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의학 석사 학위논문

Rapid induction of Type III interferon
can resist to initial Influenza virus in
Allergic asthma

알레르기 천식에서 인플루엔자 바이러스
감염 초기에 유도되는 제 III형
인터페론에 의한 바이러스 저항성

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안 수 진

Abstract

Rapid induction of Type III interferon can resist to initial Influenza virus in allergic asthma

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In general, Asthmatic have been known as being vulnerable to attack by respiratory viral infection. That is because asthmatic lungs are damaged by inflammation. So they can get worse more easily by a viral infection. But, here we found a few experimental results could demonstrate that asthmatic mice are not highly susceptible to Influenza A virus(IAV) during the early

stage of infection. C57BL/6 mice with asthma were infected by IAV(WSN/33: H1N1) and from these model we measured body weight, viral mRNA level, viral titer, histopathological analysis and cytokines profiles including interferons(IFNs) and T helper type 2 cell(Th2) compared to IAV-infected non-asthmatic mice. Contrary to expectations, The results showed that there was a significantly decrease viral load on 7 days post infection(dpi) in IAV-infected asthmatic mice. Further, the reduction of Th2 cytokines secretion was observed and it led to decrease inflammation caused by Th2 cytokines in lung tissue. We measured for the changes in the secretion rate of IFNs and Th2 cytokines over 7 days, we found that a remarkable increase of Type III IFN in early infection accompanied with a remarked decrease of Th2 cytokines. These are thought to be related to rapid induction of Type III IFN in early stage. Actually, when neutralizing and recombinant Antibody(Ab) of Type III IFN was administered to asthmatic mice, it was observed that each of which was worse or better result. In conclusion, rapid induction of Type III IFN after IAV infection activates immune response to resist initial viral spread.

Keywords: Type III interferon, Interferon- λ , Influenza A virus, Innate immunity, Allergic asthma

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Introduction

Allergic asthma is an allergic disease caused by inflammatory response with the bronchi easily becoming sensitive to even very small stimuli due to continuous exposure of allergens (1–3). When allergens are recognized by dendritic cells at the bronchial epithelial cells, naïve T cells differentiate into Th2 cells, which induce the release of Th2 cytokines, such as Interleukin(IL)–4, IL–5 and IL–13. These Th2 cytokines play a critical role in promoting inflammation and airway hyperresponsiveness that are major characteristics of asthma (4–6). As the secretion of Th2 cytokines increases in asthma, there is imbalance between Th1 and Th2 immune response. The predominant secretion of Th2 cytokines leads to severe inflammation that causes declining of lung function, so it is thought to be vulnerable to respiratory virus (7, 8). Indeed, many documents have demonstrated that asthma is exacerbated by respiratory viral infection (9). We need to investigate the innate immune system of virus infected asthma. And then by controlling the immune response, we need to prevent the asthma from worsening by viruses and further decreasing mortality.

In the case of virus targeting air passages, respiratory epithelium could be the first barrier against viruses by producing immune substance as well as

being physical barrier (10, 11). Interferons(IFNs), discovered by Isaacs and Lindenmann in 1957, are innate immune substance produced by host cells in response to viral infection, inducing interferon stimulated genes(ISGs) which can inhibit viral spread to neighboring cells (12–14). Interferons are classified into three types. One of them is Type I IFNs mainly include IFN- α and IFN- β are essential for host defense against virus infection (15–17). Another relatively recently discovered interferon is Type III IFNs, which consist of four in humans (IFN- λ 1, IFN- λ 2, IFN- λ 3 and IFN- λ 4) and two in mice (IFN- λ 2, IFN- λ 3) (18, 19).

Although Type I IFNs and Type III IFNs share the similar signal transduction pathways to activate antiviral effect, they bind to their receptor that is distinct from each other. There are also differences in the cells in which the receptor is expressed (20). Whereas Type I IFN receptor appears broadly in most cell types, Type III IFN receptor is restrictively found on epithelial cells(21). Since Type III IFN receptor is primarily expressed on the surface of the epithelial cells, Type III IFN could work more dominant in viral infections targeting respiratory epithelial cells (22, 23). According to the experiments conducted by Mordstein (24, 25) and Ank (26) with knockout mice that lack of Type I IFN receptor (IFNAR^{-/-}) and Type III IFN receptor(IL-28RA^{-/-}), it showed that mice with lack of IL-28RA were more susceptible to IAV than with lack of IFNAR. In the case of single

knockout IFNAR^{-/-}, IFN- α (Type I IFN) failed to prevent the virus spreading due to lack of their receptors to initiate the signaling cascade, whereas IFN- λ (Type III IFN) activated the antiviral pathways (16). However, when IFN- λ receptor was knocked out (IL-28RA^{-/-}), it is vulnerable to IAV infection because even if IFN- α acted as an antiviral activity, IFN- λ that produce more dominant effect on IAV could not function in the lung. This result experimentally demonstrated that Type III IFNs play a more important role in resisting the respiratory virus than Type I IFNs.

We wondered whether the susceptibility to virus resulted from deficient in IFNs. So we observed the innate immune system at the virus-infected asthmatic mice. After conducting *in vivo* models of asthma infected with IAV, we found that the results differed from that we expected. The infected asthmatic mice were not as highly susceptible as we supposed to. Our data showed that IFN- λ 2/3, Type III IFN, was rapidly producing at early stage of the infection in the asthmatic mice. This induction of Type III IFNs accompanied by increased the secretion of IFN- γ and reduced of Th2 cytokines and then resulted in lowered the viral load.

Materials and Methods

Mice and Virus Inoculation

Male C57BL/6J(B6) mice (Orientalbio, Seoul, Korea) aged 7 weeks (average 22g) were used for experiment inducing allergic asthma.

Influenza A virus (IAV WS/33: H1N1, ATCC, Manassas, VA, USA) was inoculated into non-asthmatic and asthmatic mice by intranasal treatment (213 plaque-forming unit(pfu) in 30ul PBS). After the mice were anesthetized, bronchoalveolar lavage(BAL) fluid was collected by repeating the procedure that is instilling and retrieving the Phosphate-Buffered Saline(PBS) using the cannula into the lung. The collected BAL fluid was used to measure the level of protein secretion and viral titer each of which were analyzed by Enzyme-Linked Immunosorbent Assay(ELISA) and plaque assay. Mouse lung tissue was used for DNA microarray, histopathological analysis and real-time Polymerase Chain Reaction(PCR) analyzed the expression level of mRNA.

Allergen sensitization and Challenge protocol

Mice model inducing allergic asthma began with the first sensitization by

intraperitoneal injection(i.p) mixture of 50ug ovalbumin(OVA, Grade V; Sigma, MO, USA) and aluminum hydroxide(Alum, Sigma, MO, USA). 14 days later, OVA was injected into mice for second sensitization. And then, 4 days from 21st to 24th, 150ug of OVA mixed with PBS was administered to nasal cavity (OVA/OVA). Control mice were injected with OVA as same as asthma mice during two sensitization period, but only PBS was injected when administered with the nasal cavity (OVA/PBS).

Real-time PCR

Lung tissue were obtained from 4 groups(control mice, infected mice, non-infected asthmatic mice and infected asthmatic mice) for 0,1,3,5,7,10 and 14 days after infection, and they were used to extract total RNA by TRIzol Reagent(Invitrogen). Complementary DNA(cDNA) was synthesized from the extracted total RNA(3ug with random hexamer), and real-time PCR experimental techniques were used to amplify the synthesized cDNA. Amplification was performed using the TaqMan Universal PCR Master Mix (PE Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. To determine the level of virus replication within host cells, the expression level of the PA gene was measured. Mouse IFN primers such as IFN- α , INF- β , IFN- λ 2/3 and IFN- γ were used to measure the level of interferon expression (purchased from Applied

Biosystems, Foster city, CA, USA). All PCR data were normalized to the level of glyceraldehydes phosphate dehydrogenase(GAHDHP), which called the housekeeping gene, to correct for variation between samples.

Quantification of Secreted Cytokines

The levels of secreted protein such as IFN- α (42120-1), IFN- β (42400-1), IFN- λ 2/3(DY1789B), and IFN- γ (DY485) from BAL fluid were quantified by DuoSet ELISA kit(R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The working range of the assay was 62.5–4000 pg/ml. The levels of secreted protein such as IL-4, IL-5 and IL-13 were measured by Luminex Multiplex assay(R&D systems).

Administration of Neutralizing Antibodies

The neutralizing antibodies to IFN- λ and IFN- γ could neutralize the secreted IFN- λ and IFN- γ , inhibiting it from functioning. More than 70% of the IFN- λ and IFN- γ secretion in the BAL fluid were found to be inhibited. Isotype-control antibody (rat-IgG), anti-IFN- λ 2/3 (Cat number: MAB1789) and anti-IFN- γ (Cat number:MAB4851) neutralizing antibodies were purchased from R&D systems. Antibodies(10ug/30ul in PBS) were administered through nasal cavity.

Intranasal delivery of Recombinant IFN- λ 2/3

To determine whether IFN- λ 2/3 controls acute virus infection in our animal model, recombinant IFN- λ 2 and IFN- λ 3(30ul in PBS) were given to infected asthma mice(N=5) to intranasal delivery. The recombinant IFN- λ 2 (Cat number:4635-ML/CF) and IFN- λ 3(Cat number:1789-ML/CF) were purchased from R&D systems, and used by mixing with IFN- λ 2(1ug/10ul) and IFN- λ 3(1ug/10ul).

Histopathological Analysis

A lobe of the lung was fixed with 10% neutral buffered formalin, and cut the lung in half for observing the section of the bronchi and bronchioles and then embedded in paraffin. The paraffin-embedded tissue block was sectioned at 4um thickness using a microtome. The sectioned slides were used to examine histopathologic analysis by counting the number of the inflammatory cells through the Hematoxylin &Eosin Y (H&E) stain and by identifying the distribution of goblet cells that secret mucus through Periodic Acid-Schiff(PAS, Sigma, Deisenhofen, Germany) stain. Histopathological analysis is a scoring method which is performed by the blinded examiner by taking account of the extent and severity of inflammation in at least five sections stained H&E (27).

Plaque assay

Plaque assay is a method to measure the quantity of infectious virus particles in BAL fluid. The BAL fluid was diluted with PBS to make a 10-fold serial dilution, and add 0.5ml of the 10^{-2} to 10^{-5} dilution to one of the MDCK monolayers. After the virus particle could allow to invade the cells during 2 hours incubation, threw away the solution and covered monolayer with agarose gel(1.5%) to prevent the virus from spreading to the neighboring cells. After three to four days incubation, as the cells infected with the virus died and fell off, formed a circular shape called a plaque. By staining with crystal violet dye, uncolored died areas could be clearly observed contrast to living cells stained in purple. These circles were counted to determine the concentration of virus by calculating the infectious virus particles in BAL fluid.

Statistical Analysis

All of the results including Real-time PCR, Plaque assay and ELISA were presented as the mean value \pm standard deviation. Statistical analysis was performed with GraphPad Prism software and when P value less than 0.05, it was considered statistically significant.

Results

OVA-sensitized mice exhibit typical symptoms of allergic asthma

Based on that asthma would be vulnerable to respiratory viruses, we first developed an asthmatic mouse model induced by OVA sensitization(Figure 1A). To evaluate whether mice sensitized with OVA exhibit asthma symptoms, we initially measured airway hyperresponsiveness(AHR). When methacholine was inhaled, it was observed that the total lung resistance increased in asthmatic mice(N=3, Figure 1B). This is because the air passages have become overly sensitive to nonspecific stimuli and easily contracted (28, 29). According to histopathological analysis of H&E and PAS staining of lung tissue section slides, severe inflammation with extensive peribronchial inflammatory cell infiltration were found, along with significantly increased goblet cell metaplasia in asthmatic mice(Figure 1C). We also found that the number of eosinophils increased on the total lung tissues of asthmatic mice, and the number of lymphocytes, neutrophils and eosinophils significantly increased in the BAL fluid(Figure 1D, 1E). The above results demonstrated that allergic asthma is well-developed in OVA-induced B6 mice so that could be used to investigate the susceptibility to

influenza A virus in asthmatic mice.

Asthmatic mice are not vulnerable to initial IAV infection

In previous experiments, we found that the body weight of mice infected with IAV significantly dropped after infection and had the lowest level at the seventh day of infection(30). In this study, we analyzed the body weight of both asthmatic and non-asthmatic mice infected with IAV for 7 days after infection. The results showed that the body weight and the survival rate of non-asthmatic mice sharply decreased from 4 days after infection and 20% of the mice died at 7 days, whereas the weight of asthmatic mice infected with IAV was similar to those of uninfected control mice and all mice survived(Figure 2A, 2B). The level of viral mRNA gene expression from the lungs of asthmatic and non-asthmatic mice was analyzed by real-time PCR and the viral titer in the BAL fluid was measured by plaque assay on 7 dpi. Both the mean level of IAV mRNA gene expression and the mean viral titer were significantly elevated on 7 dpi in non-asthmatic mice(IAV PA mRNA level: 2.1×10^4 , viral titer: 3.3×10^5 pfu/ml). In contrast, the level of IAV mRNA gene expression and the viral titer were much lower in the asthmatic mice(IAV PA mRNA level: 1.4×10^3 , viral titer: 8.1×10^4 pfu/ml)(Figure 2C, 2D). In non-asthmatic lung tissue section, histopathological analysis revealed severe epithelial thickening, peribronchial inflammation and

epithelial cell shedding on 7 dpi. It was similar to histopathological analysis of uninfected asthmatic mice. However, this histopathological findings were not detected in the lungs of the virus-infected asthmatic mice, and the histological score was measured lower than the virus-infected non-asthmatic mice(the non-asthmatic mice:8.6, the asthmatic mice:1.8, Figure 2E). Also in PAS score, it was estimated that goblet cell metaplasia reduced in virus-infected asthmatic mice on 7 dpi (uninfected asthmatic mice: 13.2, IAV-infected asthmatic mice: 4.6, Figure 2F). A multiplex assay was performed to quantify the secretion of Th2 cytokines, such as IL-4, IL-5 and IL-13, from the BAL fluid. The results also showed that the level of secreted Th2 cytokines was higher in uninfected asthmatic mice(IL-4:532.385.1pg/ml, IL-5: 964.7 \pm 98.5ng/ml, IL-13: 2487.5 \pm 765.5ng/ml), and measured relatively lower in infected asthmatic mice(IL-4: 64.211.5pg/ml, IL-5: 121.7 \pm 32.6ng/ml, IL-13: 498.5 \pm 104.8ng/ml)(Figure 2G). As interpreted the data, we observed that both the secretion of Th2 cytokines and the inflammatory response related Th2 cells decreased in the lung tissue of the asthmatic mice infected with IAV. This makes it seem as if the symptoms of asthma is alleviating, even though asthmatic mice is infected with IAV.

Asthmatic mice can maintain innate immunity against IAV through Type III IFN

Experiments were conducted to investigate changes in the immune response of IAV infected asthmatic mice. The immune response related IFN was measured in the lung obtained on 0,1,3,5 and 7 dpi using RT-PCR, ELISA analysis. Comparing asthmatic and non-asthmatic mice on 7 days postinfection, the gene expression level of IFN- λ 2/3 mRNA was higher in the non-asthmatic mice (IFN- λ 2/3: $1.4 \times 10^5 \pm 5.2 \times 10^4$, IFN- γ : $2.4 \times 10^3 \pm 5.4 \times 10^2$), while the gene expression of IFN- γ mRNA was higher in asthmatic mice (IFN- λ 2/3: $4.8 \times 10^4 \pm 1.4 \times 10^4$, IFN- γ : $6.4 \times 10^4 \pm 1.4 \times 10^4$) (Figure 3A). And these tendencies were the same as the level of secretion of IFNs protein from the BAL fluid on 7 dpi. The IFN- λ 2/3 protein was higher secreted in non-asthmatic mice (IFN- λ 2/3: 3298.6 ± 869.4 pg/ml), but lower in asthmatic mice (IFN- λ 2/3: 683.4 ± 97.7 ng/ml). In contrast, the secretion of IFN- γ protein from asthmatic mice (IFN- γ : 1865.7 ± 731.4 pg/ml) was higher than non-asthmatic mice (286.3 ± 48.7 pg/ml) (Figure 3B). All of the above results were analyzed from the obtained lungs on 7 days after infection, and for more detailed analysis, the mRNA expression level and secreted protein level of IFNs were measured on 0,1,3 and 5 days after IAV infection. In asthmatic mice, the mRNA expression level and secreted protein level of IFN- λ 2/3

were the highest on 1 days after infection and then gradually decreasing, while IFN- γ was drastically induced on 7 dpi (Figure 3C, 3D). Also, the secretion of Th2 cytokines, such as IL-4, IL-5 and IL-13, began to decrease from the first day after asthmatic mice infected with IAV in comparison uninfected asthmatic mice (Figure 3E). And the decrease in the secretion of Th2 cytokines was accompanied by alleviating inflammatory response at the lung tissue of asthmatic mice, which resulted in the decrease of PAS score(0 dpi: 12.7, 1 dpi: 7.8, 3 dpi: 2.6, 5 dpi: 1.4, 7 dpi: 1.3, Figure 3F). The above results showed that the rapid induction of IFN- λ 2/3 in the early stage of infection in asthmatic mice led to rise drastically IFN- γ along with a decrease in the secretion of Th2 cytokines. According to previous study, it demonstrated that relief of inflammatory reactions was affected by normalizing the level of IFN- γ , not reducing the secretion of Th2 cytokines (31, 32). (But in this data, as Th1 immune response increased and Th2 decreased at the same time, the Th1/Th2 imbalance was resolved. So it suggests that a decrease in inflammatory reactions would have more resistance against virus.

Rapid induction of Type III IFN can control IAV infection in asthmatic mice

To assess experimentally whether the rapid induction of IFN- λ 2/3 lead to the results that we found, we conducted experiments by administering the neutralized antibody of IFN- λ 2/3 and IFN- γ . Neutralized antibody of IFN- λ 2/3 or IFN- γ (10ug/30ul) were simultaneously administered to asthmatic mice(N=5) when inject with IAV(H1N1, 213pfu/30ul). As the previous results showed, the lung tissues of infected asthmatic mice were almost relieved at normal levels. However, the mice neutralized antibody that blocks the released IFN- λ 2/3 or IFN- γ were able to observe that the inflammation worsened as IFNs did not function properly. Histological score showed the same result (IAV only Asthmatic mice: 2.6, IAV+IFN- λ 2/3-neutralizing Ab: 12.4, IAV+IFN- γ -neutralizing Ab: 12.5 ;Figure 4A). More inflammation was observed in asthmatic mice that treated neutralized IFNs antibodies. And also, the results of the plaque assay showed that the viral titer from BAL fluid of mice with neutralized IFN- λ 2/3 and IFN- γ was significantly increased in comparison to IAV infected asthmatic mice (IAV only Asthmatic mice: $2.4 \times 10^4 \pm 1.6 \times 10^3$ vs IAV+IFN- λ 2/3-neutralizing Ab: $1.3 \times 10^3 \pm 3.8 \times 10^5$, IAV+IFN- γ -neutralizing Ab: $1.4 \times 10^6 \pm 2.6 \times 10^5$;Figure 4B). Comparing the secretion levels of IFN- λ 2/3

and IFN- γ by ELISA assay, the amount of IFN- λ 2/3 secretion was not affected in mice with the neutralization IFN- γ antibody that blocked the released IFN- γ . In contrast, the amount of IFN- λ 2/3 secretion was decreased in the mice that used the neutralized antibody of IFN- λ 2/3 (Figure 4C, 4D). Thus, we suggested that the induction of IFN- γ was affected by the secretion of IFN- λ 2/3 and that there is a correlation between IFN- λ 2/3 and IFN- γ in IAV-infected asthmatic mice. When observing the histopathological features of the lung tissue on 1, 3, 5 and 7 days after IAV infection, goblet cell metaplasia and consistent the PAS score were seen in asthmatic mice with neutralization IFN- λ 2/3 antibody (Figure 4E). The virus titer was also measured high for 7 days without any decreasing (3 dpi: $3.0 \times 10^6 \pm 1.3 \times 10^6$, 5 dpi: $3.1 \times 10^6 \pm 1.8 \times 10^6$, 7 dpi: $4.3 \times 10^6 \pm 1.6 \times 10^5$, Figure 4G). In the case of secretion of Th2 cytokines, it was previously measured as decreased in infected asthmatic mice, but not reduced in mice that blocked IFN- λ 2/3 (Figure 4F). From the above data, we discovered that the blockade of IFN- λ 2/3, Type III IFN, reduced resistance to virus infection, thus the symptoms of the infection were not attenuated in asthmatic mice.

Inoculation of recombinant Type III IFN also resist to IAV infection

As observing over the 14 days both asthmatic and non-asthmatic mice infected with IAV, a sudden weight loss was exhibited from 8 days later contrary to the previous result of maintaining body weight until the first seven days (Figure 5A). And with an average weight of 15 g, all of the IAV infected asthmatic mice died between 12 and 14 dpi(Figure 5B). We found that the gene expression level of inflammatory cytokines, such as TNF- α , IL-1 β , CCL7 were gradually elevated (Figure 5C). Also the IFN- γ which had the highest level of protein secretion on 7 dpi was sharply declined since 7 days after infection (7 dpi: 31322.6 \pm 7318.4 pg/ml, 14 dpi: 2284.2 \pm 378.5 pg/ml, Figure 5D). In order to identify whether the external IFN- λ 2/3 protein produce the antiviral effect, the recombinant IFN- λ 2/3 protein (IFN- λ 2: 1 μ g/10ul, IFN- λ 3: 1 μ g/10ul, total 20ul in PBS) were simultaneously administered to the asthmatic mice(N=5) when inject with IAV(H1N1, 213pfu/30ul). Interestingly, unlike asthmatic mice, which survival rate had decreased the 8 days after infection, asthmatic mice with recombinant IFN- λ 2/3 had a 100% survival rate(Figure 5E). Furthermore, in asthmatic mice administered IFN- λ 2/3, it was observed that inflammation in the lung tissue was relieved, and that the viral titer also

decreased (Figure 5F, 5G). As a result, we could suggest that susceptibility to virus infection in asthmatic mice can be controlled by IFN- λ 2/3, Type III IFN.

Discussion

The present study was conducted to investigate how innate immune responses work when infected with respiratory viruses in asthmatic respiratory tract. The results showed a different view from what was generally accepted that asthma was vulnerable to viruses (33, 34).

In general, asthma is predominant in the Th2 immune response, resulting in the Th1 response being relatively down-regulated and exacerbating the release of IFNs that can resist to viruses. Type III IFN, one of the interferon family, is a substance that acts as host defense against virus in the respiratory epithelial cells, and the release of Type III IFN activates the transcription of ISGs from neighboring cells, thus preventing the invasion and replication of viruses. Therefore deficient of Type III IFNs was thought to be vulnerable to the virus (35–37).

In the early stage of infection, we found that Type III IFN was quickly released from the airway epithelial cells and that the secreted Type III IFN kept the viral resistance. What's even more interesting we found was that asthma symptoms were also alleviated, our data showed that the lung inflammation which one of the representative symptoms of asthma was relieved. As the expression level of Type III IFN rapidly increased on the

first day after infection, the secretion of Th2 cytokines was significantly decreased at the same time. Type III IFN was measured at the highest level on the first day after infection and showed a gradual decrease in the next six days, whereas IFN- γ slightly increased on 1,3 and 5 days and reached a peak level on the seventh day. They were in inverse proportion each other. When IFN- λ 2/3 was blocked by neutralized antibody, the resistance to IAV was not shown. And using the recombinant IFN- λ 2/3 protein showed that the antiviral effect remained relatively long. According to these results, Type III IFN plays a important role to induce Th1 immune response and to limit the Th2 immune response, which was also demonstrated by previous study of allergic airway diseases (38, 39).

Since Type III IFN is induced by virus infection, it is measured that the release of Type III IFN increases in mice infected with IAV. The mice infected with IAV released Type III IFN at the highest level on 10 days after infection (30, 40). But in the case of asthmatic mice infected with IAV, the level of Type III IFN reached a peak on the first day after infection. Therefore, we could hypothesize that asthma can act as a variable in IFNs secretion.

All of our experiments were conducted within a relatively short period of 7 days after the infection. But when we kept the experiment for 14 days, we recognized that the effect of Type III IFN showed declining, unlike above

results. Although Type III IFN did not show a lasting effect after induced on early stage of infection, it was able to be complemented by the additional administration of Type III IFN from outside. It was found to have similar antiviral effect to that of endogenous IFN- λ when the recombinant IFN- λ protein was given to mice. Thus, the method of maintaining effects of Type III IFN will be a new strategy that will enable effective defense against IAV infection. In our study, it is difficult to identify that the recombinant IFN- λ protein was directly delivered and acted on the lungs. Based on several papers, which have found that IFN- λ is recognized by receptor in upper respiratory tract epithelium such as nose and trachea (41–44), we suggest that the alleviation of viral infection can result from reducing the number of live virus particles that reach the lungs due to activate the antiviral effect of IFN- λ .

In recently, novel studies have shown that asthmatic mice are more resistant to virus infection than normal mice. According to the studies, it was discovered that removing viral-infected cells by activation of NK cells and antigen-specific CD8⁺ T cells (45) and reducing the lung inflammation by increase the TGF- β expression (46) can be the ways to have resistance to the virus. Such studies are necessary to find new target substances for the development of effective treatment that can control virus infection in asthma (47–49).

Our study provides convincing evidence that Type III IFN can have therapeutic potential to deal with IAV infection in asthma. And we should conduct a further experiment to identify whether Type III IFN actually works as controlling IAV.

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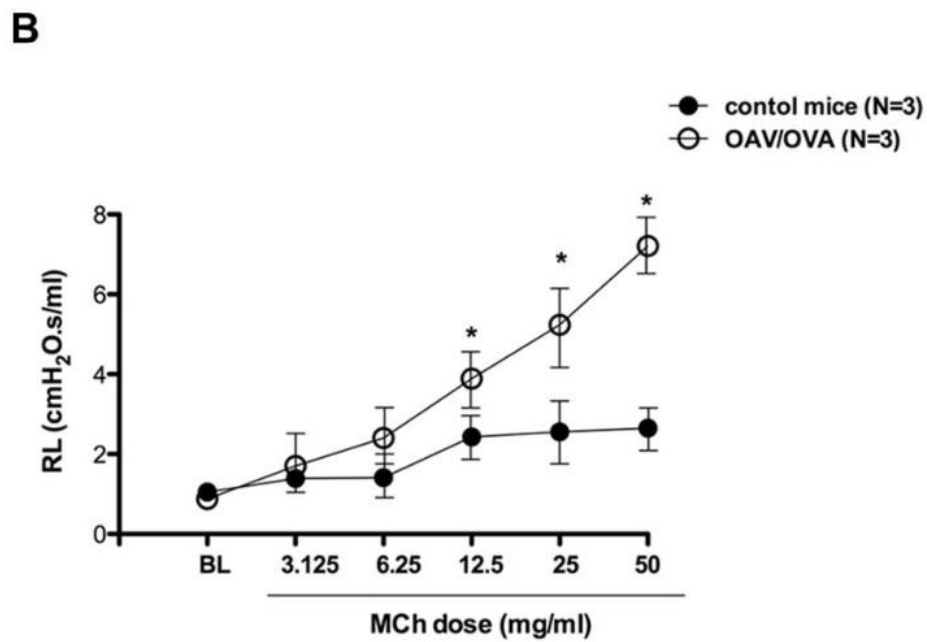
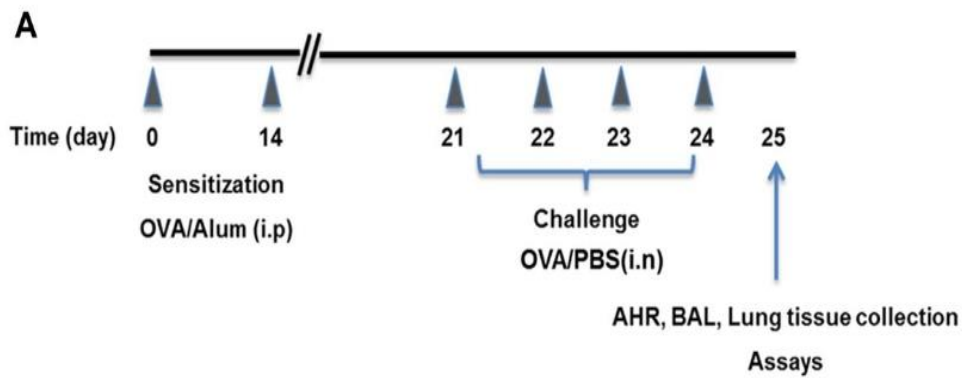
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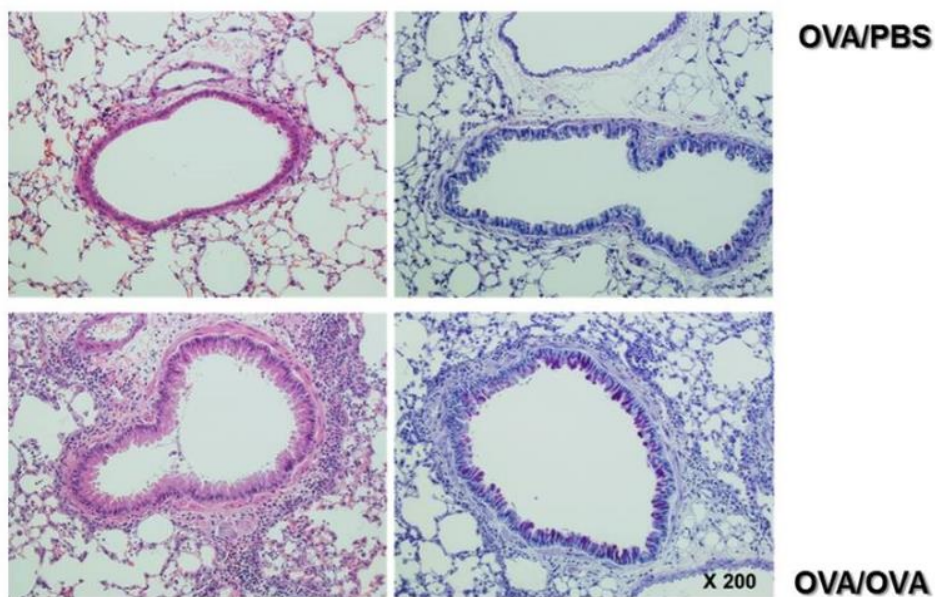
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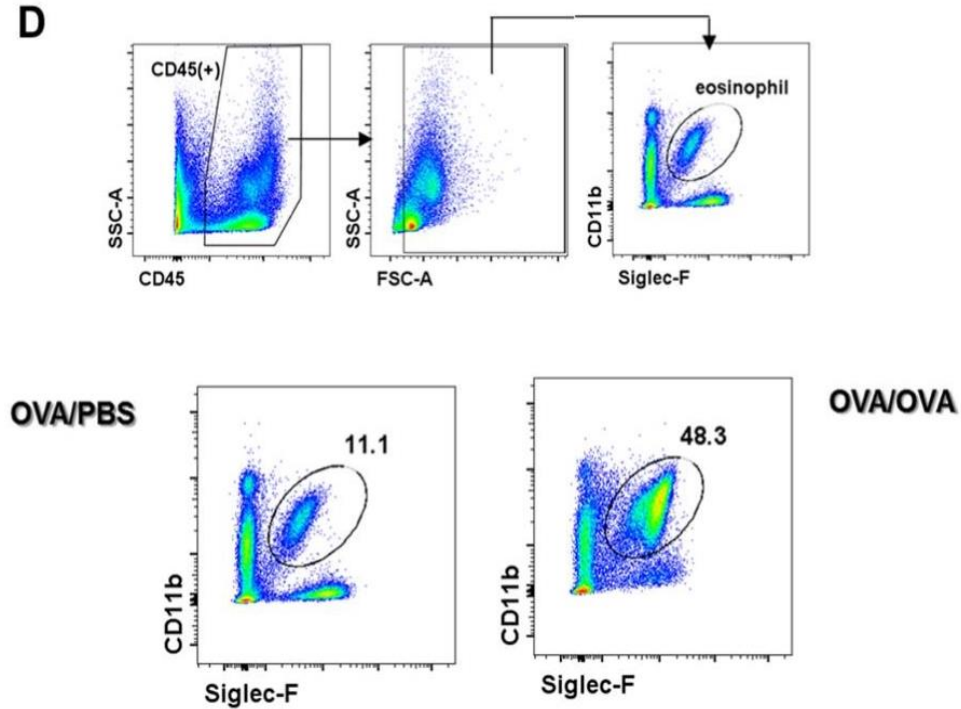
Figures



C



D



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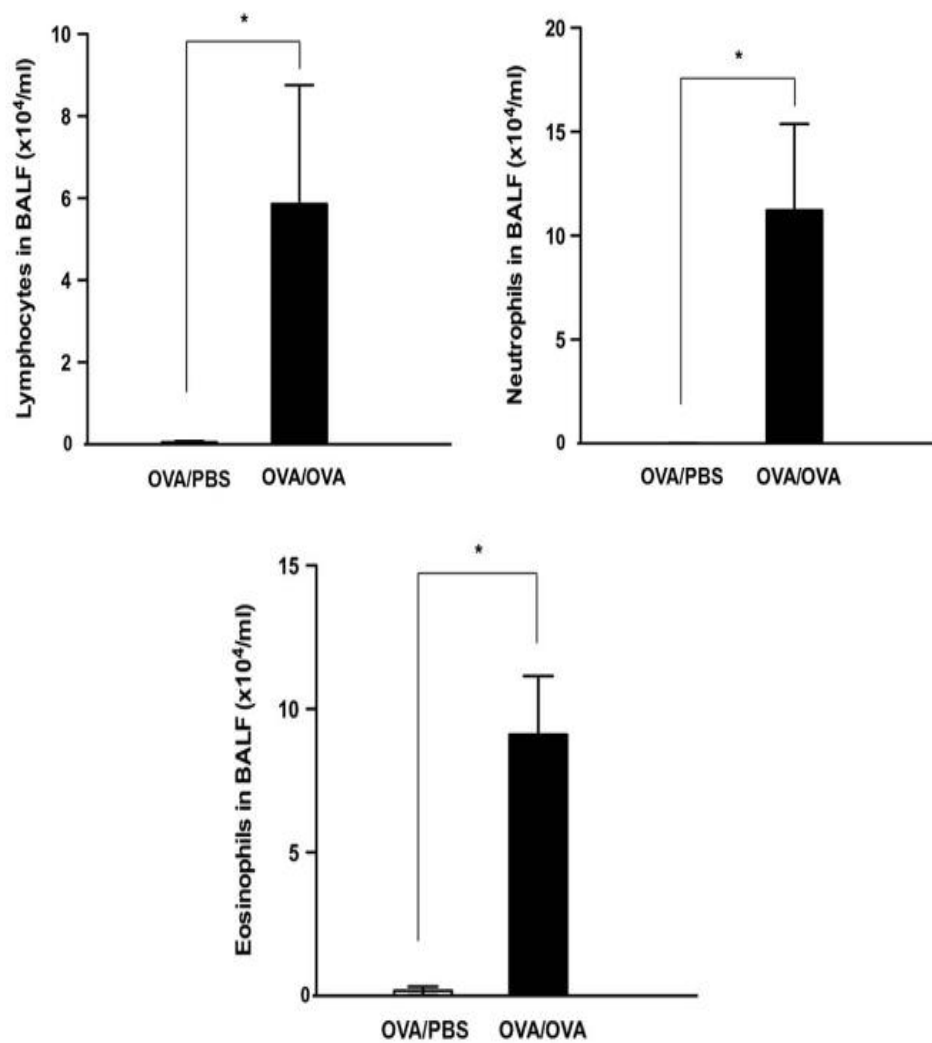
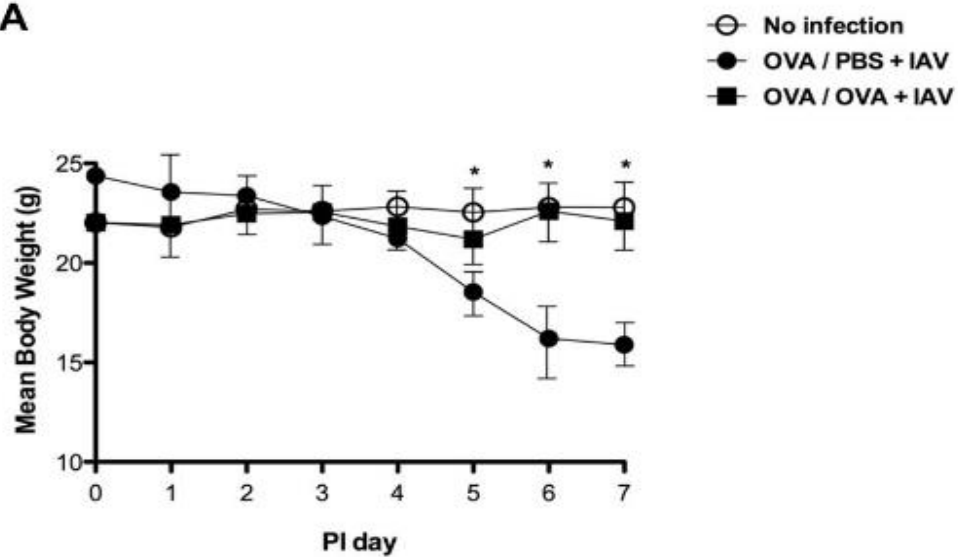
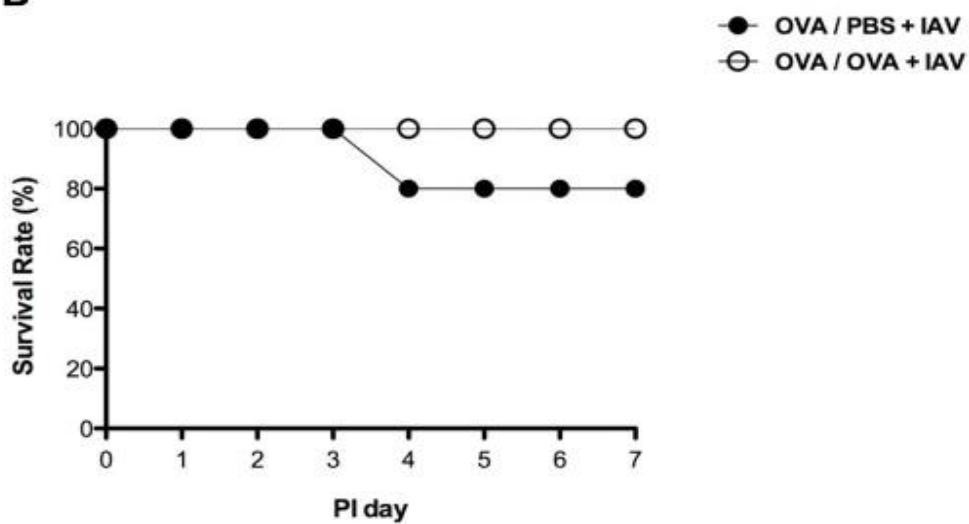
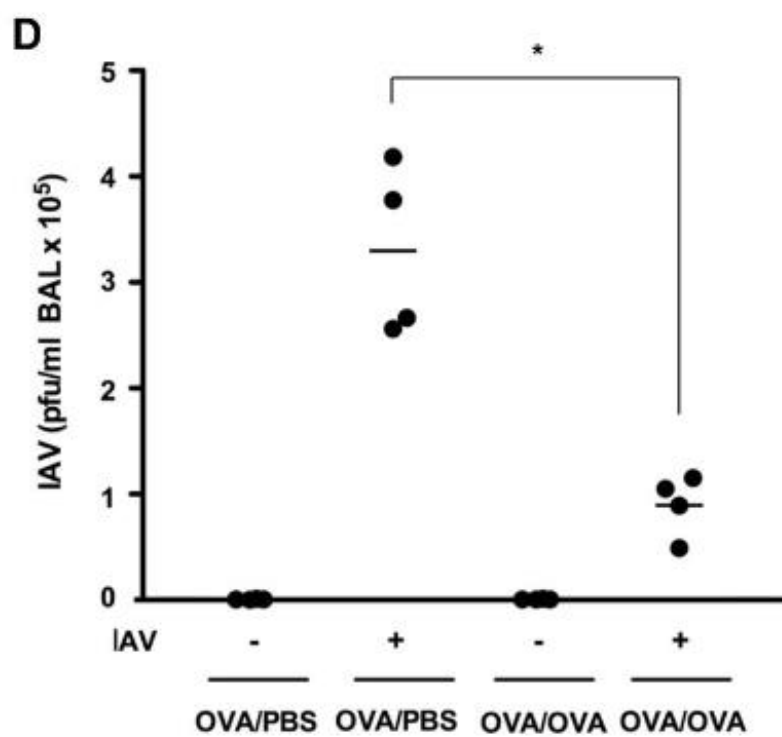
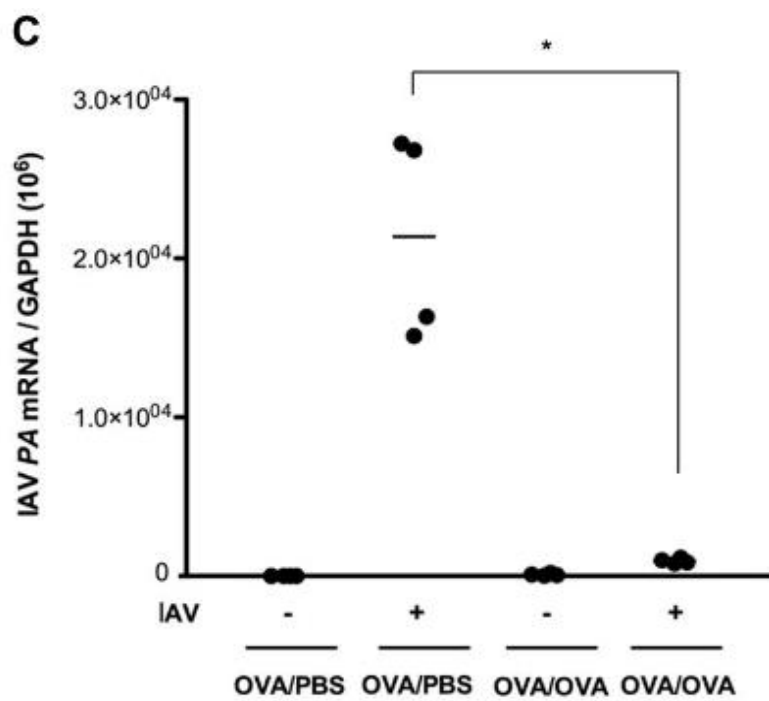


Figure 1. The phenotype of allergic asthmatic mice model

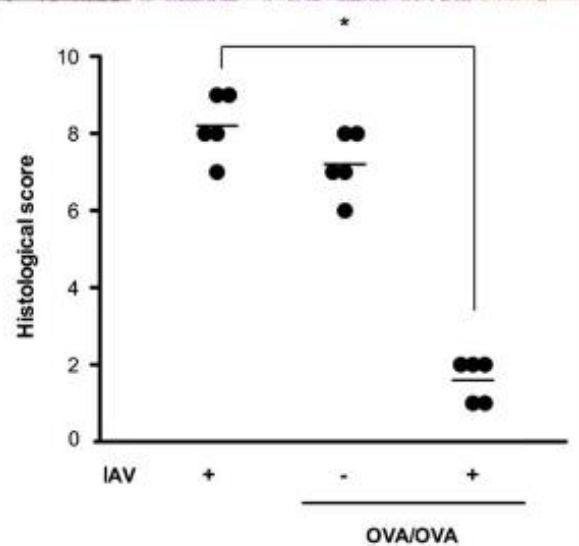
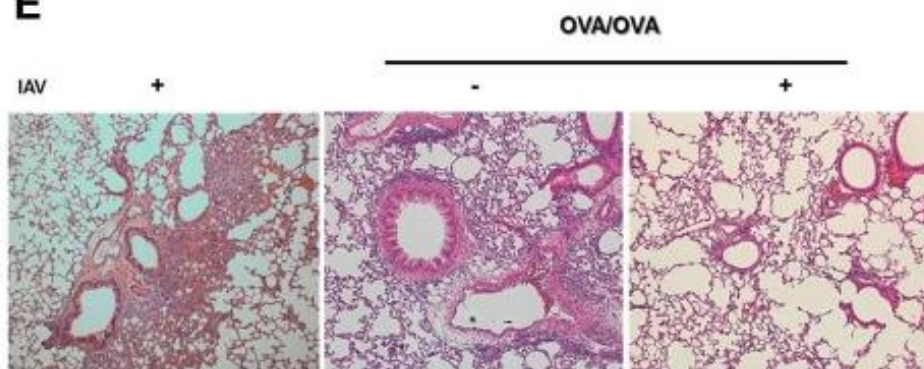
(A) The protocol for allergic asthmatic mice by sensitizing and challenging to C57BL/6(B6). *Non-asthmatic mice(OVA/PBS, N=5)

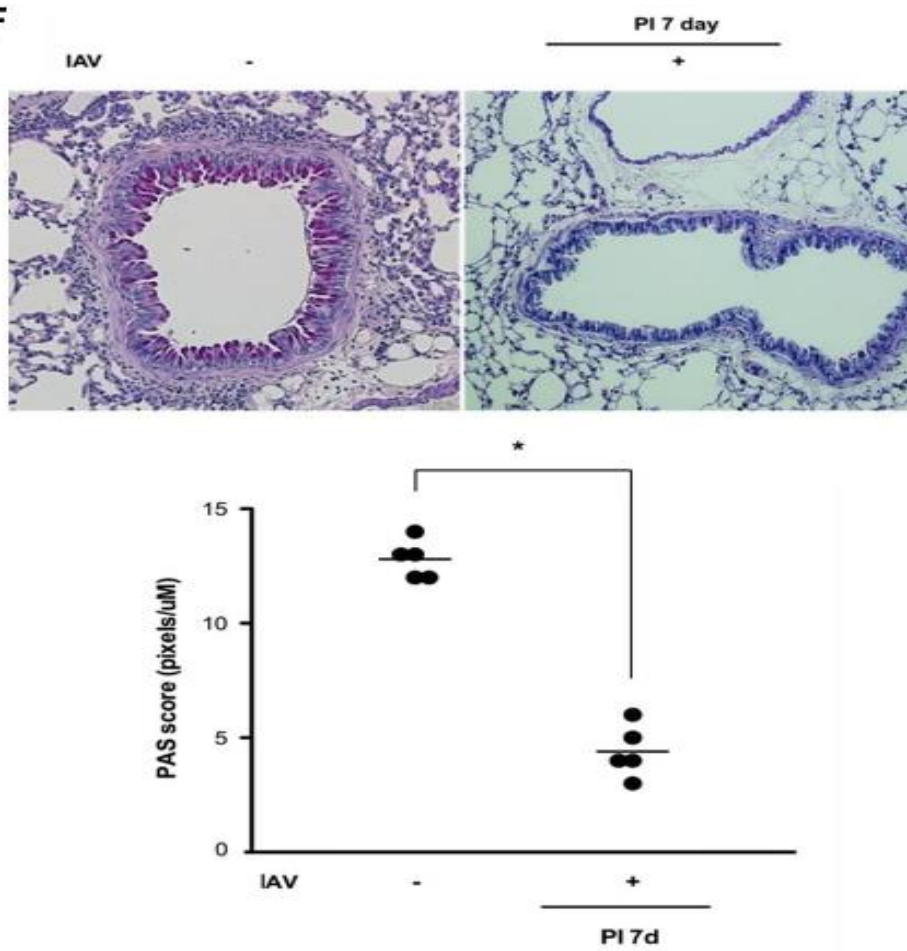
(B) Airway hyperresponsiveness was measured by exposing methacholine to induce in total lung resistance(RL) in asthmatic mice. The graph expressed mean value of increased lung resistance(RL) from base line(BL) in three mice (White dot: OVA/OVA, Black dot: OVA/PBS). (C) Histopathological analysis of mouse lung sections. Sections were stained with hematoxylin & eosin(H&E) and Periodic Acid Schiff(PAS) (Left: H&E stained, Right: PAS stained). (D) Flow cytometric analysis was performed for isolation of eosinophils by using anti-CD11c and anti-Siglec-F antibodies. (E) The graph showed count the number of lymphocytes, neutrophils and eosinophils separated from BAL fluid.

A**B**



E



F

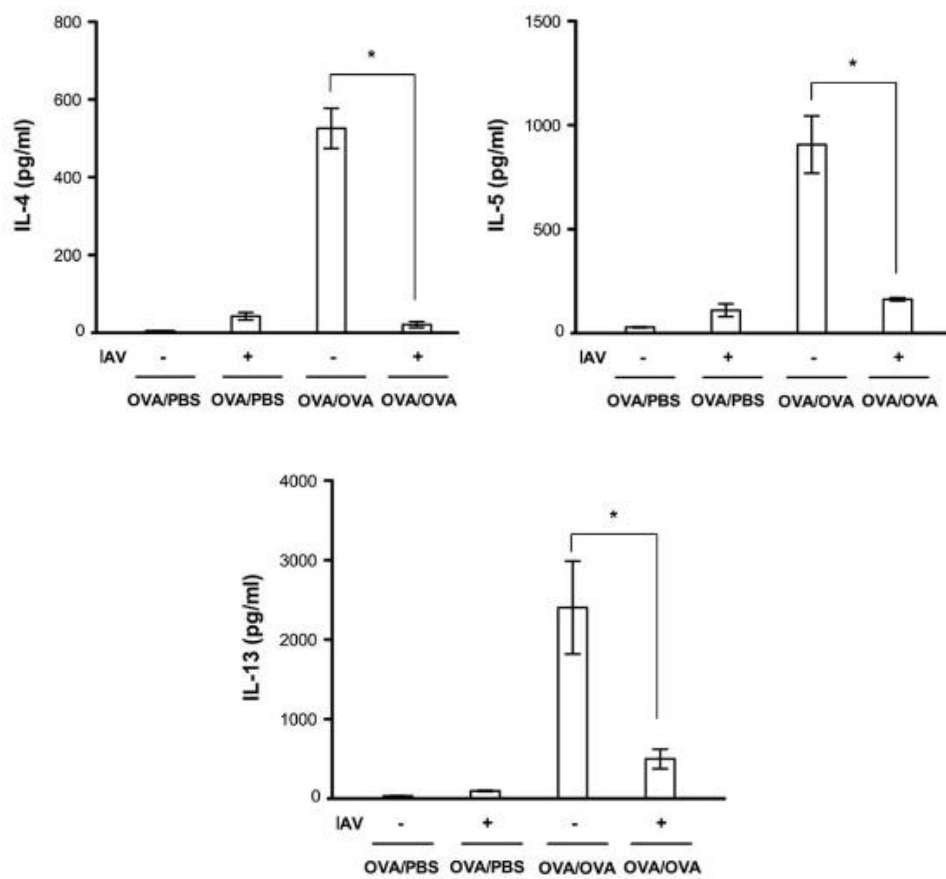
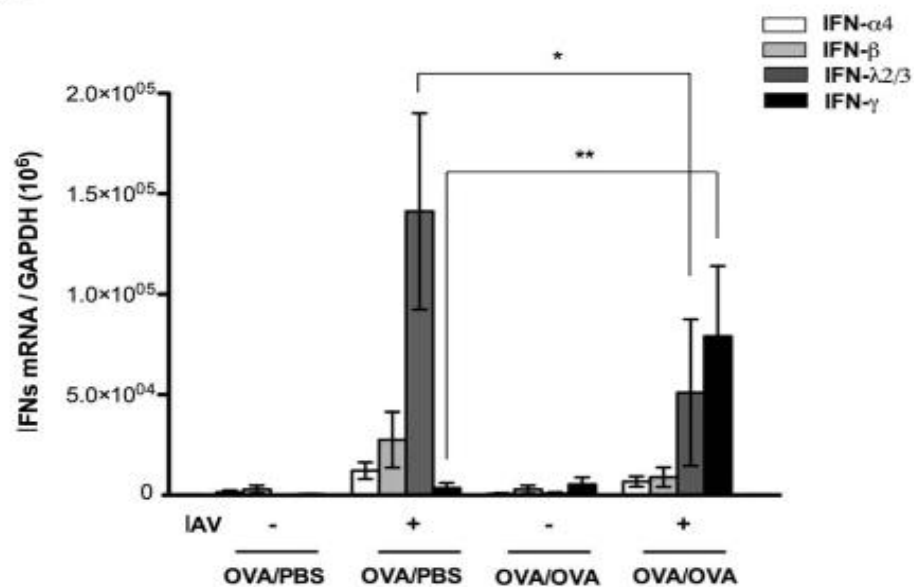
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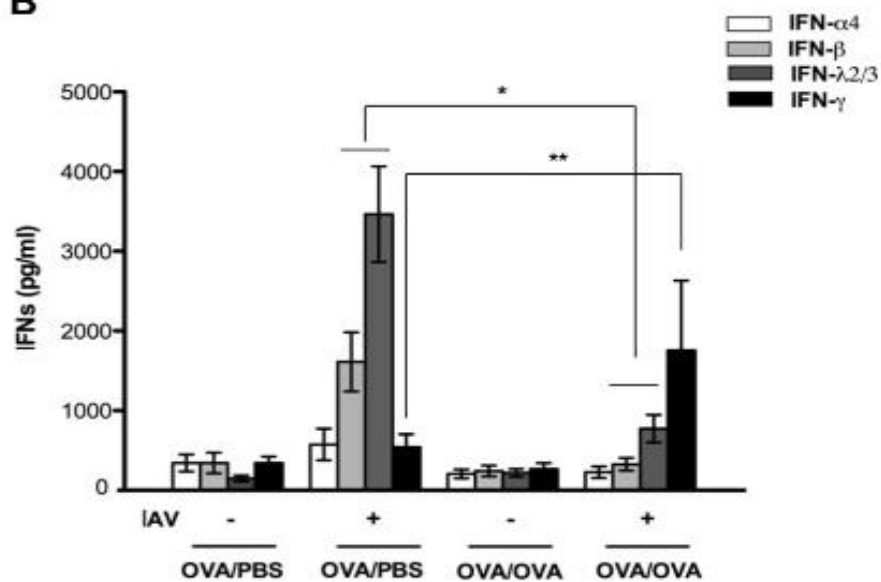
Figure 2. Difference of response from asthmatic and non-asthmatic mice after IAV infection

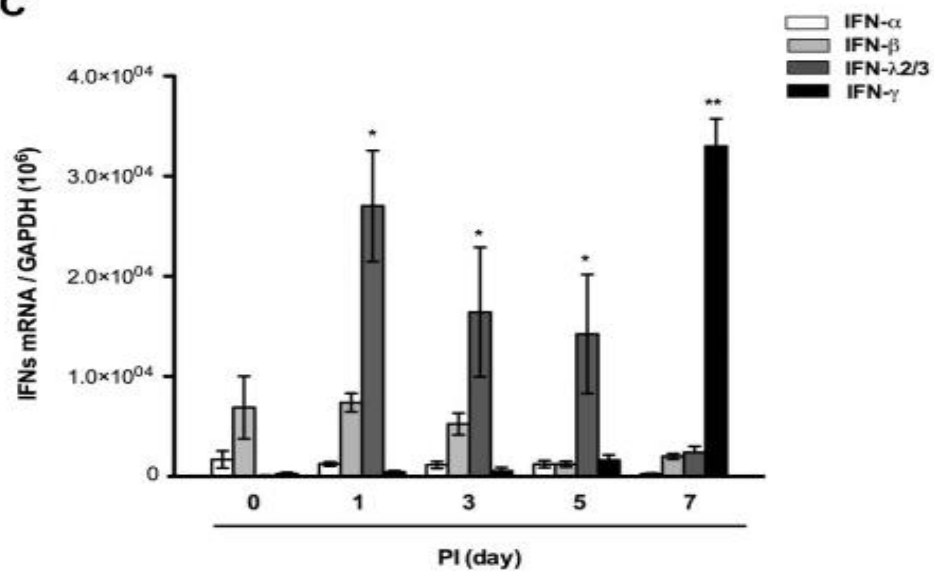
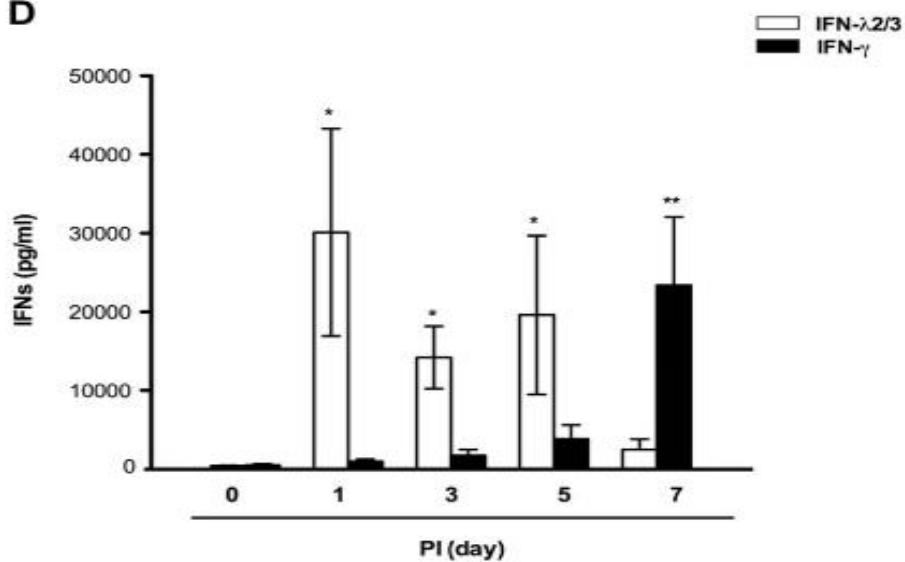
Asthmatic(OVA/OVA, N=5) and non-asthmatic mice(OVA/PBS, N=5) were infected with IAV(213pfu/30ul in PBS, WS/33, H1N1). After infection, body weight(A) and survival rate(B) were measured for 7 dpi. The gene expression level of IAV mRNA(C) and the level of viral titer(D) were measured in lung tissue and BAL fluid respectively on 7 days of infection. (E) The lung tissue section slides were stained with H&E dye, and then histological score were represented by the degree of infiltration of inflammatory cells. Also generated by PAS stain(F). (G) The secretion of Th2 cytokines such as IL-4, IL-5 and IL-13 were measured by a multiplex assay from BAL fluid of asthmatic and non-asthmatic mice. Histopathological image is one of the representative lung tissue slides from five mice. And all of the above results were represented as the average value of the five experiments (*, $P < 0.05$ compared with the levels of non-asthmatic and asthmatic mice on 7 dpi.)

A

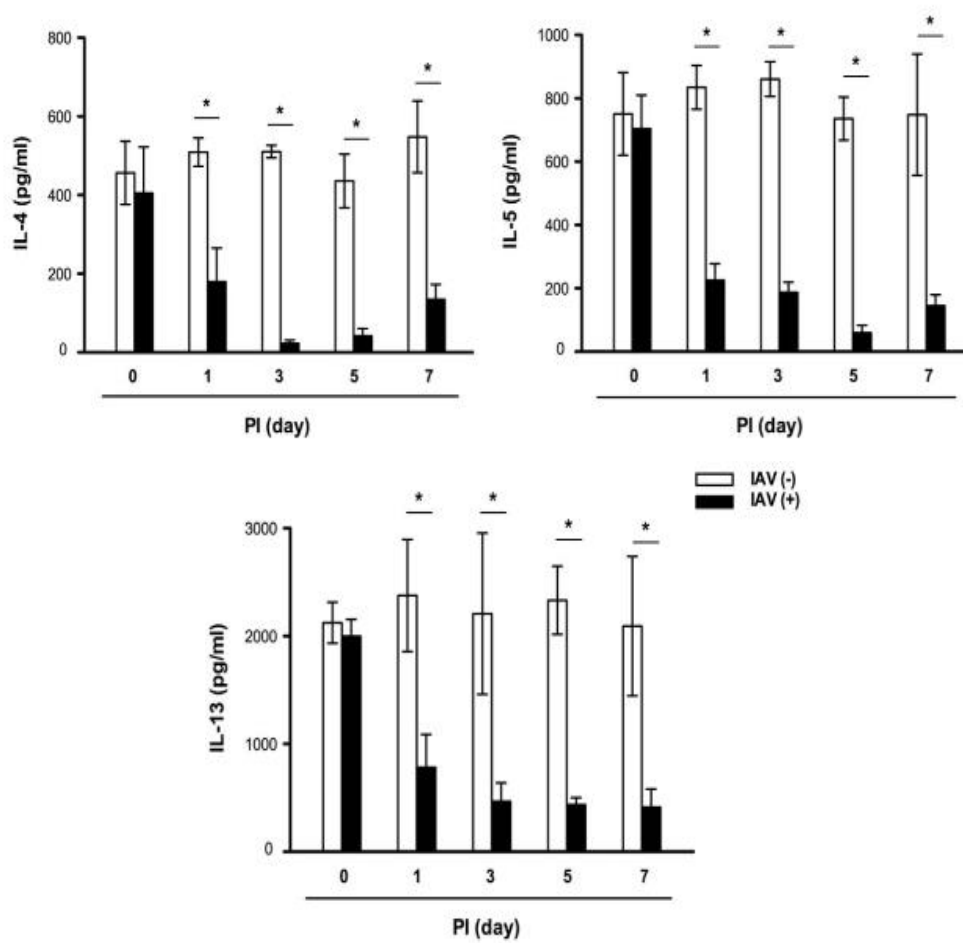


B



C**D**

E



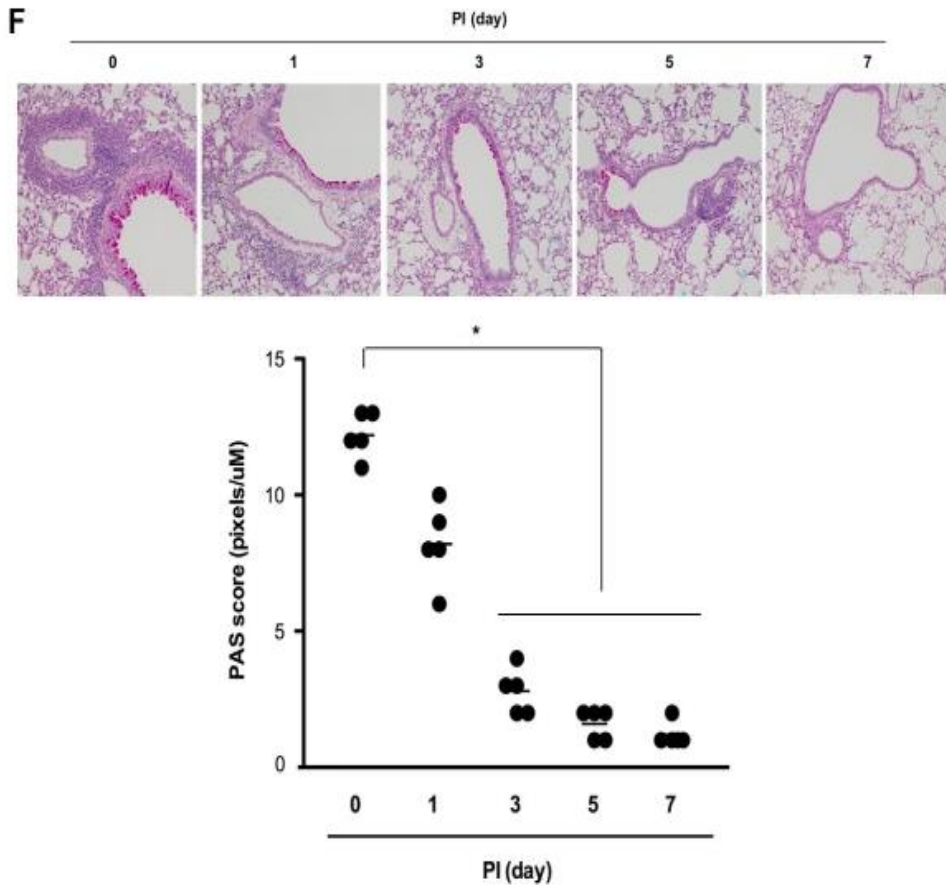
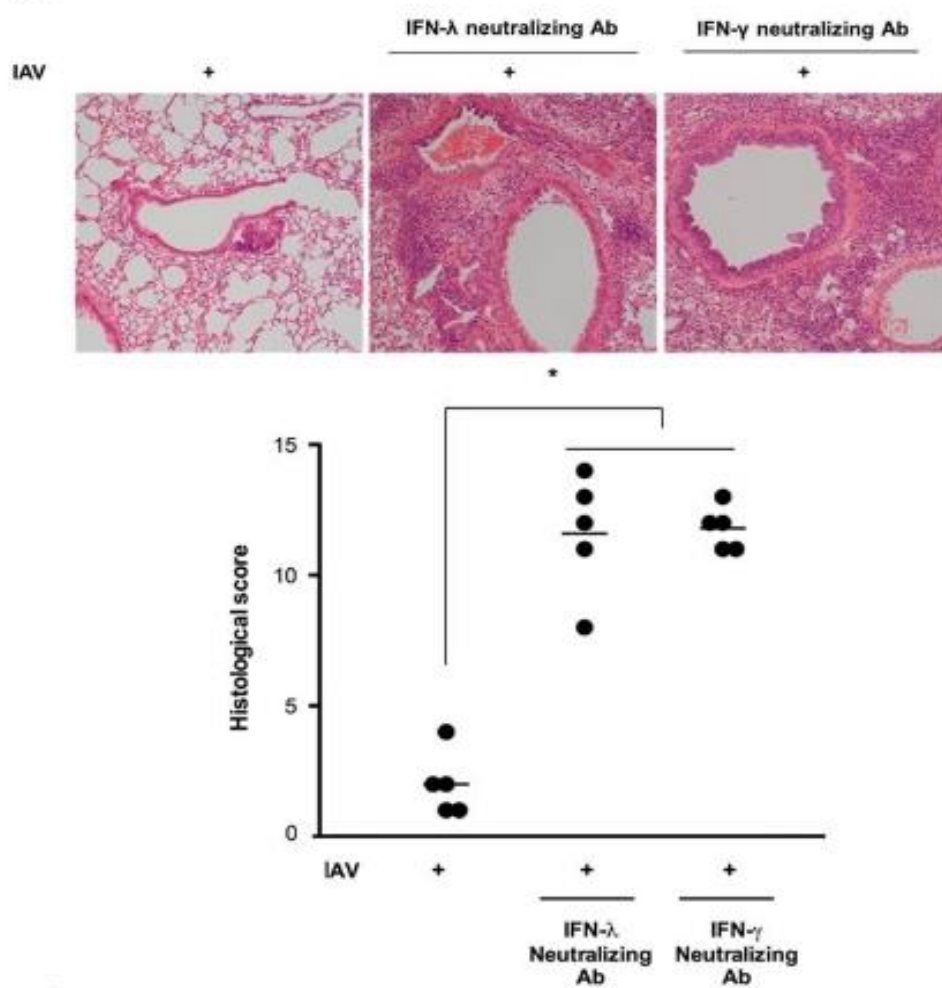


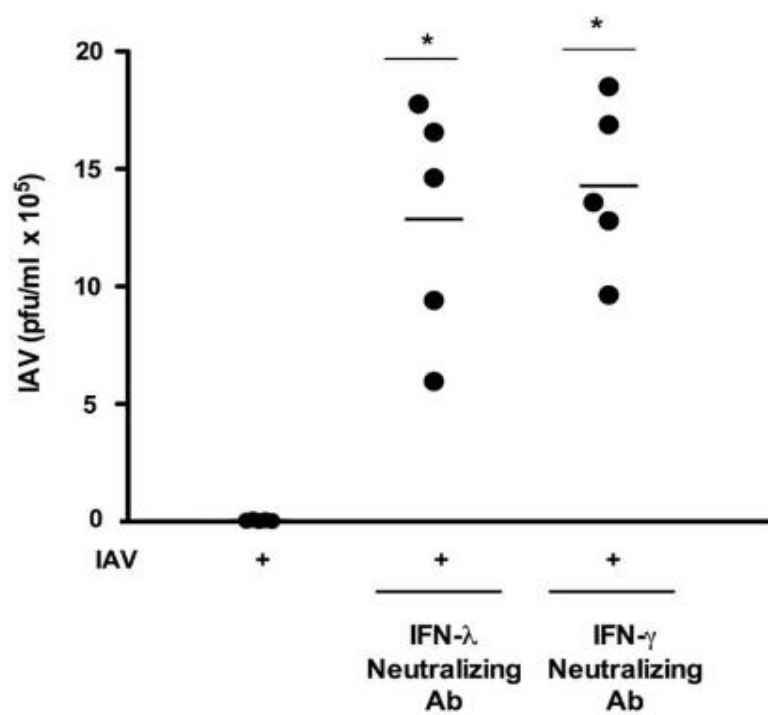
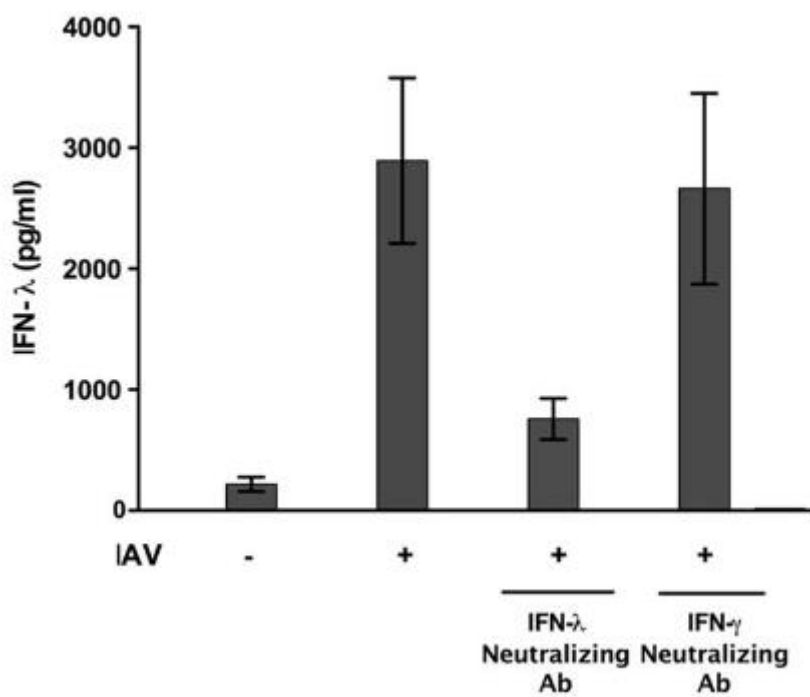
Figure 3. Expression of immune response in response to virus infection in asthmatic and non-asthmatic mice

Asthmatic(OVA/OVA, N=5) and non-asthmatic mice(OVA/PBS, N=5) were infected with IAV(213pfu/30ul in PBS, WS/33, H1N1). In each group, the gene expression level of IFNs mRNA (A) and the amount of secreted protein of IFNs (B) were measured by real-time PCR and ELISA respectively on 7 days of infection. In IAV-infected asthmatic mice group, the mRNA level (C) and the secreted protein level (D) of IFNs were

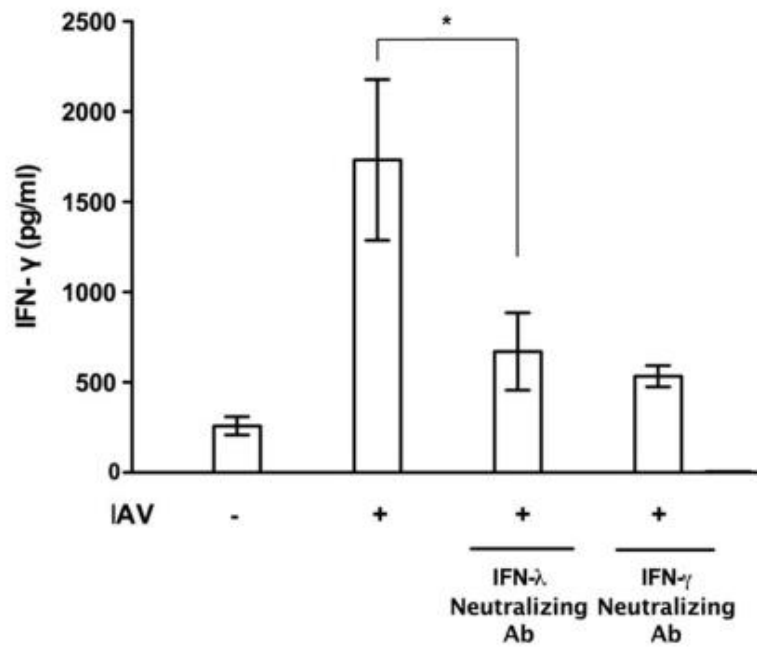
measured on 7 days after infection. (E) To quantify the level of secreted Th2 cytokines in BAL fluid, A multiplex assay was performed in IAV-infected and uninfected asthmatic mice on 7 days. (F) The PAS score was represented in count the number of mucus secreted cells in PAS-stained lung tissue section slides, which were harvested at 0,1,3,5 and 7 days after infection. Histopathological image is one of the representative lung tissue slides from five mice. And all of the above results were represented as the average value of the five experiments(*, **, $p < 0.05$ compared with the levels in asthmatic and non-asthmatic mice at 7 dpi, Figures 2B,2C,2D *:IFN- λ 2/3, **:IFN- γ).

A

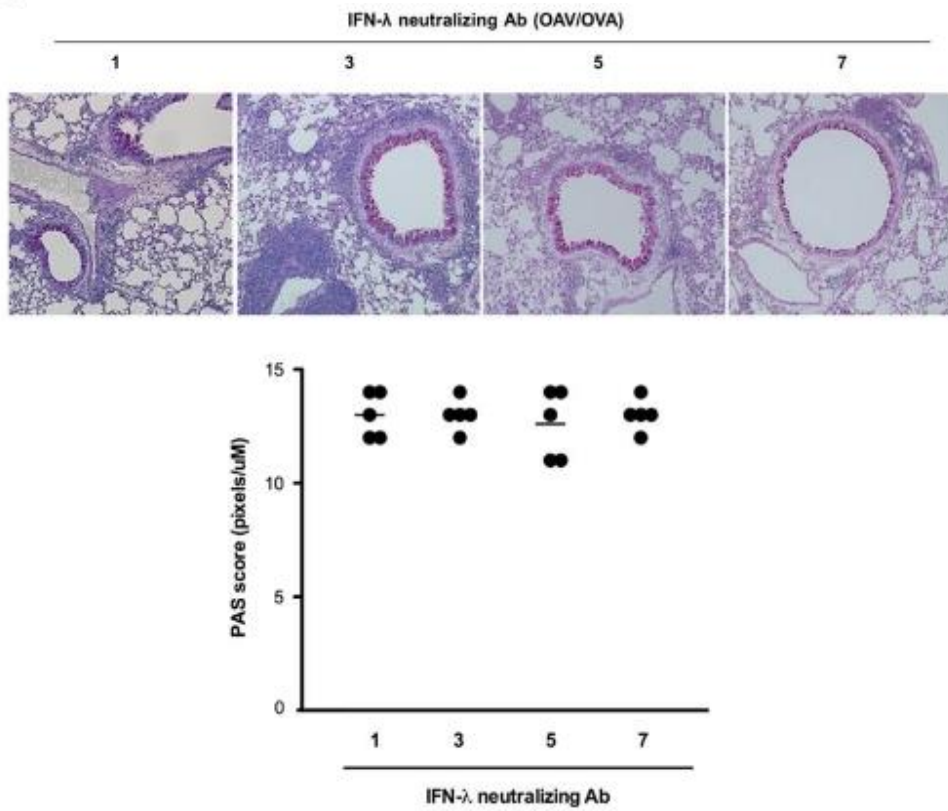


B**C**

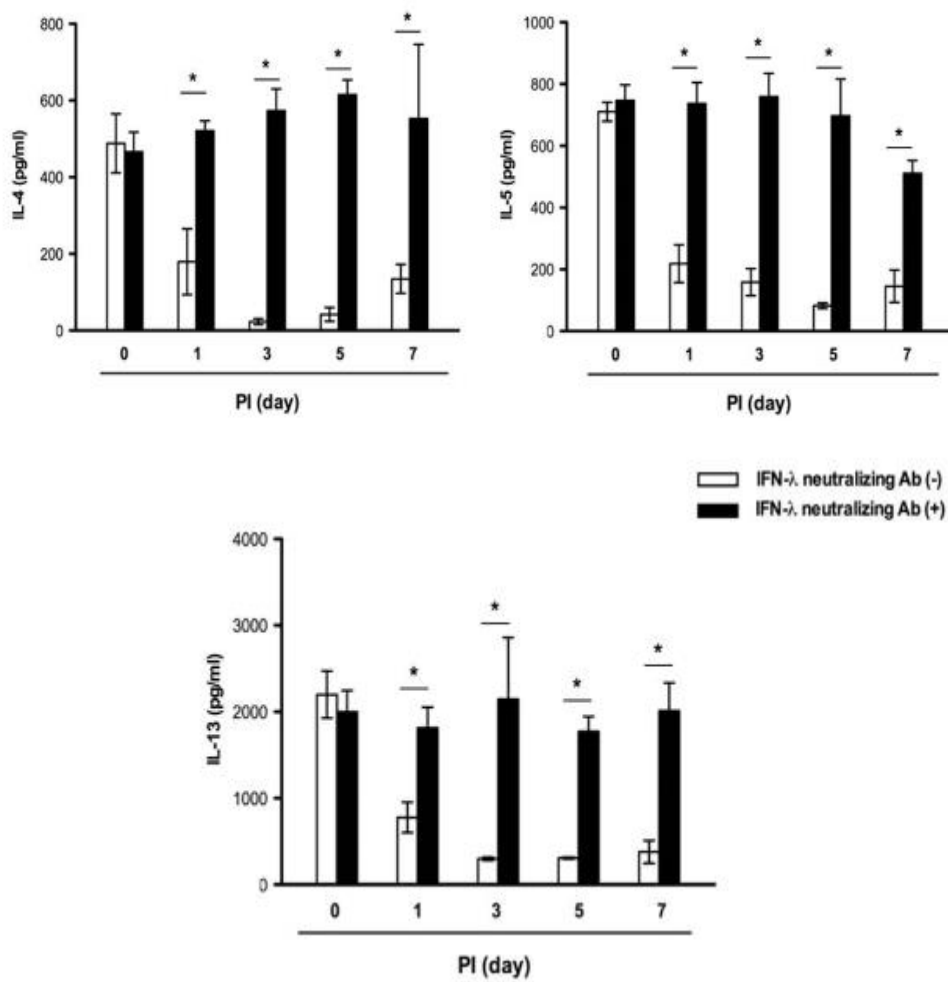
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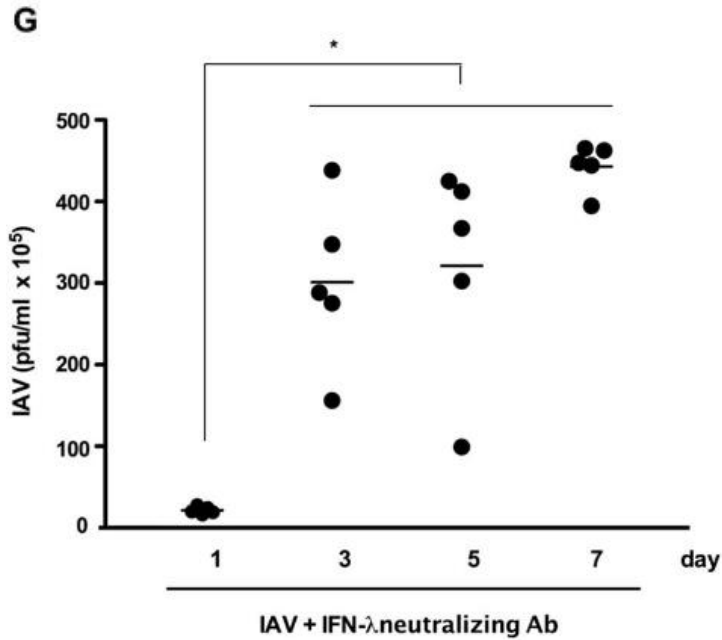
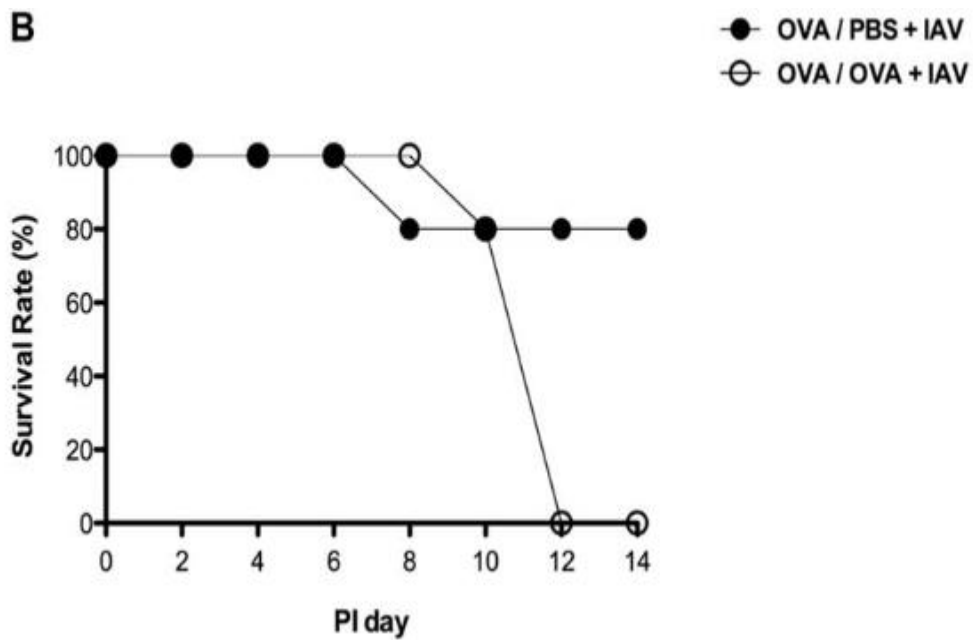
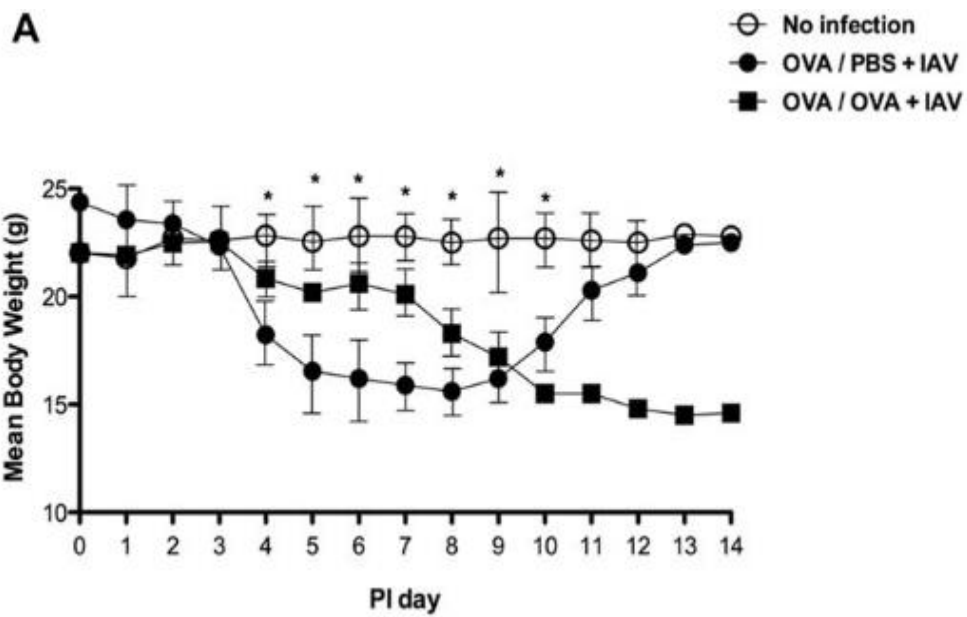
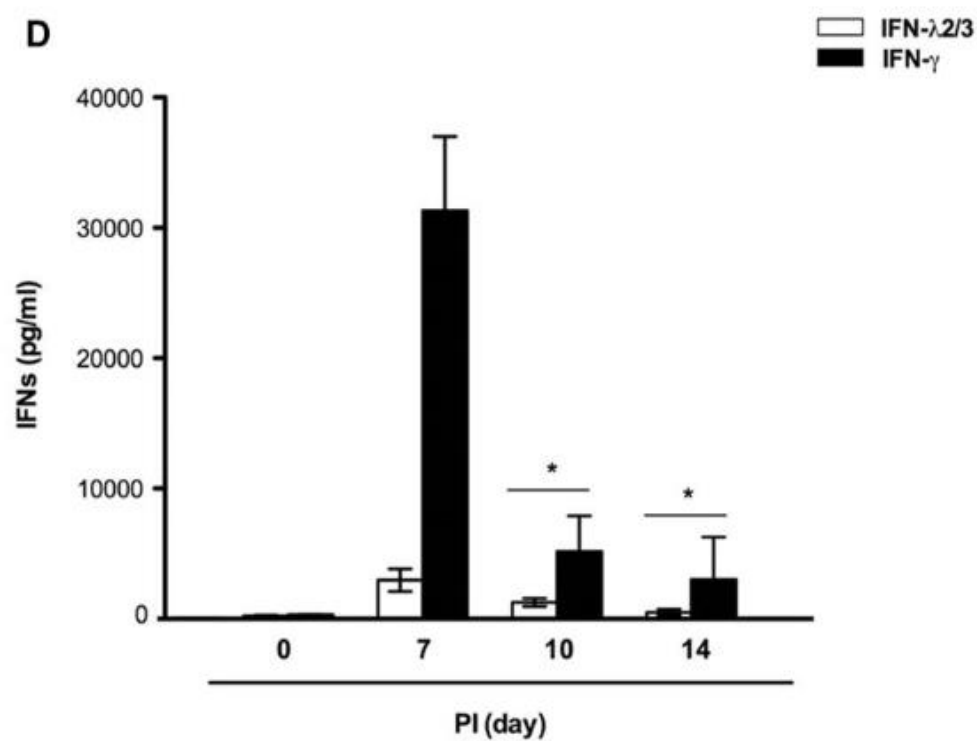
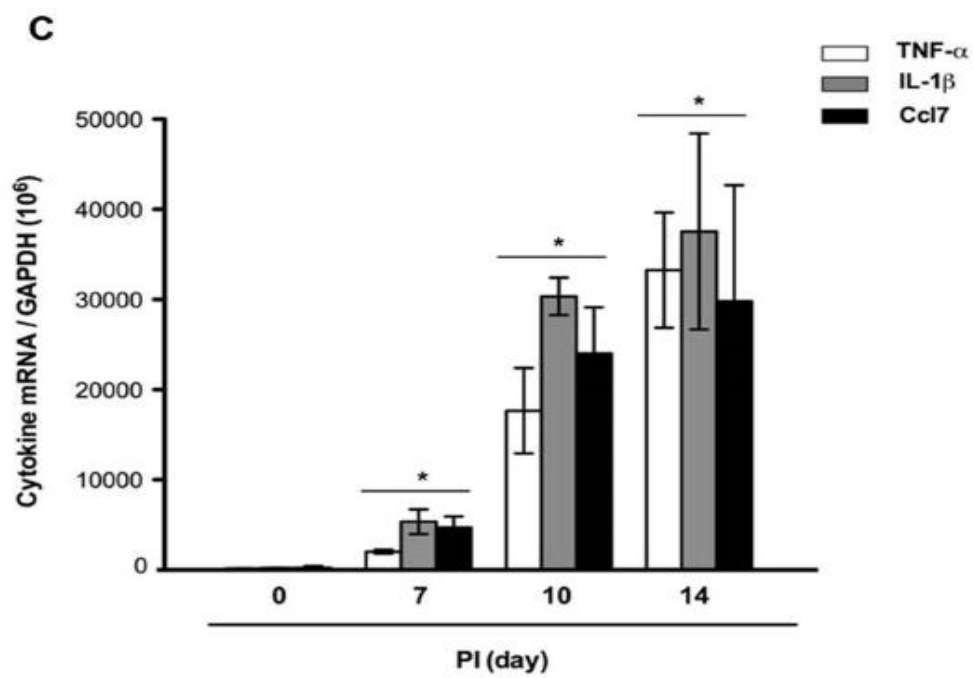


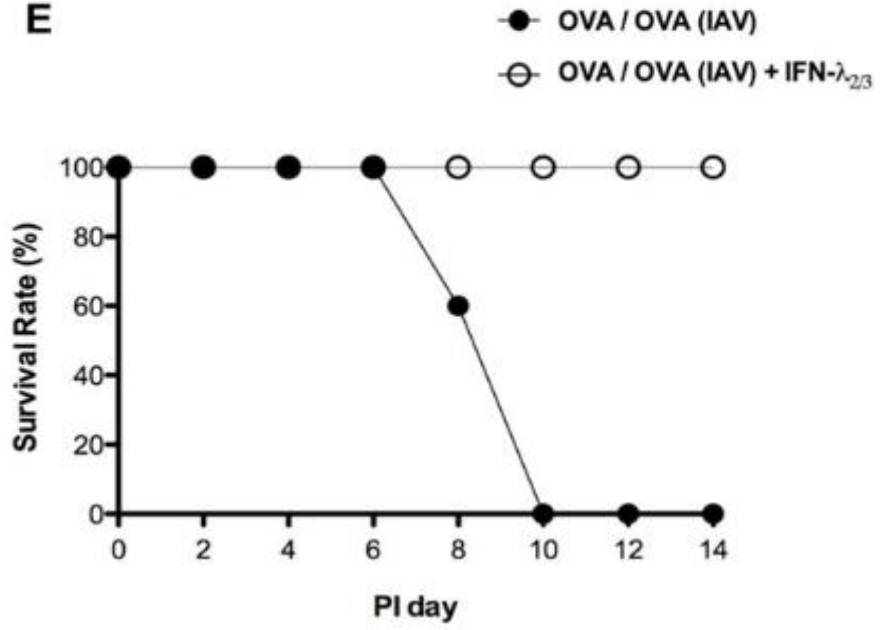
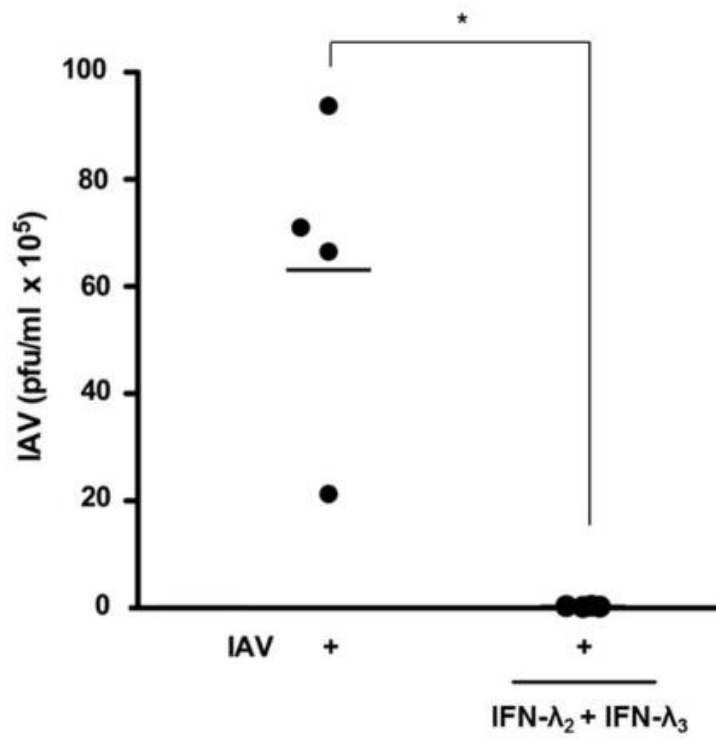
Figure 4. The administration of neutralized antibodies of IFN- λ 2/3 and IFN- γ further exacerbate the IAV infection.

Rat IgG as isotype control (N=5), IFN- λ 2/3 (N=5) and IFN- γ (N=5) neutralized antibodies were administered to IAV-infected asthmatic mice, respectively. (A) Histological score were measured in the lung tissue section slides stained by H&E on 7 days of infection in each group. Viral titer (B), the level of secreted IFN- λ 2/3 (C) and IFN- γ (D) were measured by performing the plaque assay and ELISA from BAL fluid of each group. In group of neutralized IFN- λ 2/3 antibody administered to IAV-infected asthmatic mice, PAS score (E) and viral titer (G) were measured by using PAS-stained lung section slides and the plaque assay at

1,3,5 and 7 day after infection. (G) The level of secreted Th2 cytokines, like IL-4, IL-5 and IL-13, were assessed by a multiplex assay at 0,1,3,5 and 7 day after infection. Five mice were harvested by date for each group (0 dpi: N=5, 1 dpi: N=5, 3dpi: N=5, 5dpi: N=5, 7dpi: N=5). Histopathological image is one of the representative lung tissue slides from five mice. And all of the above results were represented as the average value of the five experiments(*, $p < 0.05$ compared with the values of mice treated with IgG and neutralizing antibodies).





E**F**

G

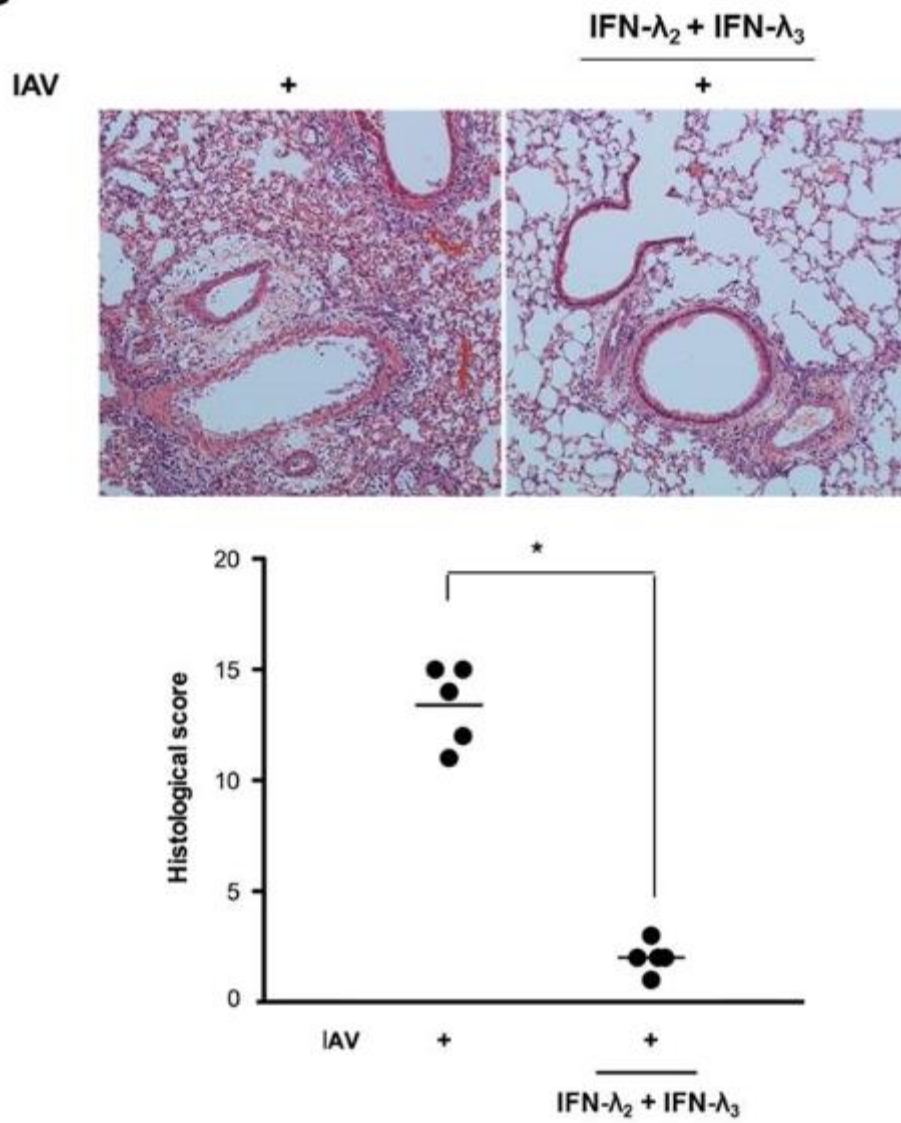


Figure 5. Exogenous IFN- λ 2/3 could also resist to IAV infection in the lung of asthmatic mice.

Asthmatic(OVA/OVA, N=5) and non-asthmatic mice(OVA/PBS, N=5) were infected with IAV(213pfu/30ul in PBS, WS/33, H1N1). After infection, body weight (A) and survival rate (B) were measured for 14 dpi. The gene expression level of cytokines mRNA, such as TNF- α , IL-1 β and Ccl7 (C), and the level of secreted protein of IFN- λ 2/3 and IFN- γ (D) were measured in the lung tissues and BAL fluid of each group. IAV-infected asthmatic mice were treated with recombinant IFN- λ 2/3(IFN- λ 2: 1ug, IFN- λ 3: 1ug in 30ul PBS) protein (N=5). As a control group, used only PBS(N=5). (E) Survival rate was measured for 14 days after infection. And histological analysis (G) and viral titer (F) were assessed on 7 days of infection. Histopathological image is one of the representative lung tissue slides from five mice. And all of the above results were represented as the average value of the five experiments(*, $p < 0.05$ compared with the values of mice treated with recombinant IFN- λ 2/3)

국문 초록

알레르기 천식에서 인플루엔자 바이러스
감염 초기에 유도되는
제 Ⅲ형 인터페론에 의한 바이러스
저항성

안 수 진

의학과 중개의학전공

서울대학교 대학원

배경 : 천식에 걸렸을 때, 기도는 염증이 생기고 부풀어 올라 좁아지게 됩니다. 천식에서는 2형 보조 T세포(Th2 cell)에서 분비되는 IL-4, IL-5, IL-13과 같은 물질에 의해 알레르기 염증 반응이 유발되는데, 2형 보조 T세포의 면역 반응 (Th2 immune response)이 상대적으로 우세하게

나타나면서 염증이 심화되게 됩니다. 2형 면역(Th2)의 과발현은 1형 면역(Th1)을 저하시켜 바이러스에 대한 방어 기전을 약화시킨다고 추정 되기 때문에, 천식은 호흡기에 감염되는 바이러스, 특히 인플루엔자 바이러스에 취약할 것이라 여겨집니다.

목적: OVA로 유도된 천식 마우스 모델을 바탕으로, 실제로 인플루엔자 A형 바이러스(IAV)를 감염시켰을 때, 정상 마우스에 비교하여 더 감염에 취약한지 알아보고, 감염 후 7일 동안 마우스의 폐에서 일어나는 면역 기전에 대해 알아보하고자 이 실험이 시행되었습니다.

방법: B6 마우스에 오브알부민(OVA)을 두 번씩 이주 간격으로 복강 주사 후, 일주일 뒤 네 번의 비강 내 주입으로 천식을 유도시킵니다(N=5). 그리고, 천식을 유도시키지 않은 마우스(N=5)와 함께 213pfu/ml의 농도로 인플루엔자 A형 바이러스를 비강 내 주입시켜 바이러스에 감염된 상태로 만들어 줍니다. 감염 후 일주일 동안 체중을 측정하고, 0,1,3,5,7일 별로 폐 조직에서의 바이러스의 유전자(viral mRNA) 발현 정도, 바이러스 수치(viral titer), 폐 조직의 조직학적 소견, 인터페론 유전자(interferon mRNA)의 발현 정도와 단백질의 분비 정도를 비교 분석해보았습니다. 또한, 천식 마우스에 인터페론 람다와 감마의 중화 항체(neutralizing Antibody)를 투여하여 세포 밖으로 분비된 람다와 감마의 기능을 억제한 다음에 바이러스 감염 정도를 확인하는 실험이 있었고, 외부에서 추가로

재조합 인터페론 람다 단백질(Recombinant protein)을 투여하고 난 후에 그 효과를 확인해보는 실험을 진행하였습니다.

결과: 천식이 유도된 마우스와 정상 마우스에 각각 인플루엔자 A형 바이러스를 감염시키고 7일 동안 관찰해보았을 때, 바이러스의 mRNA 발현 정도와 바이러스 수치(viral titer)가 천식 마우스에서 더 낮게 나타난 것을 확인할 수 있었습니다. 분비된 면역인자를 보면, 천식 마우스에서 인터페론 람다가 감염 후 1일째에 급격하게 증가하는 것과, 인터페론 감마가 7일째에 가장 높은 수치로 분비되는 것을 볼 수 있습니다. 또한, 람다의 초기 유도에 의해서 2형 보조 T세포 사이토카인(Th2 cytokines)인 IL-4, -5, -13이 유의하게 감소하였고, 폐 조직 슬라이드에서는 7일 동안 시간이 지날수록 염증 세포의 침윤 정도가 완화되는 것을 관찰할 수 있었습니다. 인터페론 람다, 감마의 중화 항체를 투여해 주는 실험을 통해서, 각각 인터페론 람다와 인터페론 감마의 효과를 억제한 상태에서의 면역 반응을 확인해 볼 수 있었습니다. 그 결과로는 람다의 중화 항체를 투여한 천식 쥐에서 바이러스 감염 정도가 낮게 나타나고, 인터페론 람다와 감마의 분비량은 줄어드는 것을 발견했습니다. 감마의 중화 항체를 투여한 쥐에서도 비슷한 결과로 염증이 심화되고 바이러스 수치도 증가하는 양상을 보였지만, 인터페론 람다의 분비량은 줄어들지 않고 유지하는 것을 알 수 있었습니다. 감염 후 7일 이후로 경과를 보았을 때, 감염 천식 쥐에서 급격한 체중의 감소와 함께 사망률이

증가하였고, 인터페론 람다와 감마 분비량의 급격한 감소와 염증 유발 사이토카인 발현의 증가가 나타났습니다. 이렇게 인터페론 람다의 분비가 감소된 상태에서 재조합 인터페론 람다 단백질을 추가로 투여해 주면, 외부에서 주어진 인터페론 람다에 의해서도 항바이러스 효과가 나타나 감염 정도가 약화되는 것을 확인할 수 있었습니다.

결론: 천식 마우스는 바이러스 감염에 취약할 것이라는 예상과 다르게, 감염 초기 빠르게 유도 되는 인터페론 람다에 의해 바이러스 저항성을 가지게 된다는 것을 알게 되었습니다. 인터페론 람다의 분비가 인터페론 감마의 분비를 증가시키고 2형 사이토카인의 분비를 감소시키면서 염증을 완화시키고 바이러스가 주변 세포로 퍼지는 것을 막아 주게 됩니다. 인터페론 람다에 의한 선천성 면역 반응은 초기에만 나타나는 것으로 관찰 되지만, 외부에서 추가적으로 주어지는 인터페론 람다 단백질에도 항바이러스 효과를 나타낸 다는 것을 알게 되었기 때문에, 인터페론 람다가 천식 환자에서 바이러스 감염의 치료제로 사용될 수 있을 것으로 보여집니다.

주요어: 제 3형 인터페론, A형 인플루엔자 바이러스, 인터페론 람다, 알레르기 천식

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