Original Article

Pathological Examination of Lung Tissues in Influenza A Virus-Infected Mice

Nilton Akio Muto[1], Yuji Sunden[2], Tomoe Hattori[3,4], Daisuke Fujikura[5], Yosuke Nakayama[6], Tadaaki Miyazaki[6,8], Mitsuo Maruyama[7], Takashi Kimura[1], and Hirofumi Sawa[1,8]*

[1]Division of Molecular Pathobiology, [3]Division of Bioresource, and [5]Division of Infection and Immunity, Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020; [2]Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818; [4]Graduate School of Life Science, Hokkaido University, Sapporo 060-0810; [6]Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815; [7]National Center for Geriatrics and Gerontology, Obu-city 474-8511; and [8]Global COE Program, Hokkaido University, Research Center for Zoonosis Control, Sapporo 001-0020, Japan

SUMMARY: This study examined pathological changes in the lung tissues of young and aged mice infected with influenza virus. Young mice inoculated with influenza virus showed body weight loss at 4 days post-infection (dpi), meanwhile body weight decrease started from 9 dpi in the aged mice. We histopathologically examined the lungs of these mice. Immunohistochemical examination revealed that viral antigen-positive bronchiolar and alveolar epithelial cell numbers at 3 dpi were significantly higher in young mice than in the aged ones. Further, viral antigen-positive cells were observed at 9 dpi in the aged mice, but not in the young ones. Diffuse and severe bronchointerstitial pneumonia characterized by the accumulation of polymorphonuclear leukocytes (PMNs) was observed in young mice at 6 dpi. Histopathological changes in the aged mice were milder than those in the young mice. Moreover, T cell and macrophage accumulation in the lungs was significantly higher in the young mice than in the aged mice at 9 dpi. These results suggest that there may be a correlation between the relatively low level of infiltration of PMNs, macrophages, and T lymphocytes and the delayed body weight loss and longer lasting infections observed in the lungs of the aged mice. These findings provide detailed insights into the age-specific course of infection in young and aged populations with associated differences in lung pathology.

INTRODUCTION

Influenza epidemics seriously affect people of all age groups annually, however, elderly individuals aged above 65 years are at the highest risk of complications (1). When compared with young adults, elderly individuals have diminished ability to protect themselves from an infection (2) and, consequently, have a greater risk of acquiring several respiratory infectious diseases and tend to experience severe and longer lasting infections (1,3). An age-associated decline in immunity is implicated as the primary cause of these problems. The decline or functional change in the immune system with aging includes changes such as decreased T-cell proliferation and repertoire diversity (4). Alterations in immune responses, including altered cytokine secretion patterns and reduced antibody production, may also contribute to increased susceptibility to respiratory infections;

however, these factors have been poorly characterized in the elderly (5). Changes in immune parameters, such as T-cell subsets and immunoglobulin concentrations, in respiratory secretions have been compared in older, healthy individuals, and younger subjects; however, the significance of these changes for protective immunity in the lungs is unknown (6). In the case of influenza virus infection, age-related changes in the effect of vaccination have been studied previously (3,7). However, immune response to virulent virus infection in aged animal models is less well characterized. A previous study describing impaired immune responses in the lungs of aged mice (8) heightened our interest in examining the lung pathological changes in aged mice infected by influenza virus.

In the present study, we assessed pathological changes—in particular, acute inflammatory infiltrates in lung tissues—in aged and young C57BL/6 mice after inoculation with influenza virus A/PR/8/34 (H1N1). Our results suggest that there are several differences between inflammatory cells involved in the immune response against influenza virus infection in young and aged mice and that these differences might affect influenza virus clearance from lung tissues and immune-mediated pathology induced by infection.

*Corresponding author: Mailing address: Division of Molecular Pathobiology, Research Center for Zoonosis Control, Hokkaido University, N20, W10, Kita-ku, Sapporo 001-0020, Japan. Tel: +81-11-706-5185, Fax: +81-11-706-7370, E-mail: h-sawa@czc.hokudai.ac.jp

MATERIALS AND METHODS

Inoculation of mice with influenza virus A/PR/8/34 (H1N1): In the present experiments, 2–4-month-old and 25–27-month-old C57BL/6 mice were selected as representative groups of young and aged mice, respectively. Survival rates and changes in body weight were assessed in 33 mice (young group, $n = 12$; aged group, $n = 13$; young and aged phosphate-buffered saline [PBS]-inoculated mice as controls, $n = 4$, respectively). Both groups of mice were anesthetized by isoflurane inhalation and were inoculated intranasally with 25 μl of viral solution (A/PR/8/34 H1N1) in each nostril. The mice were then challenged with a dose of 1,000 plaque-forming units (pfu) in 50 μl PBS. After inoculation with the virus, both groups were monitored daily for eventual weight loss (morbidity), which is a parameter of disease severity for influenza virus infection. Animal experiments were performed according to the Guidelines for Animal Experimentation at Hokkaido University. The influenza virus inoculation experiments were performed in a P2 biosafety level experiment room.

Viral load quantification using lungs of infected mice: After euthanization of the mice (young, $n = 4$; aged, $n = 4$; PBS-inoculated mice as negative controls, $n = 2$ for both groups) with isoflurane overdose, whole lung lobes were harvested at 3 days post-infection (dpi) to compare the viral load. The minced lungs were homogenized (1:2 dilution of PBS in 1% bovine serum albumin [BSA]) in a blender with a pestle (Nippi, Tokyo, Japan), followed by ultrasonication (200 W; 6 bursts each of 10 s) at 4°C. The centrifuged supernatant was serially diluted and overlaid on MDCK cells grown in 12-well dishes. Thereafter, the cells were incubated at 37°C in 5% CO_2 for 48 h. After incubation, the cells were then fixed with methanol and stained with 1% crystal violet in 20% ethanol. Virus plaques were counted, and the plaque forming units per ml (pfu/ml) were determined using the formula: (number of plaques × dilution factor)/0.1 ml.

Histopathological and immunohistochemical examinations: The lung tissues excised at 3, 6, and 9 dpi ($n = 4$ for each time point in both groups; $n = 1$ for each respective PBS-treated negative control) were stored in 20% neutral buffered formalin. After fixation for at least 1 day, the lungs were sectioned transversely both at the level of the proximal intrapulmonary main axial airway near the hilus and at the distal intrapulmonary axial airway. All the slices of lung tissues were embedded in paraffin for histological sectioning (9–11), and serial 5-μm sections were cut and mounted on glass slides for pathological and immunohistochemical examination. The sections were stained with hematoxylin and eosin (H&E) to assess general histopathology. Immunohistochemical staining was performed using paraffin-embedded sections, in accordance with the manufacturer's instructions using the following antibodies: rabbit polyclonal anti-CD3 antibody (0.05 mol L^{-1}; Dako, Glostrup, Denmark) to T lymphocytes; rat monoclonal CD45R Ab (1:1000, clone: RA3-6B2; BD Pharmigen, San Jose, Calif., USA) to B lymphocytes; rat monoclonal F4/80 Ab (1:1000, clone Cl:A3-1; Serotec, Oxford, UK) to lung macrophages; rat monoclonal Ly-6G/Ly-6C (Gr-1) (1:1000, clone: RB6-8C5; BioLegend, San Diego, Calif., USA) to polymorphonuclear leukocytes; and rabbit polyclonal anti-influenza virus antigen (IFV-Ag) hyperimmune serum (1:5000; Comparative Pathology Laboratory, Hokkaido University, Sapporo, Japan) to nuclear viral antigens (12).

For immunohistochemical staining, deparaffinized sections were treated with 20 μg ml^{-1} of proteinase K or 0.1% trypsin for 15 min at room temperature (RT) with antibodies Gr-1 or CD3, respectively, to increase the antigen binding affinity. The sections were then washed 3 times for 5 min in 0.01 M PBS (pH 7.4). Antigens were retrieved by heat treatment for 5 min at 97°C in 0.05 M sodium citrate buffer (pH 6.0). After cooling and washing in PBS, endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 20 min at RT. The sections were then washed in PBS and incubated for 30 min with normal serum (Nichirei, Tokyo, Japan) at RT and overnight with primary rabbit polyclonal antibodies (CD3 and IFV-Ag) or primary rat monoclonal antibodies (CD45R, F4/80, and Gr-1) at 4°C in a humidified chamber. The next day, the sections were incubated for 30 min at RT with a secondary antibody labeled with biotin and then with horseradish peroxidase-conjugated streptavidin (Histofine SAB-PO kit; Nichirei). Immunoreactivity was detected using a 3,3′-diaminobenzidine (DAB) substrate. We regarded only strong dark-brown signals as positive when compared to the control slides for each antibody. Nuclei were counterstained by hematoxylin or methyl green, and the stained slides were observed by performing light microscopy (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay: The TUNEL method was used to examine the apoptotic cells. In brief, 5 μm-thick lung sections were prepared, deparaffinized, and stained with 0.5 μM terminal deoxynucleotidyl transferase (TdT) (Invitrogen, Grand Island, N.Y., USA) and 10 μM biotin-16-dUTP (Roche, Basel, Switzerland) in TdT buffer for 90 min at 37°C. We randomly selected 10 high-power fields for each section, and the number of TdT-labeled cells was counted. Nuclei were counterstained with methyl green.

Statistical analyses: Data from quantitative analyses were expressed as mean ± SE (for young and aged mice). Statistical analyses were performed using Student's t test to compare significant differences between young and aged mice; a single-factor analysis of variance (ANOVA) was performed to evaluate changes over time within the same group. A P value of <0.05 was considered to be statistically significant.

RESULTS

Clinical course and detection of influenza virus infection in mouse lungs: We inoculated young ($n = 12$) and aged ($n = 13$) mice intranasally with influenza virus (1,000 pfu/50 μl). To evaluate differences in body weight change, we compared the percentage body weight loss in each group of mice until 12 dpi (Fig. 1A). Young mice inoculated with influenza virus showed body weight loss after 4 dpi, ruffled fur, and increased respiratory rate. Out of the 12 mice in the young group, 7 (58%) had died by 12 dpi, and the remaining 5 survived (Fig. 1B). On the other hand, decline in mean
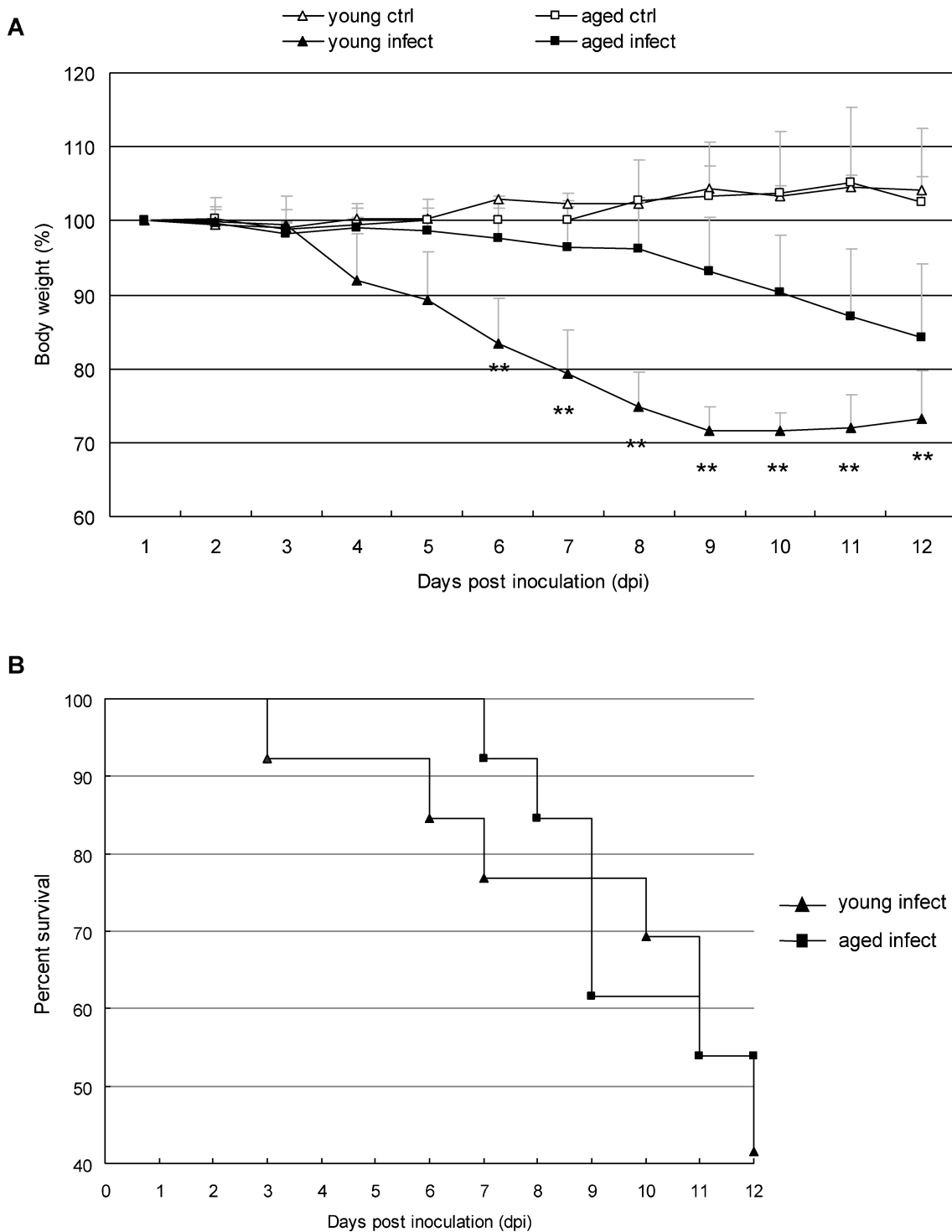
A

B

Fig. 1. Body weight change and survival rate of infected mice inoculated with influenza virus A/PR/8/34 (H1N1). (A) Body weight change of mice inoculated with influenza virus A/PR/8/34 (H1N1). The influenza virus was intranasally inoculated into both young ($n = 12$) and aged ($n = 13$) mice (Infect 1,000 plaque-forming units/mouse). The data represent the mean percent $\pm$ SE. As a control (Ctrl), PBS-treated young ($n = 4$) and aged ($n = 4$) mice were used. Statistical significance was determined by Student t test (**$P < 0.005$, infected young mice versus infected aged mice). (B) Survival rate of infected mice with influenza virus A/PR/8/34 (H1N1). No statistical difference was observed until 12 dpi between young and aged group, however, the aged mice had a relative higher survival rate until 7 dpi.

body weight started from 9 dpi in the aged group (Fig. 1A), and 6 out of 13 mice (46%) had died by the 12 dpi (Fig. 1B). The delayed clinical course observed in aged mice suggests that different respiratory pathogenesis may be involved in influenza virus infection in the young and aged mice. In PBS-inoculated control mice ($n = 4$ for both groups), no significant clinical sign or body weight change was recorded. On the basis of these findings, we attempted to compare the lung histopathology of young and aged mice inoculated with influenza
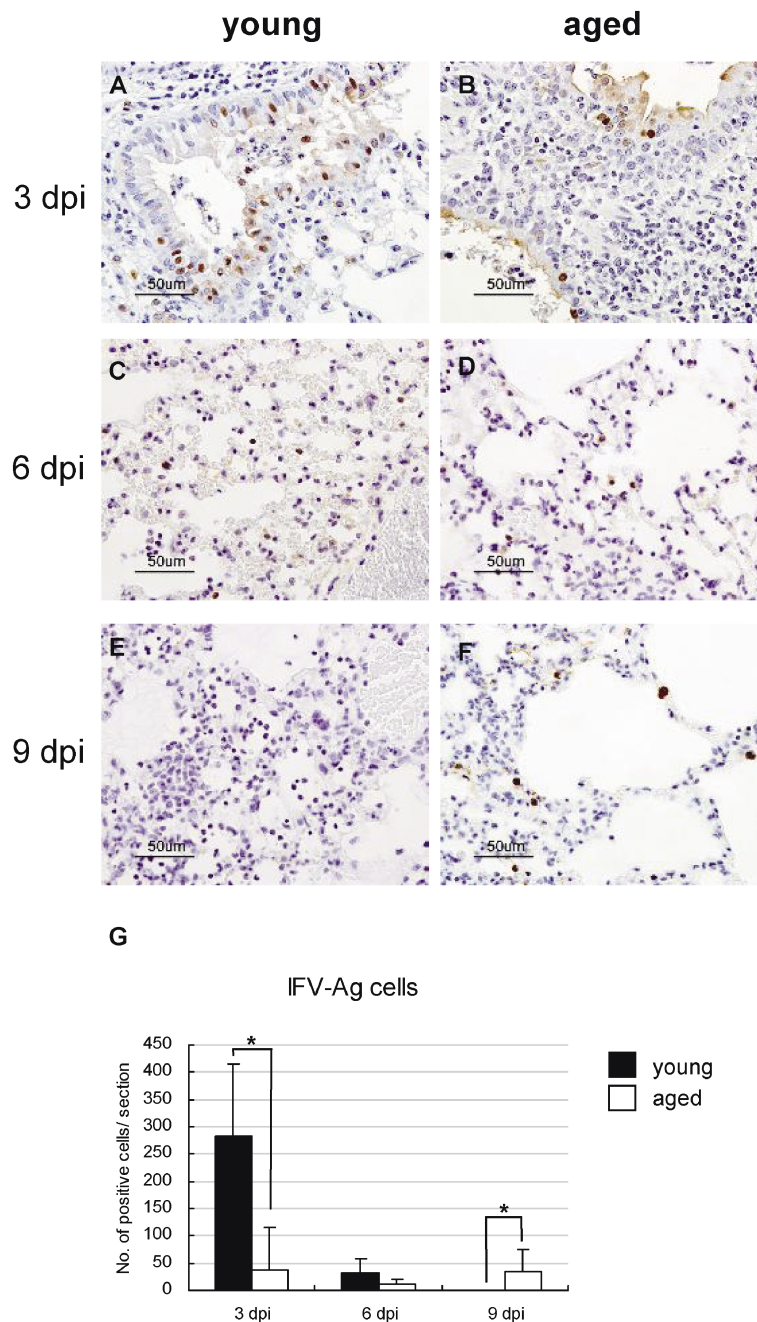
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Fig. 2. Detection of influenza viral antigen (IFV-Ag) in the lung tissues of infected mice at 3, 6, and 9 dpi. Immuno-histochemical staining for influenza antigen. (A) Many virus-positive epithelial cells were observed in the bronchi-oles in the lungs of the young mice, but (B) fewer epithelial cells were positive in the aged mice lungs. (C–D) Posi-tively stained cells were diffusely scattered in the lung parenchyma, (E) no viral antigen positivity was seen in the young mice lungs, (F) but positive cells were still detected in the aged mice at 9 dpi. (G) Positive signals were count-ed for each section and are represented as bar graphs. Statistical significance was determined by Student t test (*P < 0.05, infected young mice versus infected aged mice).

virus.

Expression of IFV-Ag in lungs of the virus-infected mice was evaluated by immunohistochemistry. The number of IFV-Ag-positive cells in the tissue sections was counted under a microscope. When compared to the aged mice, greater numbers of IFV-Ag-positive cells were detected in the bronchial and bronchiolar epithelium of the young mice at 3 dpi (Fig. 2A, B, and G). No significant difference was observed in the number of IFV-Ag-positive cells in young and aged mice at 6 dpi (Fig. 2C and D), because the number of IFV-Ag-posi-

tive cells in the lung tissues of young mice was markedly decreased at 6 dpi when compared to that at 3 dpi (Fig. 2A, C, and G). At 9 dpi, IFV-Ag-positive cells were de-tected in the aged mice (Fig. 2F and G) but not in the young mice (Fig. 2E and G). The lung viral load was as-sessed by plaque titration of infected lung tissue at 3 dpi. The viral titer in the young mice (4.5 ± 5.2 × 10^8 pfu ml^{-1}; n = 4) was almost 10 times that in the aged mice (6.1 ± 1.2 × 10^7 pfu ml^{-1}; n = 4); no significant difference was observed. Although there is no sig-nificant difference, the higher viral titers in the young

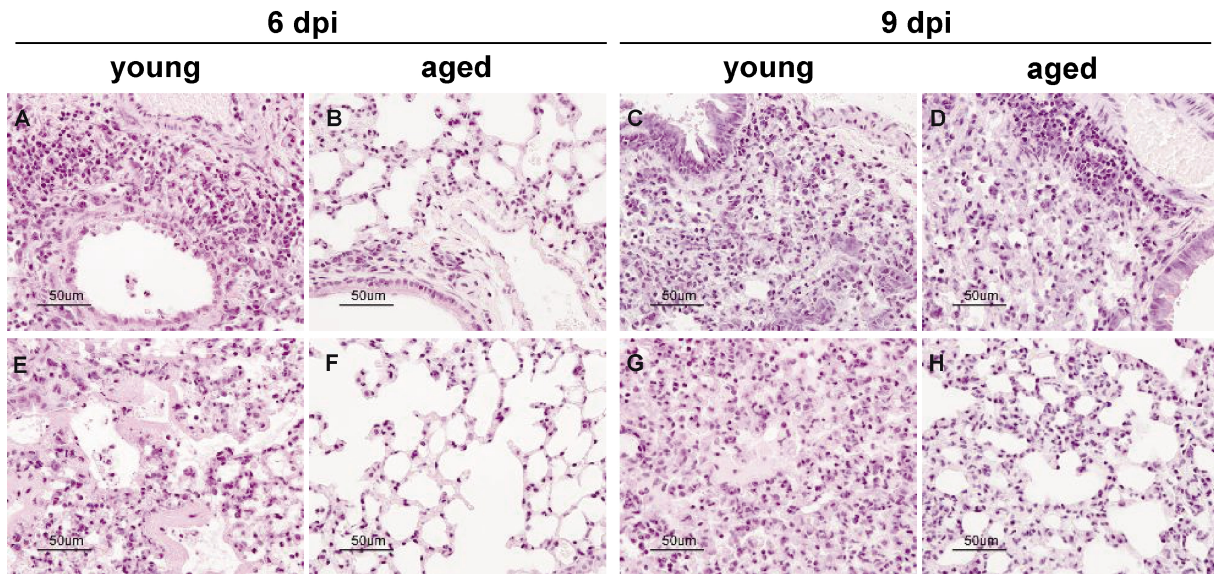|	6 dpi		9 dpi	
	young	aged	young	aged

Fig. 3. Photomicrographs of the lung sections of influenza A virus-infected young and aged mice. Distinct patho-
logic signatures were observed between young and aged mice at 6 and 9 dpi in peribronchial and perivascular
region (A–D) and lung alveoli (E–H). H&E staining showed: (A) severe peribronchial and perivascular infiltration
of granulocytes, lymphocytes, macrophages, (B) mild peribronchiolar inflammatory cell infiltration with intact
bronchiolar epithelium, (C) pulmonary edema with peribronchiolar inflammation, (D) dense mononuclear in-
filtration in the peribronchial and perivascular interstium, (E) lung alveoli with edema and hyaline membrane for-
mation, (F) no apparent alveoli damage with no infiltration of inflammatory cells, (G) alveolar edema with migra-
tion of inflammatory cells in inter-alveolar septa, and (H) alveolar thickening, still preserved air space.

mice appear to correlate with the differences in the num-
ber of IFV-Ag-positive cells in the young and the aged
mice at 3 dpi (Fig. 2G). These results are consistent with
the findings of previous studies in which plaque forma-
tion assay was used to demonstrate that virus produc-
tion was reduced in aged mice in comparison to that in
adult mice, with a longer period of infection until 9 dpi
in the aged mice (8).

Histopathological changes in mice lungs: Next, we ex-
amined the lung histopathology of young ($n = 8$) and
aged ($n = 8$) mice inoculated with influenza virus at 6
and 9 dpi, along with PBS-inoculated mice ($n = 1$) for
each respective negative control (not shown). Severe
peribronchial, perivascular, and interstitial accumula-
tion of inflammatory cells; congestion; and intra-alveo-
lar edema with hyaline membrane formation were clear-
ly evident in the lungs of young mice at 6 and 9 dpi (Fig.
3A, C, E, and G). Edema and interstitial inflammation
were milder in the lungs of aged mice than in those of
the young mice (Fig. 3B, D, F, and H versus 3A, C, E,
and G). In addition, alveolar air space in the lungs was
more preserved in the aged mice than in the young mice
(Fig. 3F and H versus 3E and G). Until 9 dpi, no diffuse
alveolar damage (DAD) was observed in the aged mice.

**Topological distribution of inflammatory cells in
mouse lungs:** To evaluate possible differences in early
inflammatory response between young and aged mice,
we initially compared the number of polymor-
phonuclear leukocytes (PMNs) in lung tissues. The
number of PMNs was significantly higher in young mice
than in aged ones at 6 dpi (Fig. 4A, B, and 5A). PMNs
were not detected in either of the groups at 9 dpi (Fig.
4C, D, and 5A). Earlier studies have indicated that mac-
rophages and PMNs play a significant role in preventing
influenza virus propagation in the lungs from the early

to plateau phase following primary pulmonary infection
with the virus (13,14). Therefore, we examined the num-
ber of macrophages in the lung tissues of both groups of
mice. The number of macrophages infiltrating the inter-
alveolar septa at 9 dpi was much higher in the young
mice than in the aged ones (Fig. 4G, H and 5B). There
were fewer macrophages at 6 dpi in both the groups,
and no significant difference was observed between the
adult and aged mice at this point (Fig. 4E, F, and 5B).

We then examined the number of T and B lympho-
cytes in the lungs of both the groups. By 6 dpi, the num-
ber of T cells that had infiltrated the lungs of young
mice was comparable to that in the aged mice (Fig. 4I,
J, and 5C). The number of T cells in the lungs of young
mice had increased by 9 dpi; no such increase was ob-
served in the aged mice (Fig. 4K, L, and 5C). The num-
ber of B cells in the lungs of aged mice was higher than
that in those of young mice at 6 dpi (Fig. 4M, N, and
5D). This number had increased significantly in young
mice when compared to aged mice by 9 dpi (Fig. 4M–P
and 5D). B cells were observed mainly in clusters sur-
rounding the peribronchial region; fewer cells were scat-
tered in the inter-alveolar septa (Fig. 4M–P).

According to previous studies (15,16), apoptosis of
lung alveolar epithelial cells and inflammatory cells un-
derlie alveolar injury and pulmonary edema, which are
hallmarks of lung injury or pneumonia following in-
fluenza A virus infection. Thus, we examined the
presence of apoptotic cells in the lung tissues of both
groups of mice using the TUNEL assay. The number of
apoptotic cells was higher in the lungs of young mice
than in those of the aged ones, but not significantly so,
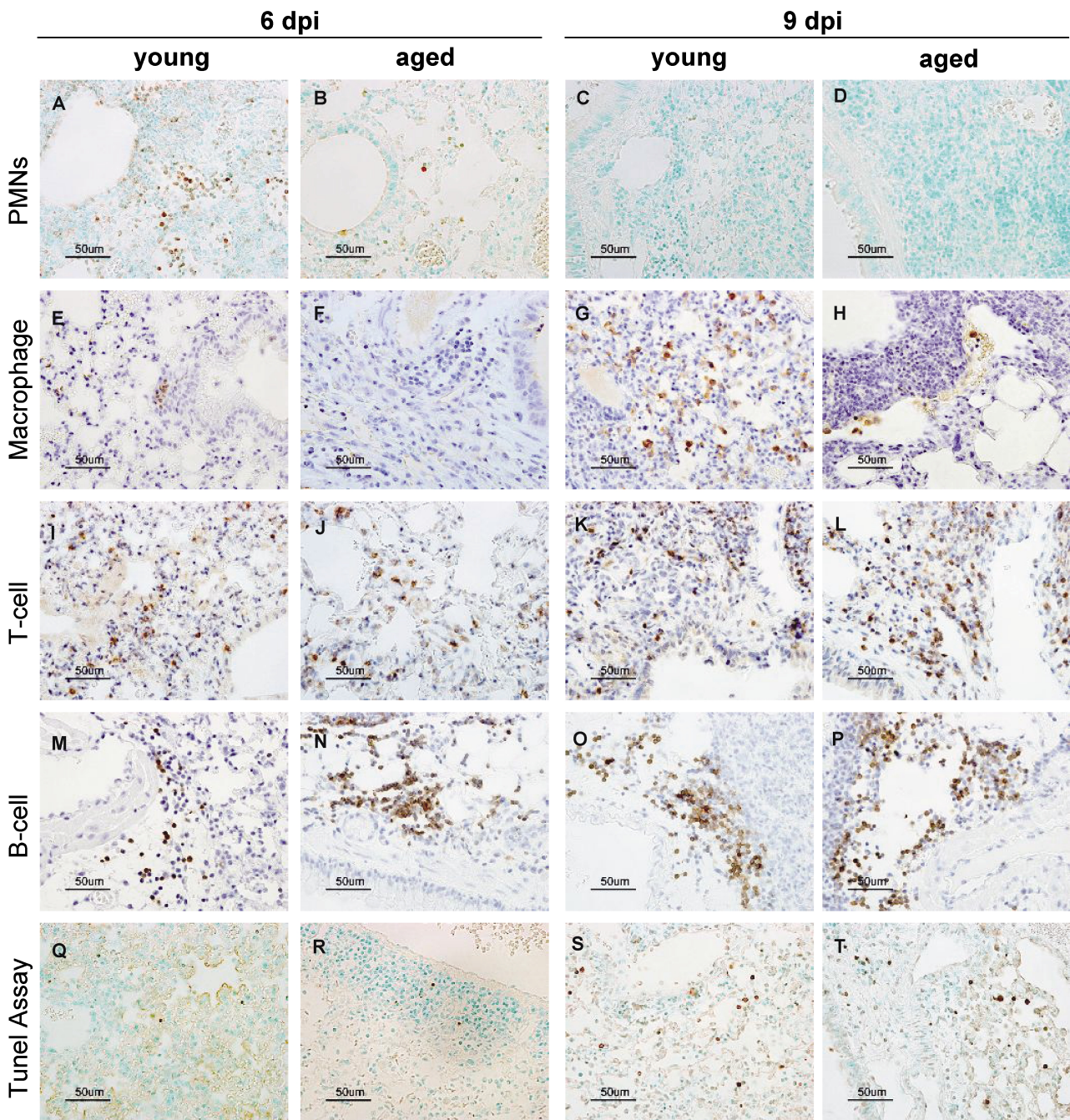at 6 and 9 dpi (Fig. 4Q–T and 5E).

Fig. 4. Topological distribution of inflammatory cells and apoptotic cells in young and aged mice after influenza A inoculation. Representative slides from lung sections of young and aged mice infected with influenza A at 6 and 9 dpi. (A–D) PMNs, (E–H) alveolar macrophages, (I–L) T lymphocytes, (M–P) B lymphocytes, and (Q–T) TUNEL assay for apoptosis. Positive cells were stained brown. Cells were counterstained with methyl green (A–D, Q–T) or hematoxylin (E–P). Sections from the spleen and thymus of unrelated mice were provided for each antibody as positive controls (data not shown). As a negative control, lung sections from non-infected mice were used (data not shown).

DISCUSSION

In the present study, we examined pathological changes in the lungs of young and aged mice after inoculation with influenza A/PR/8/34 (H1N1). In aged mice, delayed body weight loss appears to be related to low numbers of inflammatory cells during the early stages of infection with the influenza virus. Pathological changes induced by influenza virus infection were also delayed in aged mice, in accordance with the low num-

ber of IFV-Ag-positive cells detected at 3 dpi that persisted until 9 dpi. Although we did not perform virus isolation at 9 dpi, a previous study (8) indicated that the influenza virus could be recovered for a longer period of time in aged animals. In the young mice, the rapid body weight decrease appears to be related to acute lung injury induced by robust influenza virus infection, which triggers an early inflammatory response characterized by a high influx of inflammatory cells (PMNs and macrophages). This condition eventually progresses to acute
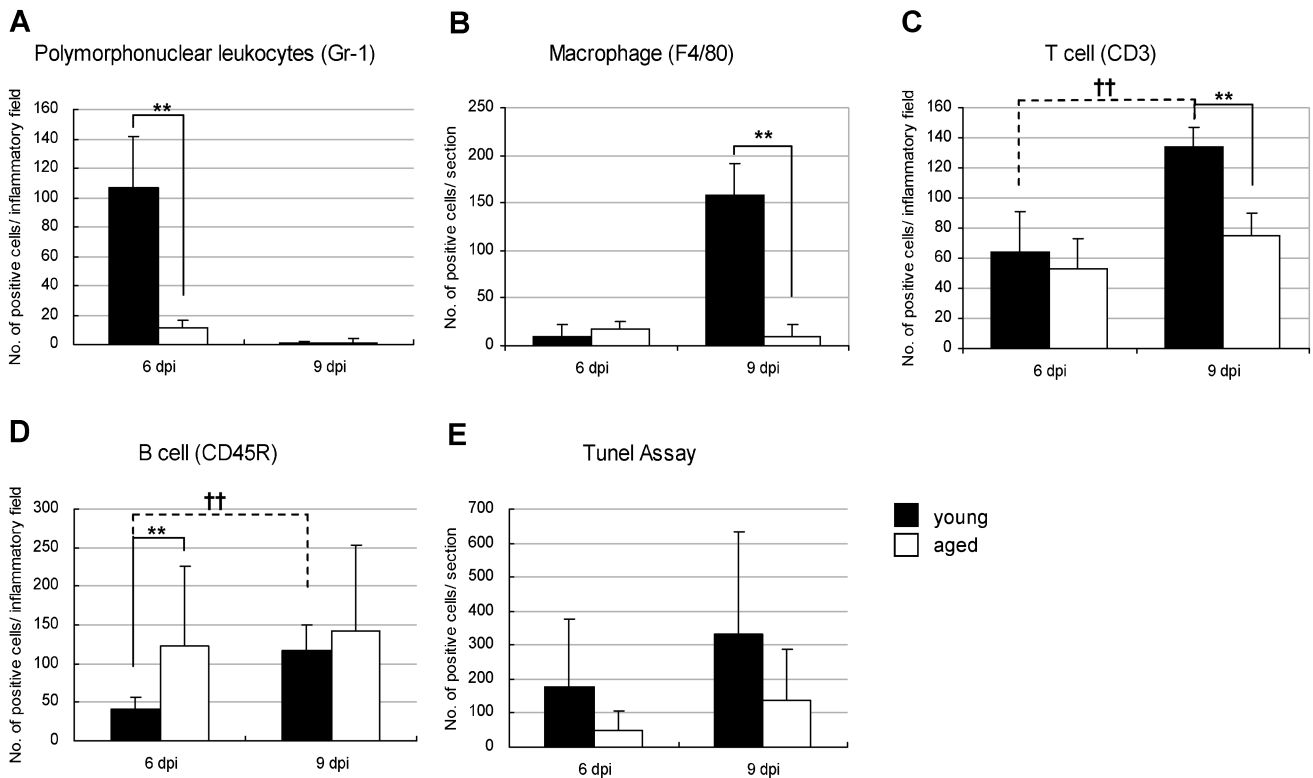
Fig. 5. The number of positive cells detected by immunohistochemical staining and TUNEL assay. (A) PMNs (rat mAb Gr-1), (B) macrophage (rat mAb F4/80), (C) T cell (rabbit pAb CD3), (D) B cell (rat mAb CD45R), and (E) TUNEL assay. The mean expression ($\pm$ SD) of each animal group (n = 4–6 mice per time point) is shown. Young (black bars) and aged (white bars) mice were intranasally inoculated with influenza virus. Statistical significance was determined by Student t test (**P < 0.005, young mice versus aged mice; black trace) and analysis of variance (one-way ANOVA, ††P < 0.005; dashed trace) for the young infected group.

respiratory distress syndrome (ARDS)-like pathological signs characterized by congested alveolar capillaries, pulmonary edema, hyaline membrane formation, and fibrin accumulation. These results are consistent with pathological findings in which ARDS was observed in mice with influenza A virus infection (17–19). In a previous study (8), both adult and aged mice showed a rapid decline in the body weight until 10 dpi. However, in our study, the delay in body weight loss and better survival rates in aged mice may be correlated to the role of host genetic background in susceptibility to influenza virus infection (20). It has been reported that antibody responses to influenza virus differed depending on the H-2 haplotype of the mouse strain (21). Furthermore, the association between a transient early protective response by CD8 T cell and interferon (IFN)-γ through Th1 cytokines in aged mice with respiratory infectious disease was reported (22,23). The present study also showed rapid body weight decrease in young mice and delayed body weight decrease and viral clearance in aged mice, suggesting that similar transient and mild immune responses might be involved in the lungs of aged mice. Taken together, these two general findings are summarized as follows: first, the time course for body weight loss was identified in young and aged mice; second, the delayed virus clearance was observed in aged mice, unlike that observed in the young mice, with rapid replication and subsequent elimination of the virus.

Alveolar macrophages and/or mononuclear phagocytes might effectively cooperate with PMNs in virus

elimination in the presence or absence of antibodies, although the presence of large numbers of these inflammatory cells can also exacerbate clinical symptoms and contribute to potentially lethal lung pathology (14,24). Our results confirmed that the significantly higher accumulation of PMNs and lung macrophages in young mice than in aged ones is related to viral clearance, which is the first line of defense of the innate response, as well as to the pathogenic effects of excessive neutrophils that occur in acute lung injury associated with influenza pneumonia. In contrast, small number of PMNs and alveolar macrophages was observed at the site of infection in the aged infected mice, with mild pathological changes of the lungs occurring during the early stages of infection (Fig. 3B, 3F, 4B, and 4F). Although we used histopathological and immunohistochemical techniques in our study, a previous report used flowcytometry analysis to demonstrate that there was a delay in the infiltration of granulocytes and dendritic cells, without any obvious difference in the infiltration of macrophages in lung cells isolated from aged mice (8).

In the present study, cytokine and chemokine levels were not measured. It has been reported that cytokines and chemokines produced by macrophages and dendritic cells showed higher concentrations of interleukin (IL)-12$_{p70}$ in the lungs of adult mice than those of the aged mice at 5 dpi, while tumor necrosis factor (TNF)-α and IL-1α continued to increase in the aged mice, with a higher concentration at 7 and 9 dpi, possibly reflecting

the increased tendency to produce proinflammatory mediators at later periods of infection (8).

Among adaptive immune system cells, the number of T and B lymphocytes was significantly higher at 9 dpi than at 6 dpi in the young mice (Fig. 5C and D). The effective response of T cells in the young mice may contribute to the protective effect in a low-dose viral challenge, but is associated with severe pathology and subsequent mortality at high viral doses (25). Although the reduction in the number of T cells in the aged mice could be related to delayed viral clearance and a longer lasting infection, the mild lung pathology (Fig. 3B and F) may have contributed to the higher survival rate during the early stages of infection (22,25–27).

It is difficult to determine immune cells that are functionally affected by age; however, it has been found that in humans, decreased functional capacity is observed in macrophages and PMNs in the elderly (3,28). We demonstrated that C57BL/6 aged mice show reduced accumulation of inflammatory cells in the lung tissues; this may be related to diminished alveolar damage during the early stages of infection. On the other hand, inflammatory cell infiltration in tissue injuries characteristic of ARDS suggest that host inflammatory response, rather than the direct cytopathic effects of the virus, plays a more significant role in lung damage in young mice. Lastly, these findings may have therapeutic implications; they may provide timely and detailed insights into the age-specific disease course in young populations and limit the inflammatory response to reduce lung pathology associated with severe influenza virus infection. Therapeutic implications in the elderly include implementation of treatment to maintain the early protective response and reduce later morbidity and mortality associated with viral infection.

It is noteworthy that elderly people have a robust antibody response because they have been exposed to various pathogens. A previous study (29) demonstrated that elderly people who had been infected with H1N1 influenza viruses—which shared structural similarity with the 2009 H1N1 pandemic influenza virus and was structurally distinct to seasonal H1N1 viruses—before the 1950s, were conferred cross-protection against the 2009 H1N1 pandemic influenza virus, contributing to the overall milder symptoms and lower-than-expected mortality rate in the elderly.

The elderly are the fastest growing segment of the population and are estimated to substantially increase to more than 2 billion by 2050 (www.who.int/ageing/global). Hence, a better understanding of changes in the immune system during the aging process is becoming a key factor for the development of strategies for prophylaxis and therapy of acute and severe infections in this population segment. Further studies are necessary to understand the balance in the immune system's positive and negative effects and improve our ability to control the viral burden by limiting pathological immune responses.

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