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Osteopontin is critical to determine symptom severity of influenza through the regulation of NK cell population

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ABSTRACT

Osteopontin (OPN) is involved in exacerbating various inflammatory diseases. A severe pulmonary inflammation is frequently found in lethal influenza A virus (IAV) infection. However, the function of OPN against the infection was poorly understood. Here, we demonstrate an importance of OPN on immune response and disease severity after IAV infection. We found that the expression level of OPN was increased in mice infected with IAV. The OPN knockout (KO) mice exhibited a severe pathological phenotype and the survival rate decreased after the lethal IAV infection, compared to the wild type mice, while the survival rate increased in OPN transgenic (Tg) mice. The population of natural killer (NK) cells significantly decreased in OPN KO mice at day 5 after the infection, whereas, it increased in OPN Tg mice. These results suggest that OPN plays an important role in host defense against IAV infection through the regulation of NK cell population.

1. Introduction

Influenza A viruses (IAVs) are well known to induce highly contagious respiratory infections in humans and several animal species. Aberrant production of inflammatory cytokines, such as TNF-α (tumor necrosis factor-α), IL-6 (interleukin-6), and IFNγ (interferon-γ), is frequently found in the course of lethal infections of IAV, and this is thought to be a prominent phenomenon linked to the viral pathogenicity [1–4]. However, the inflammatory cytokines are also important in functioning to eliminate the virus through activation of the immune system [5–8]. Therefore, simply repressing these cytokines would not always be efficient to rescue a patient from lethal infection with IAV [9]. Nevertheless, an analysis of the functions of the various inflammatory cytokines in infectious diseases caused by IAV infections would provide important information for the development of effective prevention and treatment regimes against the infectious disease.

Osteopontin (OPN), a secreted phospho-glycoprotein, is a multi-functional protein, known to be involved in biomineralization, tissue remodeling, and inflammation [10–12]. Expression of OPN is involved in the deterioration of various inflammatory diseases [13–15]. For instance, osteopontin deficiency reduced joint swelling in the mouse model for rheumatoid arthritis [16,17], and treatment with anti-OPN antibody protects a joint from the experimental rheumatoid arthritis. In addition, in acute inflammatory liver injury caused by concanavalin A (Con-A) injection, OPN plays a crucial role in the development of the hepatitis [18,19]. Despite knowledge of physiological significances of OPN to exacerbate inflammation, the function of OPN on IAV infection has been poorly understood.

Here, we report on the protective role of OPN against IAV infections. The expression levels of OPN increased in mice

Abbreviations: IAV, influenza A virus; OPN, osteopontin; rOPN, recombinant osteopontin; TNF-α, tumor necrosis factor-α; IL, interleukin; IFN, interferon; Con-A, concanavalin A; KO, knockout; Tg, transgenic; NK cells, natural killer cells; WT, wild type; DAB, 3,3′-diaminobenzidine.

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infected with IAV and the increase correlates with the virulence of the IAV strains in the mice. The OPN knockout (OPN KO) mice exhibited the more severe clinical phenotype against lethal doses of infections with IAV. In addition, lengthening of survival times and a more moderate clinical phenotype against IAV infections were observed in OPN transgenic (OPN Tg) mice. In parallel with the severity of the IAV infection, the numbers of natural killer (NK) cells decreased in the OPN KO mice during the course of the viral infection, and increased in the OPN Tg mice. These results suggest that OPN is involved in the elimination of the IAV infection at the early course of the infection through activation of innate immunity.

2. Materials and methods

2.1. Mice

Male, specific pathogen-free C57BL/6N mice were purchased from Clea Japan Inc (Tokyo, Japan). OPN deficient mice [20,21] were backcrossed 11 generations with C57BL/6N, and then used for this study. Information about OPN transgenic mice is described elsewhere [22]. All animal experiments were performed in accordance with the guidelines of the Bioscience Committee of Hokkaido University and were approved by the Animal Care and Use Committee of Hokkaido University.

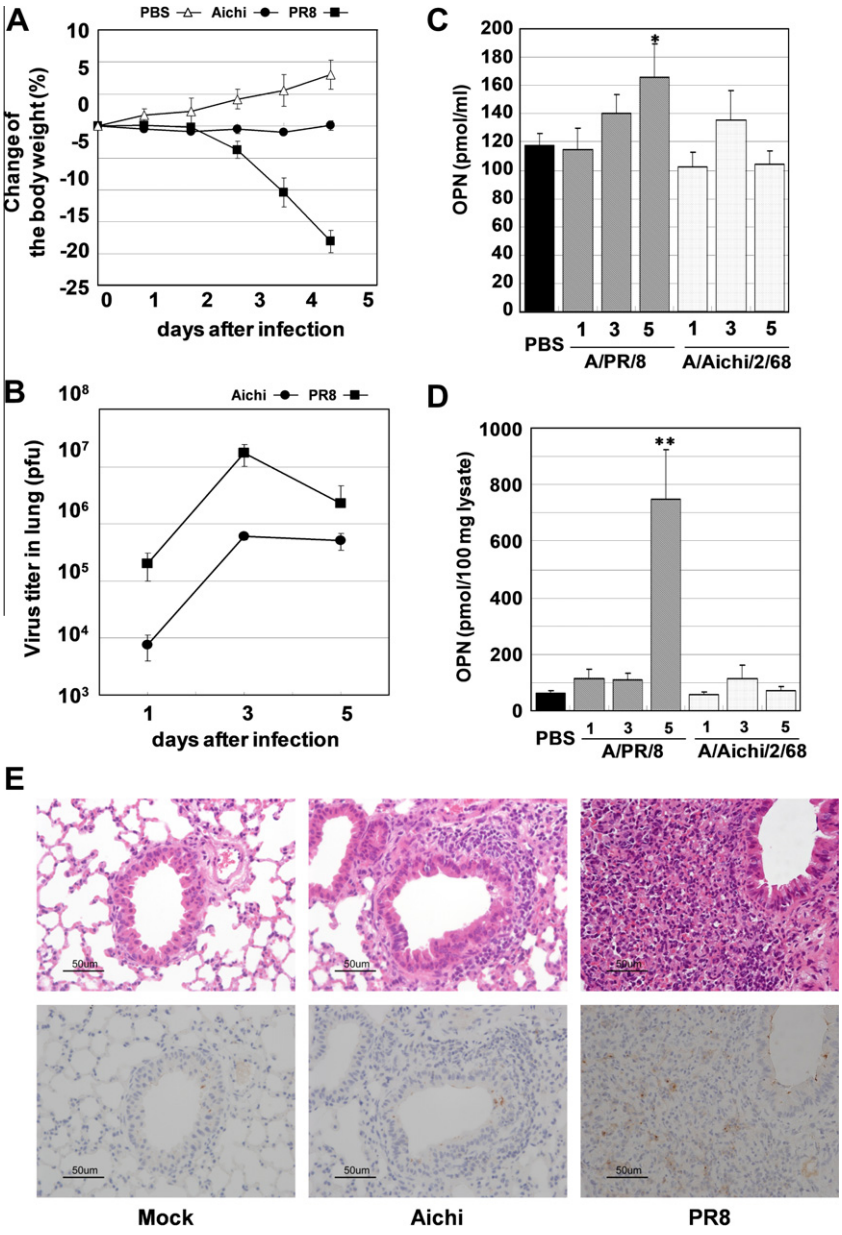

Fig. 1. OPN expression is correlated with the pathogenicity of the IAV strain. **A** and **B,** C57BL/6N mice ($n = 5$) were infected intranasally with the PR8 or Aichi strain of IAV at 10^3 pfu, and the body weight loss (**A**) and virus titer in the lung (**B**) were monitored. **C** and **D**, C57BL/6N mice ($n = 5$) were infected with the PR8 or Aichi strain of IAV, and then the plasma and the lungs were collected after the inoculation at the day indicated in the figure. The expression levels of OPN in the plasma (**C**) and the lungs (**D**) were quantified by OPN ELISA kit (IBL Co. Ltd). Asterisks indicate that the differences are statistically significant $^{*}p < 0.05$, $^{**}p < 0.001$, against the expressions of OPN in the plasma (**C**) or lung lysate (**D**) of the PBS controls. E, C57BL/6N mice were intranasally inoculated with 10^3 pfu of the PR8 (right panels) or the Aichi (center panels) strain of IAV. After 5 days the mice were sacrificed, and the lung tissue from the mice was analyzed histochemically. PBS inoculated mice (left panels) were used for the controls. The lung tissue was stained with hematoxylin-eosin (upper panels) or anti-OPN antibody (lower panels).

2.2. Viruses and virus titers

Influenza A viruses (IAVs), the A/Puerto Rico/8/34 (PR8; H1N1) and A/Aichi/2/68 (Aichi; H3N2) strains were propagated in 10-day-old embryonated chicken eggs, and used in this study. The virus titers in the lung homogenates were measured as described elsewhere [23].

2.3. Measurement of OPN expression

Lung lysates were prepared using CelLytic MT (SIGMA–Aldrich, St. Louis, MO) supplemented with the protease inhibitor cocktail (Complete Mini; Roche Applied Science, Manheim, Germany). The concentrations of OPN in the lysates and plasma were measured by ELISA using a commercially available kit (Mouse Osteopontin Assay Kit; IBL, Gunma, Japan). The measurements were performed in accordance with the manufacturer's protocol.

2.4. Histological analysis

Immunohistochemically, the detection of the cells expressing OPN was performed as described elsewhere [24,25]. In brief, tissue specimens were fixed with 10% buffered formalin and embedded in paraffin. For the antigen retrieval, deparafinized sections were pretreated in 10 mM citrate buffer, pH 6.0, in a microwave oven for 10 min. The sections were immunostained with 3,3′-diaminobenzidine (DAB) using a monoclonal antibody against OPN (clone O-17; IBL).

2.5. Flow cytometry

The number of splenocytes collected from the mice infected with PR8 virus was counted and the cells (1×10^6) were incubated with FITC-conjugated anti-NK1.1, biotinylated anti-CD3, APC-conjugated streptavidin (BD Biosciences, San Jose, CA). All analyses were performed on FACSCanto (BD Biosciences) and were analyzed by FlowJo software (Tree Star Inc., Ashland, OR). The NK cells were identified as NK1.1-positive/CD3-negative cells.

3. Results

3.1. The expression level of OPN is increased in the lung of mice infected with the IAV, correlated to the severity of the pathological phenotype

To understand functions of OPN on infection with IAV, we initially monitored the expression level of OPN during the course of the IAV infection. The C57/BL6N mice were infected intranasally with the PR8 or Aichi strain of IAV at 10^3 pfu, and then the expression levels of OPN in the plasma and lung collected from the infected mice were measured by ELISA. As shown in Fig. 1A, the body weight loss of the PR8 infected mice was more severe than that of the Aichi infected mice, in agreement with the previous study [26]. In addition, correlated with the severity of the body weight loss, the virus titers in the lungs were significantly higher in the PR8 infected mice than in the Aichi infected mice (Fig. 1B). Consistent with these results, the expression level of OPN increased in the plasma and especially in lung of the mice infected with IAV depending on the symptom severity (Fig. 1A, C and D). The expression of OPN in the plasma was increased at day 3 and significantly up-regulated at day 5 (Fig. 1C). In addition, 5 days after the infection, the expression of OPN was remarkably increased in lung homogenates (Fig. 1D). Next, the OPN distribution expressed in the IAV-infected lung tissue was analyzed by immunohistochemistry. As shown in Fig. 1E, OPN protein was predominantly present in and around areas containing acute inflammatory infiltrates at day 5. From these results, we considered that the expression of OPN is correlated to the disease severity of influenza.

3.2. Aggravation of disease severity caused by IAV infection in OPN KO mice

To evaluate the importance of OPN for the severity of influenza, the condition of disease in OPN KO mice after IAV infection was investigated. The PR8 strain of IAV was inoculated intranasally into OPN KO mice or wild-type (WT) mice, and the body weight and clinical appearance of the mice were monitored. The results show that the virus infected OPN KO mice unexpectedly exhibited more severe weight losses than WT mice (data not shown). Further, as shown in Fig. 2A, all of the OPN KO mice succumbed within 6 days of the viral infection, while all of the wild type mice were still surviving at this time. Although neither the OPN KO mice nor the WT mice survived by day 10, the OPN KO mice evidently exhibited a more severe phenotype to the infection of the IAV. As shown in Fig. 2B, the virus titers of the lung tissue increased in OPN KO mice at day 5 in comparison with that of the WT mice. These data suggest that the expression of OPN induced by the infection of IAV is crucial to repress the severity of the disease symptom.

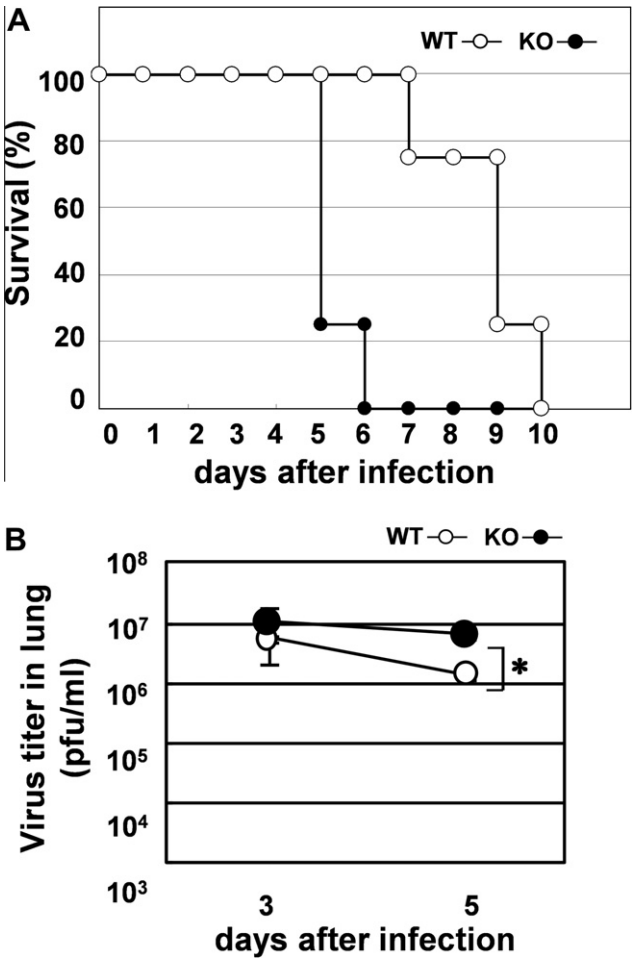

Fig. 2. OPN deficient mice exhibit more sensitive phenotype against the lethal dose infection of IAV. **A** and **B,** OPN KO mice and WT mice were infected with the PR8 strain of IAV at 10^3 pfu, and the survival rate of the mice (**A**) ($n = 5$) and virus titers in the lungs (**B**) ($n = 3$) are indicated. Open circles indicate the survival rate or the virus titer of the WT mice and closed circles indicate that of the OPN KO mice. Asterisk indicates that the difference of the virus titer in the lung homogenates between KO and WT mice is statistically significant ($^*p < 0.05$).

3.3. Reduction of morbidity caused by IAV infections in OPN transgenic mice

To confirm the protective role of OPN on infectious diseases caused by IAV, we investigated the OPN function using OPN Tg mice. The PR8 strain of IAV was infected to the OPN Tg mice or WT mice, and the overall survival and pulmonary virus titers were monitored. The results show that the survival time was apparently extended in the OPN Tg mice in comparison with that of the WT mice (Fig. 3A). In addition, the virus titer at day 3 was significantly lower in OPN Tg mice than in the WT mice (Fig. 3B). These results suggest that OPN is important for the protection against IAV replication.

3.4. The numbers of NK cells in IAV-infected mice are correlated with the OPN expression

As shown in Fig. 2A, the OPN KO mice apparently exhibited a more rapid disease progression after the IAV infection. Differences in the severity of the disease were found in the early course of the IAV infection, before any acquired immunity was adequately activated. The infiltration of macrophages and neutrophils in the lung is correlated with the disease severity after IAV infection [27]. Especially, massive infiltration of neutrophils to the lung is frequently found in lethal IAV infection. In addition, it has been reported that the disease severity after PR8 infection is not affected by depletion of airway macrophages [28]. On the other hand, the natural killer (NK) cells, an innate immune cells which exhibit cytotoxicity to the virus infected cells, are known to be crucial for the elimination of IAV [29]. Thus, to analyze the OPN function on activation of innate immunity, the numbers of NK cells in the splenocytes were monitored during the course of the IAV infection. As shown in Fig. 4A, the NK cell population was significantly decreased in the OPN KO mice in comparison with that of WT mice at day 5, and conversely, significant increments of the NK cell population were observed in the OPN Tg mice compared with the WT mice both at day 3 and day 5 (Fig. 4B). The number of NK cells was reported to be decreased in OPN KO mice [30]. However, our data indicated that the numbers of NK cells of the OPN KO and WT mice are not significantly different at day 3 after the IAV infection, while there is a significant difference at day 5 in the numbers of NK cells (Fig. 4A). Nevertheless, there still remains a possibility that the OPN expression may affect the number of NK cells respond to the IAV infection. To exclude the possibility, we examined the effect of the suppression or transient expression of OPN after the infection, using anti-OPN antibody or recombinant OPN administration. Administration of anti-OPN antibody shows slightly severe body weight losses and shortened survival time compared with the control mice (Fig. 4C and D). In contrast, administration of recombinant OPN shows the recovery of the weight losses and extended survival time compared with the control mice. Previous studies have demonstrated that the depletion of NK cells increases morbidity and mortality arising from IAV infection [29]. Therefore, the increase of the number of NK cell induced by OPN is critical for the protective role of OPN in IAV infections.

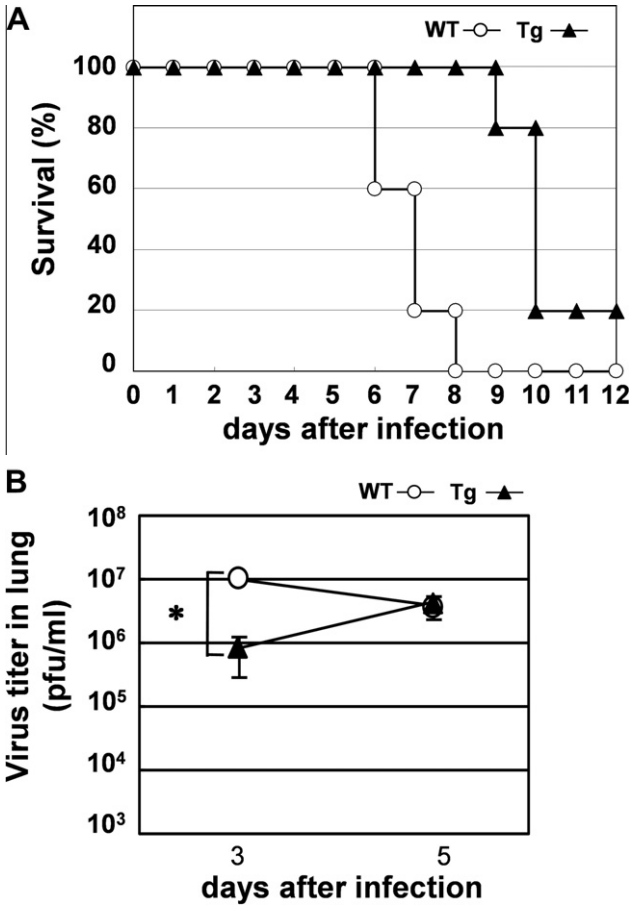

Fig. 3. OPN transgenic mice exhibit prolonged survival time and low virus titer in lung after a lethal dose infection of IAV. **A** and **B,** OPN transgenic mice and WT mice were infected with the PR8 strain of IAV at 10^3 pfu, and the survival rate of the mice (**A**) ($n = 5$) and virus titers of the lungs (**B**) ($n = 3$) are indicated. Open circles indicate the survival rate or the virus titer of the WT mice and closed triangles indicate that of the OPN Tg mice. Asterisk indicates that the difference of the virus titer in the lung homogenates between KO and WT mice is statistically significant ($^*p < 0.05$).

4. Discussion

The present study demonstrated the physiological significance of OPN in the activation of the immunoresponse to eliminate IAV for the infection. OPN is known to be involved in the worsening of various inflammatory diseases, such as rheumatoid arthritis and concanavalin A-induced hepatitis [16–19]. Our data indicate that the expression level of OPN in the lungs is remarkably increased in PR8 infected mice in comparison with that of Aichi infected mice, and that the OPN level is correlated with the pathogenicity of the strain of IAV to the mice (Fig. 1). Since massive inflammatory cell infiltration into the lungs and excessive proinflammatory cytokine production are frequently found in the course of a lethal IAV infection [31,32], we have doubted possibility that OPN exacerbates the disease severity caused by the IAV infection.

Although OPN expression was important for the survival of mice during the early period after the IAV infection, it hardly affected the final survival rates (Figs. 2 and 3). These results suggest that the protective effect of OPN expression against the IAV infection is not enough for the elimination of IAV at the late phase. A previous report has demonstrated that OPN activates the Th1 response through an activation of IL-12 production from macrophages [33,34]. On the other hand, it has also been reported that OPN expression does not influence the development of Th1 response during the course of IAV infection [35]. These results suggest that OPN function to regulate the Th balance in the acquired immunity is not effective in host defense against the IAV infection. This may be one reason why OPN expression hardly affected the final survival rates of the IAV-infected mice.

Apparent negative effects of OPN expression on the IAV infection were not able to be detected in our experimental conditions. However, there is a possibility that the pro-inflammatory functions of OPN exhibit some negative effects on the recovery from IAV infections. It has been reported that pre-treatment with TNF-α

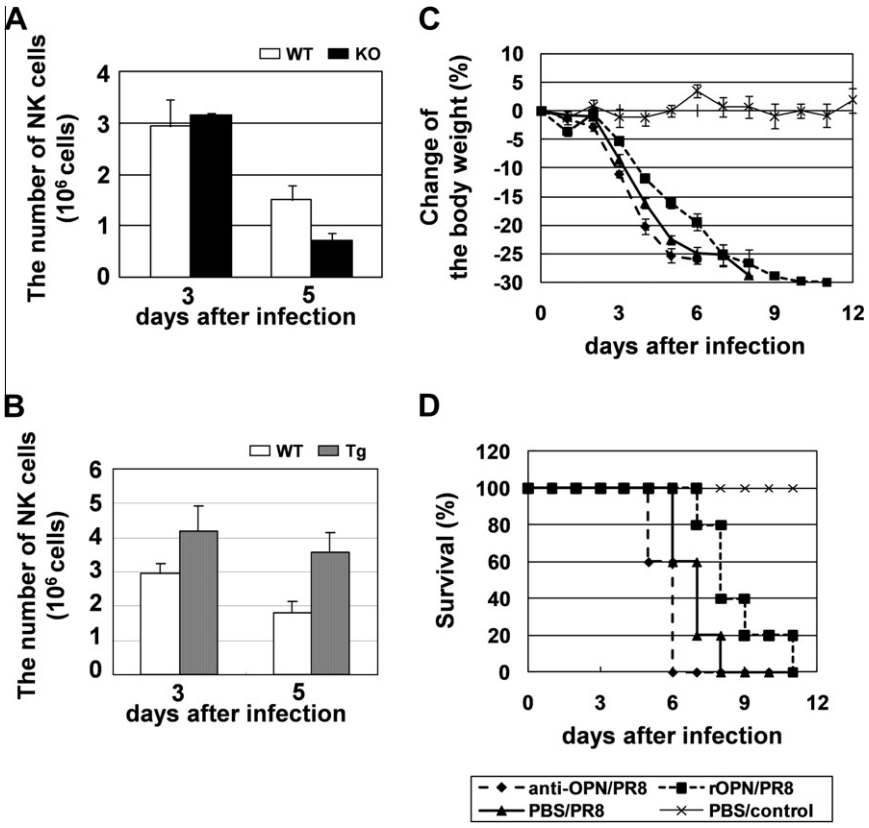

Fig. 4. The number of NK cells in IAV-infected mice is regulated by OPN. (**A**) WT and OPN KO mice were infected intranasally with the PR8 strain of IAV at 10^3 pfu, and the numbers of NK cells in the splenocyte of the WT (open bars; $n = 2$) and OPN KO mice (bold bars; $n = 2$) at the day indicated in the figure were counted by flow cytometry. (**B**) The numbers of NK cells of the WT (open bars; $n = 3$) and OPN Tg mice (hatched bars; $n = 3$) during the course of the IAV infection. Error bars indicate standard deviations calculated with three independent experiments. **C** and **D,** 1 day before the infection, C57BL/6N mice were intravenously injected with 4.5 μg of recombinant OPN (rOPN; $n = 5$), 450 μg of anti-OPN antibody ($n = 5$) or PBS ($n = 4$), and then the mice were infected with the PR8 strain of IAV at 10^3 pfu. The body weight losses (**C**) and survival rates (**D**) of the mice are indicated.

effectively represses IAV replication [6,7]. TNF-α is known to be a major inflammatory cytokine which is aberrantly expressed in lethal infections of IAV, and is considered to be related in the multiple organ disorders found in the course of lethal IAV infections [2,3]. This might suggest that the time course of the expression is crucial to determine whether a pro-inflammatory molecule exhibits a positive or negative effect on IAV infections, and the effects of OPN expression on the IAV infection might have some similarities with the TNF-α. Further investigations are required to better understand the detailed functions of OPN on IAV infections.

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