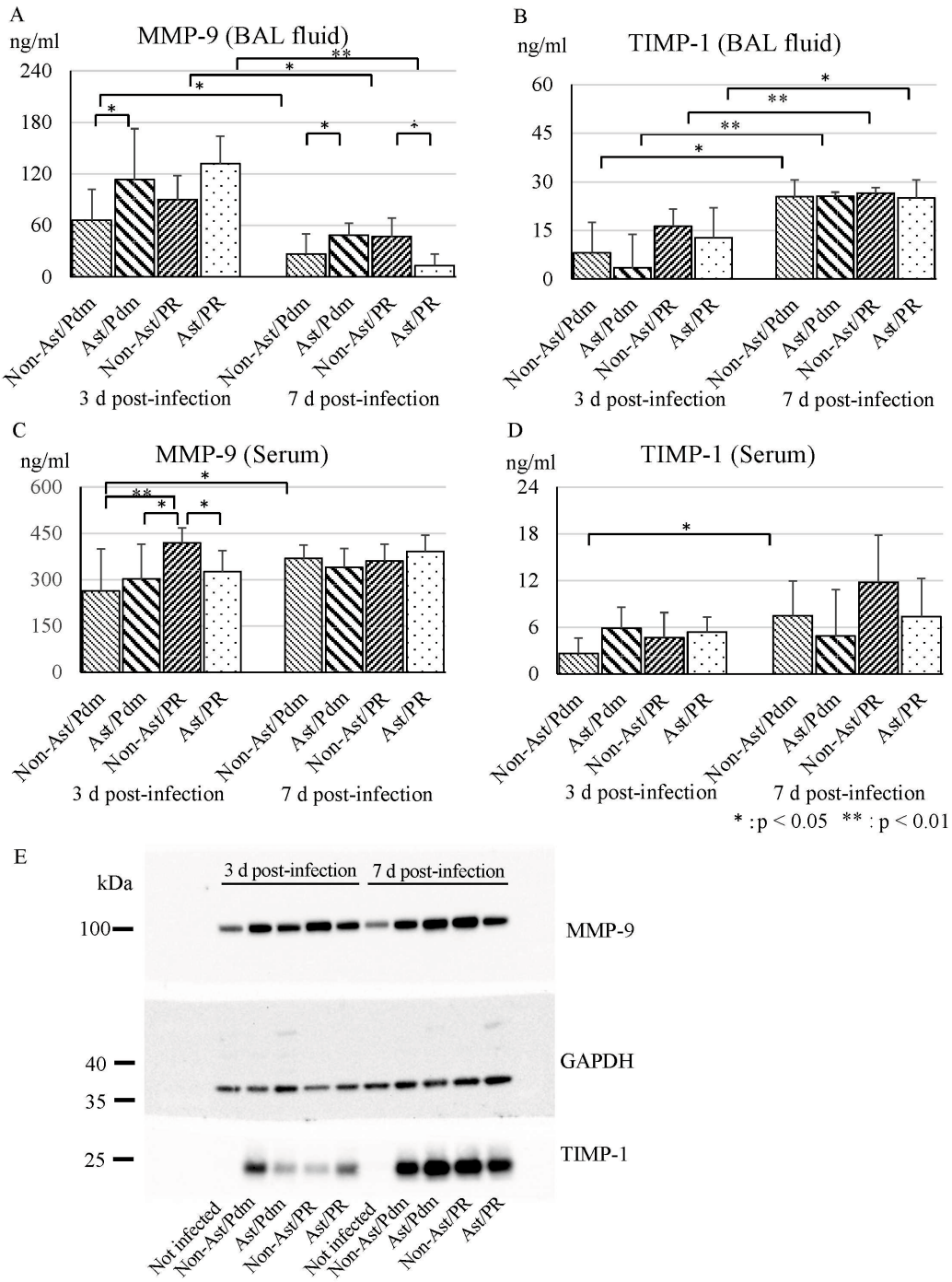
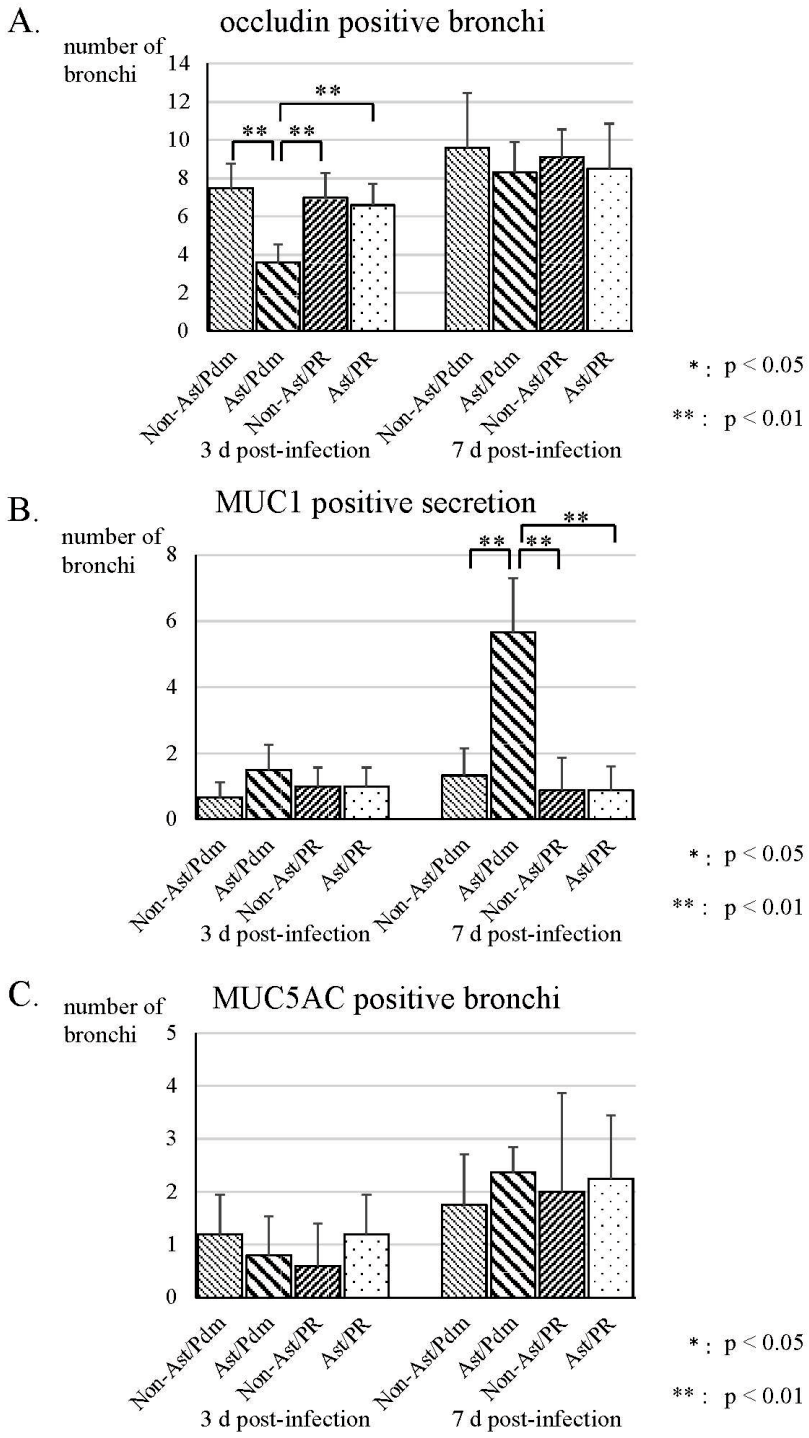


Figure 6. Immunohistochemical analysis of InfA-NP, MMP-9, and TIMP-1 expression in area of pneumonia, blood vessels, and bronchi on 7 d post-infection. The arrow indicates proximal co-expression of InfA-NP, MMP-9, and TIMP-1. Cells indicated by arrow are shown in the enlarge images as inner panels.



Supplemental Figure 1. (A-D) MMP-9 and TIMP-1 levels of bronchoalveolar lavage (BAL) fluid and serum from non-asthmatic and asthmatic mice infected with A(H1N1)pdm09 or A/PR/8/34 influenza A virus on 3 and 7 d post-infection. Non-Ast/Pdm: Non-Asthma/A(H1N1)pdm09-infected mice, Ast/Pdm: Asthma/A(H1N1)pdm09-infected mice, Non-Ast/PR: Non-Asthma/A/PR/8/34-infected mice, Ast/PR: Asthma/A/PR/8/34-infected mice. *$p < 0.05$, **$p < 0.01$. Error bars are indicating standard deviation. **(E)** MMP-9 (molecular weight: 102–105 kDa), TIMP-1 (molecular weight: 23 kDa), and GAPDH (molecular weight: 37 kDa) expression of western blotting from non-asthmatic and asthmatic mice infected with A(H1N1)pdm09 or A/PR/8/34 influenza A virus on 3 and 7 d post-infection. Since it was necessary to use different blocking solutions and immunoreaction enhancer solution, the membrane was cut according to the molecular weight to be detected.



Supplemental Figure 2. (A) Occludin positive bronchi in the area of pneumonia were counted on 3 and 7 d post infection. Using the bilateral lungs of three mice, six sites of bronchi were counted and the averages were graphed. *p < 0.05, **p < 0.01. Error bars are indicating standard deviation. **(B)** The number of bronchi with MUC1 positive secretions in the area of pneumonia were counted on 3 and 7 d post infection. Using the bilateral lungs of three mice, six sites of bronchi were counted and the averages were graphed. *p < 0.05, **p < 0.01. Error bars are indicating standard deviation. **(C)** MUC5AC positive bronchi in the area of pneumonia were counted on 3 and 7 d post infection. Using the bilateral lungs of three mice, six sites of bronchi were counted and the averages were graphed. *p < 0.05, **p < 0.01. Error bars are indicating standard deviation.

6. 考察

The results revealed that MMP-9 and TIMP-1 expression were more prominent in lung tissue of Asthma/A(H1N1)pdm09-infected mice than in that of Asthma/A/PR/8/34-infected mice. MMP-9 and TIMP-1 expression were detected in the perivascular and bronchial areas of the lung tissue. MMP-9 and TIMP-1 were strongly expressed, not only in bronchial epithelial cells, but also in bronchial secretion. Interestingly, neutrophils and eosinophils were observed at greater numbers in the bronchi of Asthma/A(H1N1)pdm09-infected mice than Non-Asthma/A(H1N1)pdm09-infected mice and A/PR/8/34-infected mice. MMP-9 and TIMP-1 expressions in the area of pneumonia and bronchi were found in close proximity to presence of Influenza A antigen.

Although a previous study using a mouse model of ovalbumin-induced asthma reported high levels of MMP-9 and TIMP-1 in BAL fluid[23], the immunohistochemical findings of current comparative study of Asthma/A(H1N1)pdm09-infected and Asthma/A/PR/8/34-infected mice showed that A(H1N1)pdm09-infected mice had more severe pneumonia and higher levels of MMP-9 and TIMP-1 expression in the lung tissue. These results suggest that the high expression of MMP-9 and TIMP-1 in Asthma/A(H1N1)pdm09-infected mice may be related to both the A(H1N1)pdm09 infection and the asthmatic conditions. The expression of TIMP-1 was also elevated, temporally following the expression of MMP-9, which is understandable in that TIMP-1 regulates the activity of MMP-9[24,25]. We speculate that the lagging rise in TIMP-1 relative to MMP-9 expression was in response to the MMP-9 expression.

Rojas-Quintero et. al. demonstrated high MMP-9 expression in bronchial epithelial cells of mice infected with pandemic influenza A virus A/California/07/2009[26], whereas we used strain A/Narita/1/09 in our study. Both A/California/07/2009 and A/Narita/1/09 induced high MMP-9 expression in epithelial cells of the bronchi, which may be one of the features of A(H1N1)pdm09-infected asthmatic mice. It has been reported that influenza A virus damages the barrier of the tracheal epithelium[27], and our study also revealed the expression of occludin, a constituent protein of tight junction was down-regulated, especially in Asthma/A(H1N1)pdm09-infected mice on 3 d post-infection. Moreover, using MMP-9 knockout mice infected with A/California/07/2009, Rojas-Quintero et al. showed that MMP-9 reduced E-cadherin expression[26]. Accordingly, reduction of occludin and E-cadherin could result in the release of adherent cells, leading to cell shedding and impaired function of the epithelial barrier resulting in epithelial and lung injury[26–28]. Therefore, A(H1N1)pdm09-infected asthmatic mice with increased expression of MMP-9 may suppress occludin and E-cadherin expression in epithelial cells, resulting in increased shedding of cells and increased secretion in the bronchi.

The presence of the influenza antigen, InfA-NP in the pneumonia and bronchi suggests that the bronchi may play an important role in A(H1N1)pdm09 infection of individuals with asthma. Since no viral antigens were found around the blood vessels, it is unlikely that influenza is directly involved in the expression of MMP-9 and TIMP-1 in the perivascular area, and the effect may be due to inflammatory cytokines transported hemodynamically from other sites. Moreover, influenza virus infection occurs in the brain with hematogenous route[29], suggesting that it causes complications hematogenously. Since we observed MMP-9 in the blood vessels, the blood vessels play a role in hematogenously spreading the inflammatory response caused by bronchus or pneumonia. Bronchial epithelia are susceptible to influenza infection and with increased viral burden can become apoptotic[30].

In our study, Asthma/A(H1N1)pdm09-infected mice showed epithelial cell shedding and secretions in the bronchial tubes compared to that of A/PR/8/34-infected mice. From the result of TUNEL assay, apoptosis was detected only in the Asthma/A(H1N1)pdm09-infected mice. The shedding of epithelial cells that filled the bronchial tubes may have resulted from tight junction fragility and apoptosis caused by the increased viral burden.

Furthermore, one of serious complications of A(H1N1)pdm09 infection is plastic bronchitis, which is known to involve the degeneration of bronchial epithelial cells, fibrin, mucin, and a large number of neutrophil and eosinophil[31]. The composition of the intratracheal contents identified in our study included tracheal epithelial cells, neutrophils, and eosinophils, as well as secretions. Mucin was previously reported to be present in the secretions of patients infected with influenza A virus[20], and limits the severity of influenza infection[32]. In this study, we identified that MUC1 was overexpressed in Asthma/A(H1N1)pdm09-infected mice with obstructing the bronchi and the components were similar to that of plastic bronchitis. Although we could not actually obtain dendritic material, the overexpression of mucin, obstruction of bronchi, and the coagulation of these contents may be associated with plastic bronchitis.

There were limitations on the current study. A technical limitation was that the slices of tissue used for MMP-9 and TIMP-1 staining were not perfectly matched as they were consecutive sections. However, the sites of MMP-9 and TIMP-1 staining were close enough to decipher the relationship between MMP-9 and TIMP-1 expressions. To compensate for this limitation, we included an assessment of the number of positive cells for MMP-9 and TIMP-1 expression. Second, it was not possible to precisely identify which kinds of cells produced MMP-9 or TIMP-1 in this study as the individual staining did not perfectly match. However, macrophages and lymphocytes at the site of pneumonia, neutrophils and lymphocytes in the blood vessels, and neutrophils and epithelial cells in the bronchi were observed around the MMP-9-positive cells. We speculate that these were the main cells responsible for producing MMP-9.

7. 結語

In conclusion, we compared the MMP-9 and TIMP-1 expressions in A(H1N1)pdm09 and A/PR/8/34 infections with asthma as an underlying disease. Severe lung pathological findings were related to high levels of MMP-9 and TIMP-1 expression in lung of mice with A(H1N1)pdm09 infection and asthma. The results of immunohistochemical examination suggest that the expression of MMP-9 in the bronchi observed in asthma model mice infected with A(H1N1)pdm09 may be relate to epithelial cell shedding, which may be a trigger for plastic bronchitis.

8. 謝辞

Inokuma Award from pediatric research in Yamaguchi supported this study.
We thank to Yuichiro Shirafuta (Obstetrics and Gynecology), Kenzo Ikemoto (Pathology), Midori Wakabayashi, Yoko Mori, and Takako Waniishi (Pediatrics) in Yamaguchi university for technical support.

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