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**Defective innate immune responses to respiratory syncytial virus infection in
ovalbumin-sensitized mice**

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ABSTRACT

Background: Respiratory viral infections have frequently been reported to closely relate with asthma exacerbations. Distinctive expression of cytokine/chemokine and anomalous responses of innate immunity induced by respiratory viral infections were suggested to play a key role. This study further evaluates the effects of airway sensitization on innate immune in respond to different viruses.

Methods: Murine sensitization was established using ovalbumin sensitization model. Mice were subsequently infected with either respiratory syncytial virus or human metapneumovirus. Type I interferon (IFN), cytokines and chemokines were measured in bronchoalveolar lavage fluid. Pulmonary tissue samples were collected for the analysis of viral titers and type I IFN signal transcriptors.

Results: Distinct expressions of cytokine/chemokine responses after viral infection were also found in mice with ovalbumin-sensitization. Significantly increase of virus replication was found in lungs of RSV-infected sensitized mice. The increment of RSV titer was associated with the decreased levels of type I IFN. Although significantly increased expression of Toll-like receptor 3 were found in the lungs, the key signal transcriptor, interferon regulatory factor 3, was significantly suppressed in the RSV-infected sensitized mice.

Conclusion: The defective anti-viral innate response was observed in murine respiratory allergy model. Suppressed expression of IFN signal transcriptor contribute to decreased production of type I IFN. The defective innate immune response might result to the acute viral exacerbations of allergic airway diseases.

INTRODUCTION

Asthma is an allergic airway disease characterized by chronic inflammatory reaction and airway hyperresponsiveness (AHR). During episodes of asthma exacerbations, respiratory viral infections can be detected in nearly 80-85% of school-age children and 75-80% in adults.^{1,2} Respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) belong to the family of *Paramyxoviridae*. They share similar clinical manifestations and seasonal epidemics in young children.³ Both are often detected from young children with asthma exacerbation.⁴⁻⁸

The question about whether respiratory virus induces asthma exacerbation or if asthmatics are more prone to respiratory viral infection still remains an enigma. Report from Wark *et al.* has shown an increased viral replication after rhinovirus inoculation in *ex-vivo* cultured primary bronchial epithelial cells collected from asthmatic adults.⁹ Decreased expression of type I interferons (IFNs) and inhibited apoptosis of infected bronchial epithelial cells were suggested to contribute to the increased viral replication.⁹ Another important antiviral immune response is the pattern-recognition receptors (PRRs)-induced type I IFNs production. Among these PRRs, retinoic acid-inducible gene I (RIGI) and Toll-like receptor 3 (TLR3) seemed to play an important role in mediating the recognition of RSV and hMPV.^{10,11} PPR domains transmit signal into the nucleus through several transcription factors. The key signal transcriptor, such as interferon regulatory factor 3 (IRF3), IRF7, and nuclear factor- κ b (NF- κ b), once activated, initiate IFNs responses.¹²

In this study, we examined how sensitization affects innate immune responses. Ovalbumin (OVA)-sensitized mice were utilized in the infection of RSV or hMPV. The study demonstrated that after RSV infection, sensitization status impaired type I IFN expression, and resulted in enhanced viral replication. Gene and protein analysis

further verified that the reduced expression of IRF3 contributed to the decline of type

I IFNs

MATERIALS AND METHODS

Mice and Viral Stocks

Six- to eight- week-old BALB/c female mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). All mice were housed in accordance with the guidelines of National Institute of Health for animal health care. The hMPV strain CAN97–83 and RSV A2 were obtained from the University of Texas Medical Branch, Galveston, TX, with kind permission from Dr. Roberto P Garofalo (Division of Clinical and Experimental Immunology & Infectious Diseases, University of Texas Medical Branch, Galveston, TX). hMPV was propagated in LLC-MK2 cells (ATCC CCL-7), and viral titer was determined by a TCID₅₀ plaque assay as described previously.¹³ RSV A2 was grown in HEp2 cells (ATCC CCL-23), and viral titer was determined by a methylcellulose plaque assay, as previously described.¹⁴

Experimental Protocol

The study protocol was outlined as Figure 1. The OVA sensitization and viral inoculation were described as below. At day 0, mice were i.p. immunized with 50 µg OVA (Sigma-Aldrich, St. Louis, MO) and 2 mg aluminum hydroxide. Mice were subsequently challenged with 1% OVA solution diluted with sterilized PBS daily using a small volume nebulizer on day 14 to 16. At day 16, mice were inoculated intra-nasally with purified hMPV, RSV, or PBS. 1×10^7 pfu hMPV or RSV was used for infecting one mouse. Analytic procedures were performed on day 17. Lung virus titers were determined at day 21 (day 5 post-infection). Mice were divided into ‘Mock’, ‘OVA mock’, ‘RSV’, ‘OVA+RSV’, ‘hMPV’, and ‘OVA+hMPV’ groups. The detailed manipulations of each group were showed in Figure 1.

Samples Collection

On protocol day 17, one day after the last OVA challenge, mice of the different

groups were sacrificed. Bronchoalveolar lavage (BAL) was performed with 1.0 ml of ice-cold PBS. BAL cells were counted, fixed, and stained using the Protocol Hema3 kit (Fisher Diagnostics, Middletown, VA). Cellular differentials of BAL fluid were determined. Supernatants of BAL fluid were collected for determining the levels of cytokine and chemokine. After lavage, lungs were collected for histopathological exam or stored at -80°C until quantitative real-time polymerase chain reaction (RT-qPCR) analysis. On protocol day 21, lungs were collected and stored at -80°C until the titration of viral titer.

Viral titration of murine lungs

Harvested murine lungs for viral titration were homogenized in 1 ml DMEM and serially log₁₀ diluted and applied onto HEp2 cells for RSV titration or onto LLC-MK2 cells for hMPV titration as described above.

RT-qPCR

Harvested lungs for RT-qPCR were homogenized in 1 ml PBS. 100 µl of lung homogenates was treated with 1ml of TRIzol reagent to extract RNA. RNA was resuspended in 10 µl of diethyl pyrocarbonate-treated water. cDNA synthesis was performed using 1 µl of the RNA suspension and a random hexamer cDNA synthesis kit (Applera, Norwalk, CT). Two µl out of 20 µl of cDNA suspension were used for amplification in real-time quantitative PCR. The mRNA expression levels of IFN- α , - β , IRF3, IRF7, NF- κ B p65, RIG-I, and TLR3 were normalized for the amount of β -actin mRNA present in each sample.

Cytokine and Chemokine Profile

BAL supernatants were detected for multiple cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-Plex panel (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. The panel including the following: IL-

1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN- γ , KC, MCP-1, MIP-1 α , MIP-1 β , RANTES, and TNF- α . The broad assay range was from 2.0 – 5,000 pg/ml. Level of IFN- α and IFN- β was measured using ELISA kits (R&D Systems Inc., Minneapolis, MN).

Nuclear protein extraction and Western blotting

Nuclear protein was extracted from lung homogenates using Nuclear Extraction Kit (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. The nuclear protein extractions were boiled in 3 $\times$ Laemmli buffer for 2 min and resolved on SDS-PAGE. Proteins were transferred for 24h onto Hybond-ECL nitrocellulose membrane and immersing the membrane in TBST blocking solution containing 5% skim milk powder for 60 minutes blocked nonspecific binding sites. After a short wash in TBST, the membranes were incubated with the primary antibody overnight at 4°C, followed by a peroxidase-conjugated secondary antibody diluted in TBST for 60 min at room temperature. After washing, the proteins are detected using ECL according to manufacturer's protocol. The primary antibodies used for Western blotting are antibodies against NF- κ B p65 (Millipore, Billerica, MA), IRF3 (Abcam, Cambridge, MA), IRF7 (Lifespan, Seattle, WA), RIG-I (Abgent, San Diego, CA), and β -actin (Millipore, Billerica, MA).

Statistical Analysis

Statistical analysis was performed using InStat software (GraphPad, San Diego, CA). Data are presented as mean $\pm$ SEM values from 2 to 3 independent experiments. When two groups are compared, the values are analyzed using Mann-Whitney U test. The p value for significance was set at 0.05.

RESULTS

Viral infection attenuates the recruitment of airway eosinophil in OVA-sensitized mice

To determine the effect of respiratory viral infection on ovalbumin challenge, we analyzed the cellular spectrum of BAL fluid in OVA-sensitized/challenged mice after viral inoculation. Neutrophil was the predominant cell of BAL fluid in mice after viral inoculation. Although the ratio of airway eosinophil of OVA-sensitized mice significantly decreased after viral inoculation, especially after RSV infection (Figure 2A), the absolute number did not change (Figure 2B).

Distinct expressions of cytokine and chemokine in OVA-sensitized mice after viral infections

The concentrations of Th1/Th2 cytokines, pro-inflammatory cytokines, and chemokines in BAL fluid were measured on day 1 post-infection. Increased expression of IFN- γ after viral infection was seen in all groups. However, OVA+RSV mice expressed higher IL-4 and lower IL-5 than OVA mock mice ($p < 0.05$) (Figure 3A). Similar trend was also found in OVA+hMPV group. Regarding to the pro-inflammatory cytokine and chemokine responses, OVA+RSV group induced lower IL-1 α , IL-1 β , G-CSF, but higher MCP-1 response than the RSV group. Nevertheless, reduced GM-CSF production in the OVA+hMPV mice was the only difference observed between the hMPV groups (Figure 3B and 3C). In general, for other cytokine/chemokine responses, such as TNF- α , RANTES, MIP-1 α , and -1 β , RSV elicited stronger responses than hMPV (Figure 3B and 3C).

Increasing viral replication in OVA-sensitized mice correlates with the reduction of type I interferon production

To clarify if OVA-sensitization can change host's innate immune response to viral infection, mice with OVA immunization were infected with RSV or hMPV during the last day of OVA challenge. OVA-sensitized mice had higher peak viral titer than non-sensitized mice after RSV infection (36260 ± 4518 pfu/gm lung tissue vs 21240 ± 1306 , $p < 0.05$) (Figure 4A). On the contrary, there was no difference in peak hMPV viral titer between mice with and without OVA sensitization (Figure 4B). Due to the fact that the production of type I IFNs peaked at around 18 to 24 hours after RSV infection,¹¹ the levels of IFN- α and - β in BAL fluid were measured 24 hours after infection. Significant reduction of IFN- α and - β expression was found in OVA-sensitized mice with RSV infection (Figure 5A) ('RSV' vs 'OVA+RSA'; IFN- α : 1263 ± 194 vs 282 ± 78 pg/ml, $p < 0.005$; IFN- β : 1226 ± 125 vs 73 ± 43 pg/ml, $p < 0.005$). Although decreased expression of IFN- β was also found in OVA-sensitized mice with hMPV infection, there was no significant change in IFN- α production (Figure 5B) ('hMPV' vs 'OVA+hMPV'; IFN- α : 2506 ± 286 vs 2255 ± 212 pg/ml; IFN- β : 4585 ± 428 vs 3181 ± 395 pg/ml, $p < 0.05$).

Distinct expressions of IFN signal transcripts in OVA-sensitized mice responding to different respiratory viral infections

According to previous reports, the signal transcripts of type I IFN production began to rise at around 6hr post RSV and hMPV infection, and peaked after 15-24 hr [10, 11]. To elucidate the distinct expression of type I IFN between OVA-sensitized mice infected with RSV and hMPV, we extracted RNA from murine lung homogenates 24hr post-infection. RNA expressions were determined by using RT-qPCR, and

normalized by internal RNA expression of β -actin. The RNA expressions of TLR3, NF- κ b p65, IRF3, IRF7, and RIG-I were similar between allergic and mock mice after hMPV infection (Figure 6A). However, OVA-sensitized mice of the RSV group showed significant suppression of IRF3 RNA expression (Figure 6B) (expression of 'OVA+RSV' / expression of 'RSV' $\times 100\% = 17 \pm 4\%$, $p < 0.005$), and an increased TLR3 RNA expression (Figure 6B) (expression of 'OVA+RSV' / expression of 'RSV' $\times 100\% = 819 \pm 283\%$, $p < 0.05$). In addition, there were no significant differences for the expressions of NF- κ b p65, IRF7, and RIGI after RSV infection between the OVA-sensitized and non-sensitized mice. To further evaluate the protein expression of IFN signal transcriptors, we extracted nuclear protein from the murine lung homogenates. The result further proved that after RSV infection, OVA-sensitized mice exhibited diminished level of nuclear IRF3 protein, but not nuclear NF- κ b p65 and IRF7 (Figure 6C).

DISCUSSION

Asthma exacerbation is an exaggerated lower airway in response to an environmental exposure, which includes allergen, viral infection, pollutant, or certain medication. Airway inflammation is the critical component of the lower airway response in asthma exacerbation. The pattern of cellular profile is different depending on the trigger of asthma exacerbation. Instead of the eosinophilic bronchitis found in allergen-induced exacerbation, neutrophilic bronchitis is usually found in viral related asthma exacerbation.² In the murine OVA sensitization model applied to asthma study, airway inflammation characterized by eosinophil recruitment and type 2 cytokine responses were suggested to be the key component of allergen-induced airway hyper-responsiveness.¹⁵ However, Peebles and colleagues concluded differently, they have shown that deteriorated AHR of allergic mice during viral infection was not associated with type 2 cytokine response.^{16, 17} Currently, most of our knowledge regarding the mechanism of viral asthma exacerbation derived from the study of rhinovirus infection. Reports showed several pro-inflammatory mediators, including IL-1 β , IL-6, IL-8, RANTES, and eotaxin, to be related with rhinovirus-induced asthma exacerbation.^{18, 19} Increased viral replication with decreased type I IFN production after rhinovirus inoculation were also found in bronchial epithelial cells collected from asthmatics.⁹ Thus, we hypothesized that viral asthma exacerbation triggered by other respiratory virus, such as RSV and hMPV, would also demonstrate a distinctive cytokine/chemokine response and an impaired anti-viral innate immune responses.

Our study showed that neutrophil is the predominant inflammatory cell of BAL fluid in all infected mice (RSV, hMPV, OVA+RSV, and OVA+hMPV group). Although the percentage of eosinophil had decreased significantly after infection in

the OVA-sensitized groups (OVA+RSV and OVA+hMPV), the absolute cellular number was comparable to the OVA mock group. This finding is compatible with several reported studies.^{20, 21} However, in comparison with the results of Aeffner *et al.*,²¹ our study showed a more profound neutrophil response. The difference might be caused by a higher inoculum of virus used in our study.

In the OVA-sensitized mice, our result revealed conflicting responses in Th2 cytokine after viral infection, displaying a decreased IL-5 but an increased IL-4 expression. Peebles *et al.* have described similar finding and concluded that the enhanced AHR of RSV infected OVA-sensitized mice was not related to Th2 cytokine responses.¹⁷ Currently, no reports are available regarding the effects of hMPV infection on allergic mice, our results indicated that hMPV elicited Th1/Th2 responses similar to that of RSV infection. However, in the aspect of chemokine expression, RSV induced more profound responses of MIP-1 α and MIP-1 than hMPV. Significant reduction of IL-1 α , IL-1 β , G-CSF and RANTES was observed which might partially be contributed to the decreased ratio of eosinophil in OVA-sensitized mice after infection. On the contrary, expression of KC was increased in the OVA-sensitized mice after viral infection, especially that of RSV. In the study of Aeffner *et al.*, the enhanced response of KC further attenuated the response of AHR in OVA-sensitized mice.²¹

Our study demonstrated that after RSV infection, viral replication was significantly augmented in the OVA-sensitized mice. Hassantoufighi *et al.* had also shown prolonged viral clearance in aspergillus-sensitized cotton rats after low-dosage RSV infection ($10^{4.5}$ pfu/animal),¹⁶ which was speculated to be related to inhibited IFN- γ expression. Although our study showed decreased IFN- γ □□□□□□□□ in both OVA+RSV and OVA+hMPV group, no difference was observed in the viral titer

between OVA+hMPV and hMPV group. Type I IFNs, including IFN- α and - β , are the most important anti-viral pro-inflammatory cytokine against viral replication. In the mice model, hMPV is a better IFN- α inducer than RSV.²² Our study further showed that hMPV also elicited stronger IFN- β response. However, there was no difference in the expression of type I IFNs between hMPV and OVA+hMPV group. Reduced type I IFN production was indeed found only in the OVA+RSV group, which resulted in enhanced viral replication. Bronchial epithelial cells taken from asthmatics also expressed worsened type I IFNs production in response to rhinovirus infection, thus, contributed to increased viral replication.⁹ These findings suggested that different viral infections trigger variable type I IFNs expressions among allergic hosts.

RSV infection induces type I IFNs mainly through two signal transduction pathways. First, replication products of RSV can be recognized by cytosolic RNA helicases, RIG-I and melanoma differentiation antigen 5 (MDA-5),¹¹ which then ubiquitylate tumor-necrosis factor receptor-associated factor 3 (TRAF3) and TRAF6 and subsequently activates the NF- κ B and IRF3 pathway. Alternatively, TLRs are also important in the recognition of the viral nucleic acids after RSV infection. Although TLR4 and TLR2 have been shown to involve in the activation of proinflammatory cytokine,^{23, 24} TLR3 is the most important sensor among TLRs in the induction of IFNs production.¹¹ TLR3 pathway also converges on a common TRAF3 adaptor complex. TRAF3 then activates IRF3 kinases, including IRF3 kinase I κ B kinase ϵ (IKK ϵ) and TANK binding kinase-1 (TBK-1),^{25, 26} which cause phosphorylation and homodimerization of IRF3. IRF3 then translocates into the nucleus, where, together with NF- κ B, activates type I IFN production. Liao *et al.* reported similar signaling pathway after hMPV infection for the induction of IFN production, except that RIG-I

seemed to play a more important role than TLR3 in the recognition of viral nucleic acid.¹⁰ Based on previous reports and our results, all evidences point to NF- κ b and IRF3 to be the key signaling transcriptors in IFN production after RSV and hMPV infection.

The expression of upstream sensors such as NF- κ b and RIG-1 did not differ between the OVA-sensitized and non-sensitized mice. Although RSV significantly increased TLR3 expression in OVA-sensitized mice, such trend was not observed in the OVA+hMPV group. Next, the study regarding the downstream signal transcriptors showed a significantly reduced IRF3 expression in OVA-sensitized mice after RSV infection (OVA+RSV group), both in the protein and mRNA level. However, OVA-sensitized mice did not exhibit similar defective IRF3 expression after hMPV infection (OVA+hMPV group). These evidences implied that allergic mice expressed distinct signaling pathway of IFN production in respond to different viral infections.

IRF3 is constitutively expressed in the cytoplasm. Viral infection triggers IRF3 phosphorylation, which allows IRF3 to form homo-dimer that translocates into the nucleus and binds to DNA. IRF3 is regulated by ubiquitination and proteasome-dependent degradation. Several viruses have evolved certain ability to interfere with the activation and function of IRF3. Zheng *et al.* found that the non-structural protein 3 of mouse hepatitis virus A59 can cause de-ubiquitination of IRF3 and prevent its nuclear localization.²⁷ It is well known that the non-structural protein of RSV, NS1 and NS2, reduces the activation of IRF3 and NF- κ b, which subsequently inhibits the induction of type I IFN.^{28, 29} Our report also showed RSV to induce less type I IFN expression than hMPV. However, the exact mechanism of the interference of IFN induction by NS still remains an enigma.

Several cellular factors have been suggested to involve in the negative regulation

of IRF3. Cytoplasmic peptidyle-prolyl-isomerase (PIN1) can bind to phosphorylated IRF3 to trigger its ubiquitination and subsequent degradation.³⁰ However, PIN1, a *cis-trans* isomerase, does not have any effect on NF- κ b activation.³⁰ PIN1 was also shown to play a critical role in allergic pulmonary eosinophilia in rats.³¹ Our data also revealed that the expression of IRF3, but not NF- κ b, was reduced after RSV infection in the murine allergic pulmonary model. It is possible that PIN1 may play a key role in the mechanism of defective activation of type I IFN signal transduction of allergic hosts.

In summary, OVA-sensitized mice showed distinct cytokine-chemokine expression after viral infection. Inhibited production of type I IFN resulted in increased viral replication after RSV infection in the allergic mice. We found that the reduction of type I IFN was closely related to the decreased expression of nuclear signal transcriptor, IRF3. These findings suggest that allergic mice exhibit distinct signaling transcription of innate immunity after different viral infection.

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LEGEND OF FIGURES

Figure 1

Time Line of experimental protocol and the treatment. Day 0 is shown as the day of intraperitoneal OVA injection. 14 days later, mice are challenged for 3 consecutive days with nebulized OVA aerosols then inoculated with virus or PBS in day 16. Lungs for histopathological exam and BAL fluid were collected in day 1 post-infection. Virus titers of lung are titrated in day 21 (5 days post-infection). The treatments in each groups are shown in the below table.

Figure 2

Cellular differential in BAL fluid. Mice were immunized intraperitoneally with OVA and challenged with OVA aerosols for 3 consecutive days. Mock group was sensitized and challenged with PBS following the same protocol. In the last day of challenge, mice were inoculated with RSV, hMPV or PBS. In day 1 post-infection, BAL was performed. Cellular differential, in relative percentage (A) and absolute count (B), were analyzed. Data presented are means of 6 mice per group $\pm$ SEM and representative of three different experiments. * $p < 0.05$ relative to 'OVA mock' group.

Figure 3

Cytokine-chemokine levels. Mice were immunized, challenged then inoculated as protocol described in Figure 1. BAL fluid was collected in day 1 post-infection. Th1/Th2 cytokines (A), pro-inflammatory cytokines (B), and chemokines were measured. Data presented are means of 6 mice per group $\pm$ SEM and representative of three different experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ comparing

between two groups.

Figure 4

Virus titer in lungs of OVA-sensitized and non-sensitized mice after infection.

Mice were immunized, challenged, and infection as protocol of Figure 1. Five days after infection with RSV (A) and hMPV (B) (1×10^7 pfu/mice), lungs were collected and homogenized. Virus titers of homogenized lungs were then determined by using plaque assay. Data presented are means of 6 mice per group $\pm$ SEM and representative of three different experiments. * $p < 0.05$ relative to RSV or hMPV group.

Figure 5

Type I interferon production after viral infection in OVA-sensitized and non-sensitized mice.

Mice were immunized, challenged, and infection as Figure 1. Twenty-four hours after RSV (A) or hMPV (B) inoculation, BAL fluid was collected. IFN- α and - β were measured by using ELISA assay. Data presented are means of 6 mice per group $\pm$ SEM and representative of three different experiments. * $p < 0.05$, *** $p < 0.005$ relative to RSV or hMPV group.

Figure 6

Expression of signal transcripts of type I interferon after viral infection OVA-sensitized and non-sensitized mice.

Mice were immunized, challenged, and infection as Figure 1. Twenty-four hours after infection, lungs were collected for extraction of mRNA and nuclear protein. The relative mRNA expressions of signal transcripts after RSV (A) or hMPV (B) infection are normalized for the amount of β -actin mRNA. Signal transcripts, which having translocated into nucleus, are

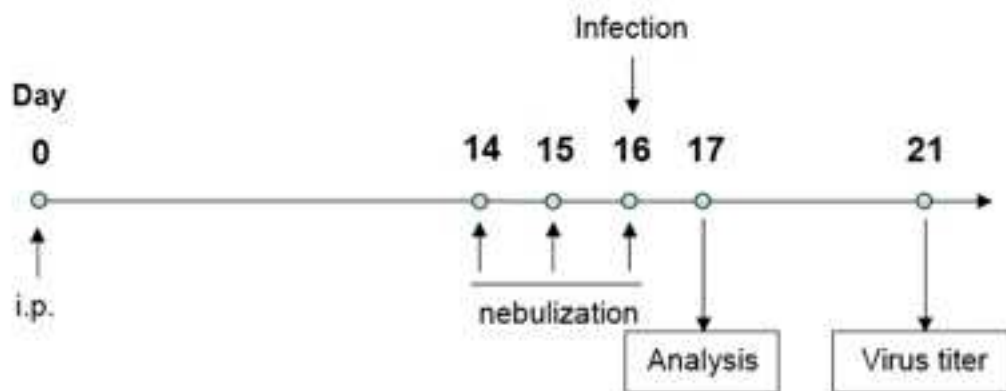
assayed using Western blotting (C). Data presented are means of 5 mice per group $\pm$ SEM and representative of three different experiments. * $p < 0.05$ relative to RSV or hMPV group.

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Group	i.p.	nebulization	infection
<i>Mock</i>	PBS	PBS	PBS
<i>OVA mock</i>	OVA	OVA	PBS
<i>RSV</i>	PBS	PBS	RSV
<i>OVA+RSV</i>	OVA	OVA	RSV
<i>hMPV</i>	PBS	PBS	hMPV
<i>OVA+hMPV</i>	OVA	OVA	hMPV

