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

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Background/Purpose: Respiratory viral infections have frequently been reported to closely correlate 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 immunity in response to different viruses.

Methods: Murine sensitization was established using an ovalbumin (OVA) sensitization model. Mice were subsequently infected with either respiratory syncytial virus (RSV) or human metapneumovirus (hMPV). Type I interferon (IFN), cytokines, and chemokines were measured in bronchoalveolar lavage (BAL) 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 OVA sensitization. A significant 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 Toll-like receptor 3 (TLR3) expression was significantly increased in the lungs, the key signal transcriptor, IFN regulatory factor 3, was significantly suppressed in the RSV-infected sensitized mice.

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

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Introduction

Asthma is an allergic airway disease characterized by a chronic inflammatory reaction and airway hyper-
responsiveness (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. 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. Both are often detected in young children with asthma exacerbation.

Whether respiratory virus induces asthma exacerbation, or if asthmatics are more prone to respiratory viral infection still remains an enigma. A report from Wark et al showed an increased viral replication after rhinovirus inoculation in ex vivo cultured primary bronchial epithelial cells collected from asthmatic adults. Decreased expression of type I interferons (IFNs) and inhibited apoptosis of infected bronchial epithelial cells were suggested to contribute to the increased viral replication. Another important antiviral immune response is the pattern-recognition receptors (PRRs)-induced type I IFNs production. Among these PRRs, retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3) domains transmit signals into the nucleus through several transcription factors. The key signal transcriders, such as IFN regulatory factor 3 (IRF3), IRF7, and nuclear factor-kb (NF-kb), once activated, initiate IFNs reponses.

In this study, we examined how sensitization affects innate immune responses. Ovalbumin (OVA)-sensitized mice were utilized in the infection with 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

BALB/c female mice (6–8 weeks old) were purchased from the 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 and RSV A2 were obtained from the University of Texas Medical Branch, Galveston, TX, USA with kind permission from Dr. Roberto P Garofalo (Division of Clinical and Experimental Immunology and Infectious Diseases, University of Texas Medical Branch). hMPV was propagated in LLC-MK2 cells (ATCC CCL-23), and viral titer was determined by a methylcellulose plaque assay, as previously described.

Experimental protocol

The study protocol is outlined as in Fig. 1. The OVA sensitization and viral inoculation are described below. On Day 0, mice were immunized with 50 μg OVA (Sigma-Aldrich, St. Louis, MO, USA) and 2 mg aluminum hydroxide. Mice were subsequently challenged with 1% OVA solution diluted with sterilized Phosphate Buffered Saline (PBS) daily using a small volume nebulizer on Days 14–16. On Day 16, mice were inoculated intranasally with purified hMPV, RSV, or PBS. A total of 1 × 10^7 plaque-forming unit (pfu) hMPV or RSV was used for infecting one mouse. Analytic procedures were performed on Day 17. Lung virus titers were determined on Day 21 (Day 5 postinfection). Mice were divided into Mock, OVA mock, RSV, OVA + RSV, hMPV, and OVA + hMPV groups. The detailed manipulations of each group are shown in Fig. 1.

Sample collection

On protocol Day 17, 1 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, USA). Cellular differentials of BAL fluid were determined. Supernatants of BAL fluid were collected for determining the levels of cytokines and chemokines. After lavage, lungs were collected for histopathological exam or stored at –80°C until real-time quantitative 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 Dulbecco’s Modified Eagle Medium (DMEM) and serially log_10 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. A total of 100 μL of lung homogenate was treated with 1 mL 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

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suspension and a random hexamer cDNA synthesis kit (Applera, Norwalk, CT, USA). A total of 2/20 μL of cDNA suspension was used for amplification in RT-qPCR. The mRNA expression levels of IFN-α, -β, IRF3, IRF7, NF-κb 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, USA), according to the manufacturer’s instructions. The panel included the following: interleukin (IL)-1α, IL-1β, 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, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), IFN-γ, keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 (MIP-1) α, MIP-1 β, regulated in activation, normal T cell expressed and secreted (RANTES), and tumor necrosis factor (TNF)-α. The broad assay range was from 2.0 pg/mL to 5000 pg/mL. The levels of IFN-α and IFN-β were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN, USA).

Nuclear protein extraction and Western blotting

Nuclear protein was extracted from lung homogenates using the Bio-Plex Mouse Cytokine 23-Plex panel (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. The nuclear protein extractions were boiled in 3°C Laemmli buffer for 2 minutes and resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred for 24 hours onto a Hybond-ECL nitrocellulose membrane and immersing the membrane in Tris Buffered Saline with Tween (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 minutes at room temperature. After washing, the proteins were detected using enhanced chemiluminescence (ECL) according to the manufacturer’s protocol. The primary antibodies used for Western blotting are antibodies against NF-κb p65 (Millipore, Billerica, MA, USA), IRF3 (Abcam, Cambridge, MA, USA), IRF7 (Lifespan, Seattle, WA, USA), RIG-I (Abgent, San Diego, CA, USA), and β-actin (Millipore).

Statistical analysis

Statistical analysis was performed using InStat software (GraphPad, San Diego, CA, USA). Data are presented as mean ± standard error of the mean values from two to three independent experiments. When two groups are compared, the values are analyzed using the 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 OVA challenge, we analyzed the cellular spectrum of BAL
fluid in OVA-sensitized/challenged mice after viral inoculation. Neutrophils were the predominant cells 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 (Fig. 2A), the absolute number did not change (Fig. 2B).

Distinct expressions of cytokines and chemokines in OVA-sensitized mice after viral infections

The concentrations of T helper (Th)1/Th2 cytokines, proinflammatory cytokines, and chemokines in BAL fluid were measured on Day 1 postinfection. 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; Fig. 3A). A similar trend was also found in the OVA + hMPV group. Regarding the proinflammatory cytokine and chemokine responses, the OVA + RSV group induced lower IL-1α, IL-1β, and G-CSF, but higher MCP-1 responses than the RSV group. Nevertheless, reduced GM-CSF production in the OVA + hMPV mice was the only difference observed between the hMPV groups (Fig. 3B and C). In general, for other cytokine/chemokine responses, such as TNF-α, RANTES, MIP-1α, and -1β, RSV elicited stronger responses than hMPV (Fig. 3B and C).

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

To clarify if OVA-sensitization can change the 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 a higher peak viral titer than nonsensitized mice after RSV infection (36,260 ± 4518 pfu/g lung tissue versus 21,240 ± 1306 pfu/g lung tissue, p < 0.05; Fig. 4A). By contrast, there was no difference in peak hMPV viral titer between mice with and without OVA sensitization (Fig. 4B). Due to the fact that the production of type I IFNs peaked at around 18–24 hours after RSV infection,11 the levels of IFN-α and -β in BAL fluid were measured 24 hours after infection. A significant reduction of IFN-α and -β expression was found in OVA-sensitized mice with RSV infection (Fig. 5A) (RSV vs. OVA + RSA; IFN-α: 1263 ± 194 pg/mL vs. 282 ± 78 pg/mL, p < 0.005; IFN-β: 1226 ± 125 pg/mL 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 (Fig. 5B; hMPV vs. OVA + hMPV; IFN-α: 2506 ± 286 pg/mL vs. 2255 ± 212 pg/mL; IFN-β: 4585 ± 428 pg/mL vs. 3181 ± 395 pg/mL, p < 0.05).

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

According to previous reports, the signal transcriptors of type I IFN production began to rise at around 6 hours post RSV and hMPV infection, and peaked after 15–24 hours.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 24 hours postinfection. RNA expressions were determined by using RT-qPCR, and normalized by internal RNA expression of β-actin. The RNA expressions of TLR3, NF-κB p65, IRF3, IRF7, and RIG-I were similar between allergic and mock mice after hMPV infection (Fig. 6A). However, OVA-sensitized mice of the RSV group showed significant suppression of IRF3 RNA expression (Fig. 6B; expression of OVA + RSV/ expression of RSV × 100% = 17 ± 4%, p < 0.005), and an increased TLR3 RNA expression (Fig. 6B; expression of OVA + RSV/expression of RSV × 100% = 819 ± 283%, p < 0.05). In addition, there were no significant differences for the expressions of NF-κB p65, IRF7, and RIG-I after RSV infection between the OVA-sensitized and nonsensitized mice. To further evaluate the protein expression of IFN signal transcriptors, we extracted nuclear protein from the murine lung homogenates. The results further proved that after RSV infection, OVA-sensitized mice exhibited diminished levels of nuclear IRF3 protein, but not nuclear NF-κB p65 and IRF7 (Fig. 6C).
Figure 3. Cytokine-chemokine levels. Mice were immunized, challenged then inoculated as the protocol described in Fig. 1. Bronchoalveolar lavage (BAL) fluid was collected on Day 1 postinfection. Th1/Th2 cytokines (A), proinflammatory cytokines (B), and chemokines were measured. Data presented are means of six mice/group ± standard error of the mean and representative of three different experiments. *p < 0.05, **p < 0.01, and ***p < 0.005 comparing between two groups.
Discussion

Asthma exacerbation is an exaggerated lower airway response to environmental exposure, including allergen, viral infection, pollutant, or certain medication. Airway inflammation is the critical component of the lower airway response in asthma exacerbation. The pattern of the 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. In the murine OVA sensitization model applied to the asthma study, airway inflammation characterized by eosinophil recruitment and type 2 cytokine responses was suggested to be the key component of allergen-induced airway hyper-responsiveness. However, Peebles and colleagues concluded differently; they showed that deteriorated AHR of allergic mice during viral infection was not associated with a type 2 cytokine response. Currently, most of our knowledge regarding the mechanism of viral asthma exacerbation is derived from the study of rhinovirus infection. Reports showed several proinflammatory mediators, including IL-1β, IL-6, IL-8, RANTES, and eotaxin, to be related with rhinovirus-induced asthma exacerbation. Increased viral replication and decreased type I IFN production after rhinovirus inoculation were also found in bronchial epithelial cells collected from asthmatics. Thus, we hypothesized that viral asthma exacerbation triggered by other respiratory viruses, such as RSV and hMPV, would also demonstrate a distinctive cytokine/chemokine response and an impaired antiviral innate immune response.

Our study showed that neutrophil is the predominant inflammatory cell of BAL fluid in all infected mice (RSV, hMPV, OVA + RSV, and OVA + hMPV groups). Although the percentage of eosinophils 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. However, in comparison with the results of Aeffner and Davis, 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 cytokines after viral infection, displaying a decreased IL-5 but an increased IL-4 expression. Peebles et al described a similar finding and concluded that the enhanced AHR of RSV-infected OVA-sensitized mice was not related to Th2 cytokine responses. 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-1α and MIP-1 than hMPV. Significant reductions of IL-1α, IL-1β, G-CSF and RANTES were observed which might partially be contributed to the decreased ratio of eosinophil in OVA-sensitized mice after infection. By contrast, expression of KC was increased in the OVA-sensitized mice after viral infection, especially that of RSV. In the study of Aeffner and Davis, the enhanced response of KC further attenuated the response of AHR in OVA-sensitized mice.

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), which was speculated to be related to inhibited IFN-γ expression. Although our study showed decreased IFN-γ in both the OVA + RSV and OVA + hMPV groups, no difference was observed in the viral titer between the OVA + hMPV and hMPV groups. Type I IFNs, including IFN-α and -β, are the most important antiviral proinflammatory cytokines against viral replication. In the mice model, hMPV is a better IFN-α inducer than RSV. Our study further showed that hMPV also elicited a stronger IFN-β response. However, there was no difference in the expression of type I IFNs between the hMPV and OVA + hMPV groups. 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 respond to rhinovirus infection, and 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 5,11 which then ubiquitylated TNF receptor-associated factor 3 (TRAF3) and TRAF6 and subsequently activates the NF-κb 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 be involved in the activation of proinflammatory cytokines,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 IkB kinase ε (IKKε) and TANK binding kinase-1,25,26 which cause phosphorylation and homodimerization of IRF3. IRF3 then translocates into the nucleus, where, together with NF-κb, it activates type I IFN production. Liao et al10 reported a similar signaling pathway after hMPV infection, except that RIG-I seemed to play a more important role than TLR3 in the recognition of viral nucleic acid. Based on previous reports and our results, all evidence points to NF-κb and IRF3 to be the key signaling transcriptors in IFN production after RSV infection. Although TLR4 and TLR2 have been shown to be involved in the activation of proinflammatory cytokines,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 IkB kinase ε (IKKε) and TANK binding kinase-1,25,26 which cause phosphorylation and homodimerization of IRF3. IRF3 then translocates into the nucleus, where, together with NF-κb, it activates type I IFN production. Liao et al10 reported a 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. Based on previous reports and our results, all evidence points to NF-κb and IRF3 to be the key signaling transcriptors in IFN production after RSV and hMPV infection.

The expression of upstream sensors such as NF-κb and RIG-1 did not differ between the OVA-sensitized and nonsensitized mice. Although RSV significantly increased TLR3 expression in OVA-sensitized mice, such a 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 a distinct signaling pathway of IFN production in response to different viral infections.

IRF3 is constitutively expressed in the cytoplasm. Viral infection triggers IRF3 phosphorylation, which allows IRF3 to form a homo-dimer that translocates into the nucleus and binds to DNA. IRF3 is regulated by ubiquitination and proteasome-dependent degradation. Several viruses have evolved a certain ability to interfere with the activation and function of IRF3. Zheng et al27 found that the nonstructural protein 3 of mouse hepatitis virus A59 can cause de-ubiquitination of IRF3 and prevent its nuclear localization. It is well known that the nonstructural protein of RSV, NS1 and NS2, reduces the activation of IRF3 and NF-κb, which subsequently inhibits the induction of type I IFN.28,29 Our report also showed that RSV induces 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 be involved in the negative regulation of IRF3. Cytoplasmic peptidyl 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-κb 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-κb, 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.

Figure 5. Type I interferon (IFN) production after viral infection in ovalbumin (OVA)-sensitized and nonsensitized mice. Mice were immunized, challenged, and infected as in the protocol of Fig. 1. Twenty-four hours after respiratory syncytial virus (RSV) (A) or human metapneumovirus (hMPV) (B) inoculation, bronchoalveolar lavage (BAL) fluid was collected. IFN-α and -β were measured by using the enzyme-linked immunosorbent assay assay. Data presented are means of six mice/group ± the standard error of the mean (SEM) and representative of three different experiments. *p < 0.05, ***p < 0.005 relative to RSV or hMPV group.
Figure 6. Expression of signal transcriptors of type I interferon after viral infection in ovalbumin (OVA)-sensitized and nonsensitized mice. Mice were immunized, challenged, and infected as in the protocol of Fig. 1. Twenty-four hours after infection, lungs were collected for extraction of mRNA and nuclear protein. The relative mRNA expressions of signal transcriptors after respiratory syncytial virus (RSV) (A) or human metapneumovirus (hMPV) (B) infection were normalized for the amount of \( \beta \)-actin mRNA. Signal transcriptors, which had translocated into the nucleus, were assayed using Western blotting (C). Data presented are means of five mice/group ± the standard error of the mean (SEM) and representative of three different experiments. *\( p < 0.05 \) relative to RSV or hMPV group.
Defective innate immune responses

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 transciption of innate immunity after different viral infections.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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