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Altered mast cell activity in response to rhinovirus infection provides novel insight into asthma

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ABSTRACT

Objective: Human rhinoviruses (RVs) are a type of common respiratory virus capable of inducing an asthma attack. Although mast cells are important effector cells involved in allergic disease, little is known about the direct effects of an RV infection on mast cells. The aim of this study is to investigate mast cell behavior in response to RV infection and gain insight into the effects of RVs on mast cells. **Methods:** Viral replication, cell viability, apoptosis and cytokine release were quantified in Human mast cell-1 (HMC-1) cells following RV16 infection. **Results:** The results revealed that the viral RNA copy number increased substantially over time. Intercellular cell adhesion molecule-1 (ICAM-1) transcripts were significantly up-regulated from 1.79 to 6.37 times following RV16 infection compared to the controls ($p \leq 0.05$). Lactate dehydrogenase (LDH) activity was significantly increased, whereas the cell viability decreased following RV16 infection. Examination of the early cellular response to infection revealed that RV16 increased caspase 3 activity and aggravated apoptotic responses. Furthermore, detection of the innate immune response to RV infection revealed that the release of IL-6, IL-8, TNF- α , and IFN- α by HMC-1 cells increased significantly compared to the control groups. **Conclusions:** RV infection influences mast cell functionality and promotes the innate immune response of mast cells following viral infection. These results provide a novel insight which mast cells have the potential to be involved in the pathogenesis of RV-induced exacerbations of asthma.

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Asthma; rhinovirus; mast cells; allergic disease; innate immune response

Introduction

Human rhinoviruses (RVs) are small non-enveloped viruses with a positive sense single-stranded RNA genome belonging to the *Picornaviridae* family [1]. RVs are the causative agent of the common cold and are well recognized as important and frequent triggers of respiratory disease [2], which lead to increased lower airway inflammation and bronchial hyper-responsiveness [3]. Although it has been observed that RV infection is closely associated with acute asthma exacerbations, the precise mechanism behind this clinical observation remains unclear.

In asthma, mast cells have been found to infiltrate the airway mucous glands and may lead to disordered airway smooth muscle function [4]. During RV infection, increased numbers of mast cells have been observed in the bronchial epithelium of asthmatics [5], and it is thought that they may contribute to viral

immunity during RV-induced asthma exacerbations [6]. Mast cells are central effector cells involved in the elicitation of early- and late-phase allergic inflammatory reactions [7–9]. Upon activation, mast cells degranulate to release an array of mediators, including histamine, leukotrienes, and prostaglandins, which lead to allergic symptoms [10]. RV infection promotes histamine secretion in the nasal passages of allergic subjects [11], as well as the plasma histamine content [12]. In addition, an RV infection may promote cytokine and histamine production from mast cells and basophils, which causes the airway inflammation associated with asthma [13]. These findings indicate that infection with RVs activate mast cells, and it is believed that RV-induced pathological damage is the result of the virally-induced release of inflammatory mediators [14]. Moreover, cells recruited into the airway are activated by the infection and secrete a variety

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of cytokines and inflammatory mediators that may have antiviral effects, but also contribute to airway inflammation and respiratory symptoms [15]. RV infection activates lymphocytes to secrete INF- γ , IL-13, and GM-CSF [16] and promotes the production of several cytokines (e.g. TNF- α , IFN- α , and IL-6) [17] by epithelial cells, which reflect the activation of host defense and inflammatory responses.

Furthermore, mast cells have been reported to express intercellular adhesion molecule-1 (ICAM-1), which induces an allergic response by mediating mast cell accumulation into inflammatory sites [18]. ICAM-1 has been well-established as a molecule that serves as the major group RV receptor. Thus, RVs may be able to directly interact with ICAM-1 and alter mast cell functionality during RV infection, resulting in an asthma attack or exacerbation. Since the direct interaction between RVs with mast cells remains poorly understood, we have investigated the innate immune response of mast cells using the human mast cell-1 (HMC-1) line following RV16 infection, a major group of RV, in the present study.

Methods

Cell lines and RV

HMC-1 cells were maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% Fetal bovine serum (FBS) (Gibco Laboratories), 100 U/mL penicillin, and 50 μ g/mL streptomycin (Sigma-Aldrich, St Louis, MO). The cells were cultured under a humidified atmosphere of 5% CO₂ at 37°C. The RV16 was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA), and passaged five times in HeLa cells before use. The RV16 were grown in HeLa cells in serum-free medium until they reached 80–90% confluence under a humidified 5% CO₂ atmosphere at 33°C for one to seven days. When 90% of the culture exhibited cytopathic effects (CPE), the cells in culture medium were collected through three freeze-thaw cycles and centrifuged at 80,000 rpm, 4°C for 30 min. The supernatant was then collected as the viral stock, aliquoted, and frozen at –80°C. The viral titers were determined by 50% tissue culture infectious doses per mL (TCID₅₀) as previously described [19]. The TCID₅₀ of the viral stocks was 10^{6.3} per 0.1 ml, and 10⁴ TCID₅₀ was used as the inoculum. UV-inactivated RV16 was obtained by exposing the virus to UV light at 1200 mJ/cm² for 30 min. To test viral inactivation, the cells were exposed to UV-inactivated RV16 and the CPE were

Table 1. Primers used for RT-qPCR analysis of gene expression.

Genes	Direction	Sequence	Product (bp)
ICAM-1	Forward	GGCTGGAGCTGTTTGAGAAC	202
	Reverse	ACTGTGGGGTTCAACCTCTG	
RV16	Forward	GGAGATTGCATCATGGCTTT	192
	Reverse	AGAACACACAGAGGGGCACA	
β -actin	Forward	AGAGCTACGAGCTGCCTGAC	184
	Reverse	AGCACTGTGTTGGCGTACAG	

assessed. As a control, the cells were treated with either media alone or UV-inactivated RV16.

Viral infection

HMC-1 cells were suspended in 2 ml of RPMI 1640 medium with 100 U/mL penicillin, and 50 μ g/mL streptomycin were infected with RV16 (10⁴ TCID₅₀) or UV-inactivated RV or exposed to medium alone for 2 h at 33°C in 5% CO₂ incubator. The cells were then washed with Phosphate buffer saline (PBS) and seeded into six-well plates at a density of 2 $\times$ 10⁶ cells/mL and cultured in a 5% CO₂ incubator at 33°C. Successful RV16 infections in virally-infected cells were confirmed by RT-qPCR. The specific primers are listed in Table 1.

Analysis of RV16 replication and ICAM-1 expression by Real-time quantitative polymerase chain reaction (RT-qPCR)

Real-time polymerase chain reaction (RT-PCR) was performed to measure the level of ICAM-1 expression and RV16 replication. Total RNA was extracted from HMC-1 cells using a total RNA extraction kit (Tiangen, Beijing, China) and treated with DNase I (Promega, Madison, WI) to remove any contaminating DNA. Subsequently, 1.5 μ g of RNA was used to synthesize cDNA in a 20 μ L reaction mixture. The cDNA was analyzed by using qPCR with SYBR green Premix Ex Taq reagents (TaKaRa, Dalian, China). The specific primers were designed using Primer Express software v3.0 (Applied Biosystems, Foster City, CA) and are listed in Table 1. qPCR was performed as follows: 95°C for 5 min, and 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by one cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. All experiments using standard dilutions, controls, and experimental samples were performed in triplicate on the same plate. The level of the target gene expression was calculated using the 2^{– $\Delta\Delta$ Ct} method and the β -actin gene as a reference. The target gene expression was expressed as the fold-change relative to the control samples. The replication of RV16 was presented

as the $\log_{10}$ copy numbers using absolute quantification with the standard curve method. The correlation coefficient (r^2) of the standard curves was greater than or equal to 0.99.

Determination of cell lysis

The release of lactate dehydrogenase (LDH) into the culture supernatant is an indicator of the degree of cell death caused by a viral infection. LDH activity was measured using the LDH assay kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions.

Assessment of cell viability and apoptosis by flow cytometry

Cell viability and apoptosis were assessed by flow cytometry using Annexin V conjugated with CF488 and propidium iodide (PI) (Biotium, Hayward, CA) following the manufacturer's instructions. The active forms of caspase 3 were detected using a Nucview 488 Caspase 3 assay kit for live cells (Biotium, Hayward, CA). Caspase activity was determined via the Nucview 488 Caspase 3 substrate uptake technique. HMC-1 cells were collected by centrifugation at 150 g for 10 min, followed by three washes with PBS. For apoptosis analysis, the cells were incubated with CF488-conjugated Annexin V and PI at 4°C for 30 min in the dark. For caspase-3 staining, the cells were incubated Nucview 488 Caspase 3 substrate at room temperature for 30 min in the dark. Finally, the cells were resuspended in fluorescence-activated cell sorting (FACS)-flow solution and analyzed with a FACS verse flow cytometer (BD Biosciences, San Jose, CA). A total of 10,000 events were analyzed per population for each sample. Data were analyzed using CellQuest software (BD Immunocytometry systems).

Determination of cytokine production

To determine the level of cytokine production, cell culture supernatants were collected, centrifuged to remove cellular debris, and assayed immediately or stored at -80°C until future use. The level of IL-6, IL-8, TNF- α , and IFN- α in the culture supernatant was measured with a commercial ELISA kit (Biolegend, San Diego, CA) following the manufacturer's protocol, and absorbance was measured at 450 nm with a Bio-Tek kinetic ELISA reader (Bio-Tek Instruments, Winooski, VT). All measurements were performed in duplicate and the mean values of three

independent experiments were used for statistical analysis.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.1 software (SPSS Inc., Chicago, IL). Data are expressed as the mean $\pm$ SEM from three independent experiments. Statistical significance between means was analyzed with a one-way analysis of variance or a Student's *t*-test. A *p* values of ≤ 0.05 was considered to be statistically significant.

Results

RV infection increases viral replication and ICAM-1 expression in HMC-1 cells

Since the VP2 gene encodes one of the major RV16 structural proteins, the level of VP2 gene expression is considered to be a measure of viral load. To detect viral replication in HMC-1 cells, the number of copies of the VP2 gene in HMC-1 cells infected with RV16 were examined by absolute quantification PCR. Following infection with RV16, the number of VP2 gene copies in HMC-1 cells increased substantially at the different time points, reaching approximately three orders of magnitude at 48 h postinfection (Figure 1(A)). In contrast, the number of copies of the viral VP2 gene were barely detected in UV-inactivated RV infected cells and cells treated with medium alone. Since ICAM-1 functions as a cellular receptor for RV16, the transcription of ICAM-1 presented as the fold-change. Following RV16 infection, the level of ICAM-1 transcription in HMC-1 cells was markedly upregulated at 12, 24, 48 and 72 h compared to the group treated with medium alone at the corresponding time points (Figure 1(B)). However, there were no significant differences between the UV-inactivated RV group and the group treated with medium alone ($p > 0.05$). UV-inactivated RV did not influence the expression of ICAM-1 in HMC-1 cells.

LDH activity increases after viral infection

LDH activity was evaluated by examining the level of LDH in the culture medium of HMC-1 cells following RV16 infection. LDH activity was found to be significantly increased at 24, 48 and 72 h following infection compared to the medium control group ($p < 0.05$) (Figure 2). On the other hand, there were

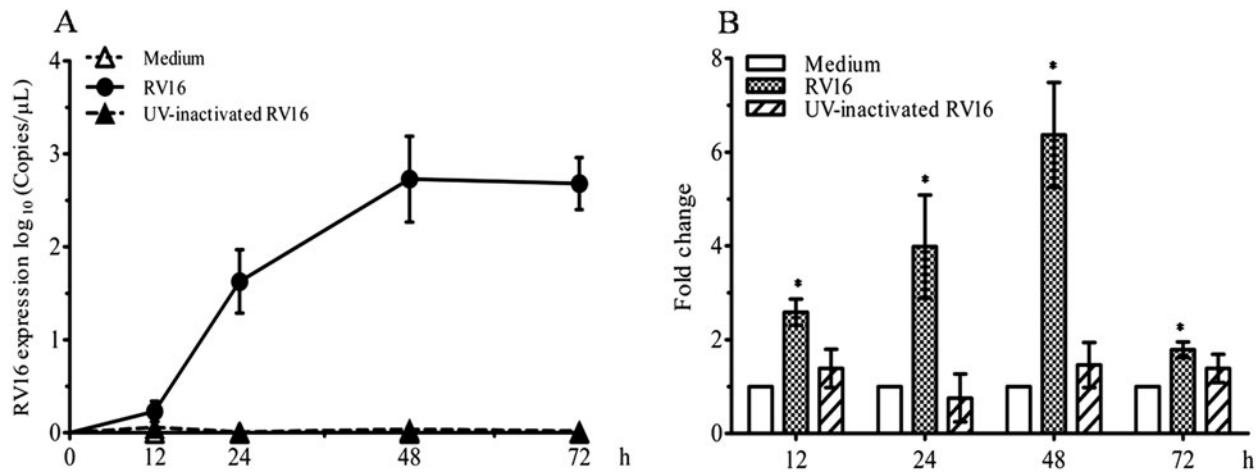


Figure 1. ICAM-1 expression in HMC-1 cells following RV16 infection. (A) The number of RV16 copies in HMC-1 cells was determined by absolute quantification PCR at 12, 24, 48 and 72 h following RV16 infection. The correlation coefficient (r^2) of the standard curves exceeded or equaled 0.99. Copy numbers are represented as the $\log_{10}$ (copy numbers/ μ L). (B) Changes in ICAM-1 expression were analyzed by using RT-qPCR at 12, 24, 48 and 72 h after RV16 infection and expressed as the fold-change compared to HMC-1 cells treated with medium control. Data are presented as the means $\pm$ SEM from three independent experiments. Asterisks (*) indicate significant differences compared to the medium control group ($p \leq 0.05$; Student's t -test).

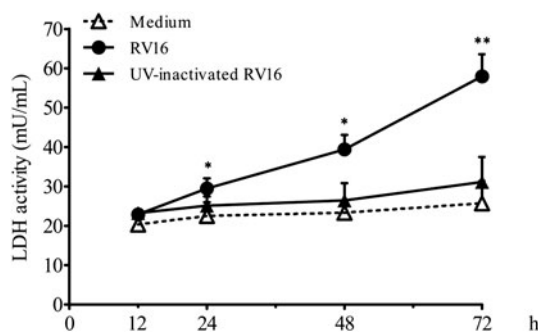


Figure 2. LDH activity in HMC-1 cells infected with RV16. LDH release was evaluated by determining the quantity of LDH released into the culture supernatants at 12, 24, 48 and 72 h post-RV16 infection. Data are reported as the means $\pm$ SEM from three independent experiments. Significant differences compared to treatment with medium control are indicated as * ($p \leq 0.05$) and ** ($p \leq 0.01$), respectively.

no differences between LDH activity in the culture supernatants in the UV-inactivated RV group and the medium control group at each of the indicated time points ($p > 0.05$).

Cell viability and apoptosis after RV16 infection

To further understand the influence of an RV infection on mast cell function, we investigated the level of cell viability and apoptosis from RV16-infected HMC-1 cells by flow cytometry. Viable cell numbers were determined following viral infection and expressed as the percent viability compared to cells treated with medium alone. After RV16 infection, the number of

viable cells had an approximately 20% reduction in the cell populations compared to both the groups treated with medium alone and with the UV-inactivated RV16 ($p < 0.05$) (Figure 3(A)). Furthermore, to investigate natural defense ability of HMC-1 cells against virus replication, we analyzed the changes in apoptotic and necrotic cells following RV16 infection. The results are expressed as the fold-change relative to the samples treated with medium alone. As shown in Figure 3(B), a significant increase in apoptosis was observed at 12 h and 24 h following RV16 infection ($p < 0.05$), which was not observed in the cells treated with UV-inactivated RV16 ($p > 0.05$). This finding confirmed that the induction of apoptosis was directly related to viral replication.

Induction of caspase 3 activation by RV16 infection

To confirm that the activation of caspase 3 was involved in RV16-induced apoptosis, we examined the level of activated caspase 3 in HMC-1 cells following RV16 infection using a caspase 3 assay kit. As shown in Figure 4, caspase 3 activity was significantly enhanced in HMC-1 cells infected with RV16 at 6 h, peaked at 8 h, and remained elevated at 12 h postinfection ($p < 0.05$). However, there were no differences observed in the cells treated with UV-inactivated RV16 ($p > 0.05$). These results demonstrate that RV16 infection triggers caspase 3 activity and RV16-induced

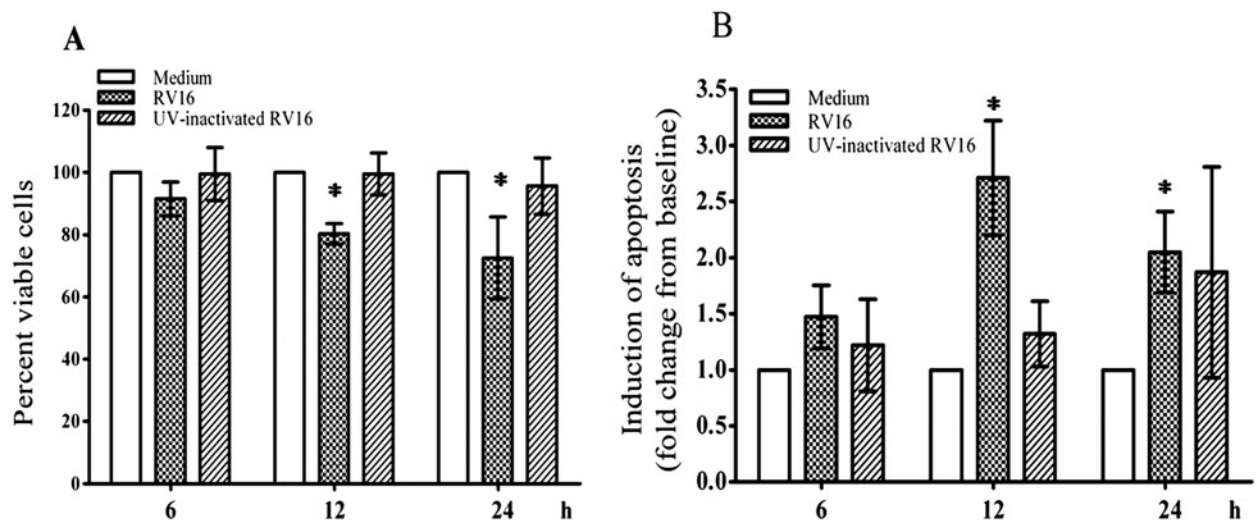


Figure 3. Cell viability and apoptotic response of HMC-1 cells following RV16 infection. (A) The number of viable cells (AxV/PI) was examined at 6, 12, and 24h following RV16 infection, and the results were expressed as the percent viability compared with the cells treated with medium alone. (B) Apoptotic cells (AxV⁺/PI) were also determined at 6, 12, and 24 h after RV16 infection and the data are expressed as the fold-change compared to medium control group. Data are presented as the means $\pm$ SEM from three independent experiments. Asterisks (*) indicate significant differences compared to the medium control group ($p \leq 0.05$; Student's *t*-test).

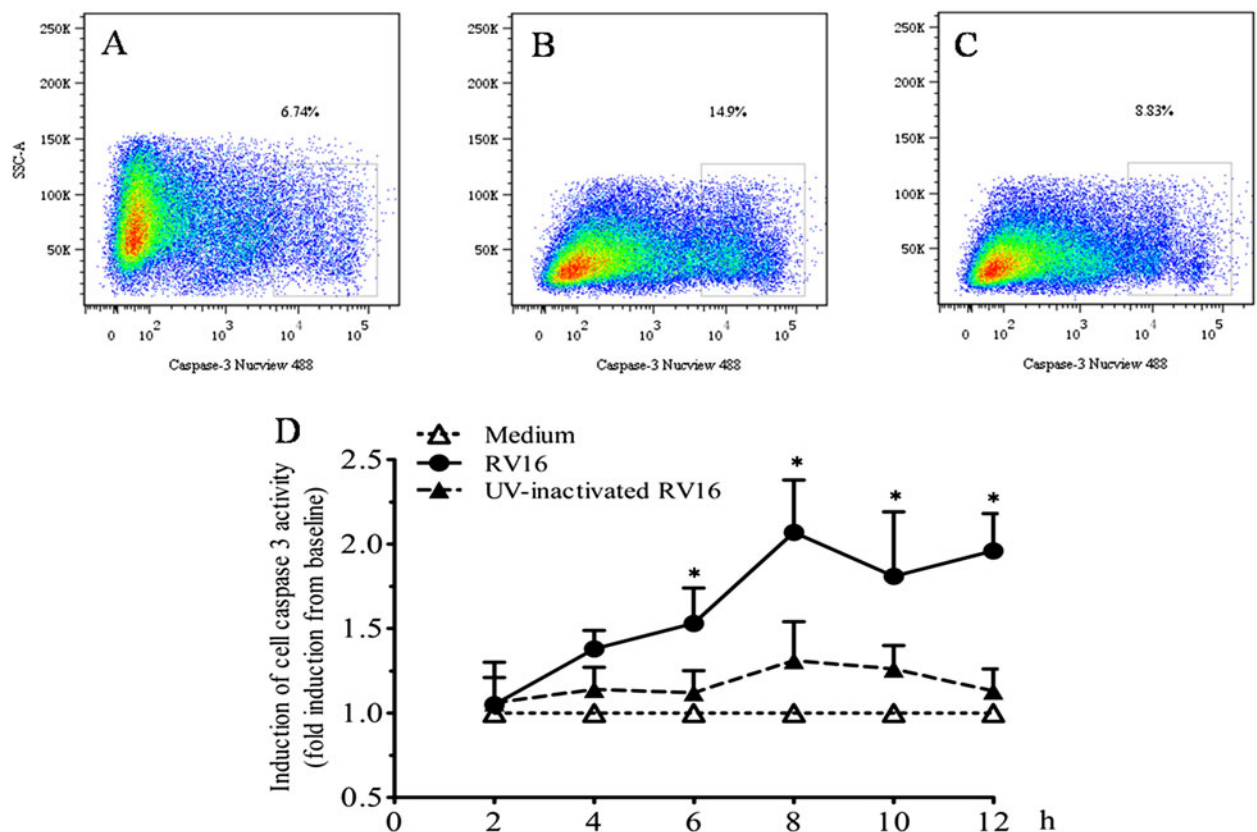


Figure 4. Flow cytometric analysis of caspase 3 activation in HMC-1 cells infected with RV16. Representative graphs at 8h following RV16 infection are presented (A–C). (A) Medium control group; (B) RV16 infected group; (C) UV-inactivated RV16 treated group. The statistical results of each time point are presented as the fold-change relative to the group treated with medium control at the corresponding time points (as baseline, D). Data are presented as the means $\pm$ SEM from three independent experiments. Asterisks (*) indicate significant differences compared to medium control group ($p \leq 0.05$; Student's *t*-test).

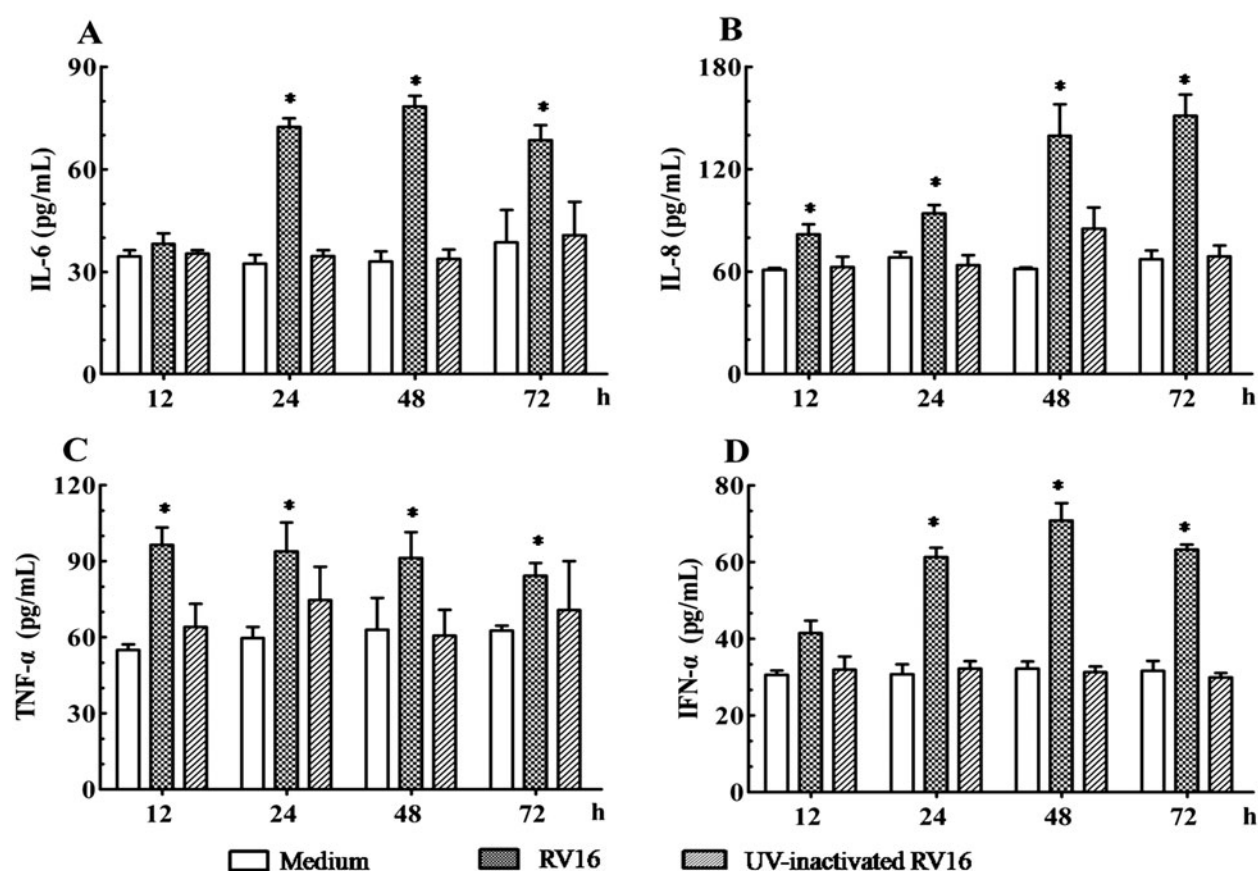


Figure 5. Cytokine production from HMC-1 cells after RV16 infection. Cytokine production from HMC-1 cells was assessed by ELISA, (A: IL-6; B: IL-8; C: TNF- α ; D: IFN- α). Results are reported as the means $\pm$ SEM from three independent experiments. Asterisks (*) indicate significant differences compared to the group treated with medium alone ($p \leq 0.05$; Student's t -test). (A) IL-6; (B) IL-8; (C) TNF- α ; and (D), IFN- α .

apoptosis might occur via a caspase 3-associated signaling pathway.

RV16 induce cytokine release from HMC-1 cells

Pro-inflammatory cytokines have been well-established to play an important role in innate immunity. As a primary effector cell, mast cells are actively involved in the pathogenesis of allergic diseases and can release a variety of cytokines. To further evaluate the effect of RV16 infection on mast cells, we measured the level of cytokines present in the culture supernatants of HMC-1 cells following an RV16 infection with a commercial ELISA kit. As shown in Figure 5, the RV16-infected HMC-1 cells produced significantly larger amounts of IL-6, IL-8, TNF- α , and IFN- α following infection than the cells treated with either medium alone or UV-inactivated RV16 ($p < 0.05$), especially IL-8 and TNF- α . These results suggest that RV16 infection triggers the release of cytokines from HMC-1 cells, and indicates that the viral infections may cause an exacerbation of asthma or Chronic

obstructive pulmonary disease (COPD) by inducing cytokine release from mast cells.

Discussion

The innate immune system is highly conserved and the first line of defense for protecting the host from invading microbial pathogens [20]. Mast cells reside in peripheral tissues (e.g. skin, lungs, gut, and mucosal surfaces) and play a critical role in the innate immune response. As first line cells of the innate immune system and major effector cells [10, 21], mast cells play an important role in the pathogenesis of virally-induced asthma exacerbations. In the present study, we report for the first time that HMC-1 cells can be infected by RV16. In addition, a variety of early anti-viral host defenses were activated following RV16 infection, including apoptosis, cytokine production, and modulation of host transcription. Moreover, HMC-1 cells were found to be permissive for the replication and release of RV16. Similar results were obtained from a previous study that used a mature LAD2 cell line and primary mast cells following

infection with both major and minor group RVs [6]. These data indicate that the innate immune response of mast cells was insufficient to limit RV replication. Moreover, mast cells may contribute to viral immunity during RV-induced asthma exacerbations.

An increase of three orders of magnitude in the copy number of viral RNA in HMC-1 cells following RV16 infection demonstrated that RV16 had a strong capability for replicating and spreading in the HMC-1 cells, which is similar to that observed in P815 cells infected with influenza A viruses [22]. In addition, ICAM-1 expression was markedly upregulated during RV16 infection. The results suggest that elevated ICAM-1 expression was associated with a higher viral load in the HMC-1 cells. It has been shown that the upregulation of ICAM-1 expression can promote mast cell accumulation into inflammatory sites to induce an allergic response [23]. This finding agrees with a previous report by Wark et al. [24] who studied viral replication and ICAM-1 expression following viral inoculation and found a significant association between higher viral RNA levels and ICAM-1 transcription in asthmatic bronchial epithelial cells. However, viral replication, factors influencing the timing of ICAM-1 expression in HMC-1 cells, and the relationship between viral entry into cells and the host response are unclear from the present observations. Thus, future experiments should be conducted to assess ICAM-1 expression in variety of cell types during the course of viral infection.

It has been well-established that apoptosis is resistant to the innate immune response to viral infection [25]. To investigate the mechanism for this increased susceptibility of mast cells to viral replication and cytolysis, we studied the innate antiviral responses of HMC-1 cells. Our results indicate that RV16 tends to induce an apoptotic response via the activation of the caspase 3/7 pathway. Similarly, it has been found that the early apoptotic response in virally-infected cells was a key protective mechanism required for the inhibition of apoptosis, which resulted in enhanced viral replication [26]. However, asthmatic bronchial epithelial cells infected with RV16 have been found to be resistant to early apoptosis and associated with enhanced viral replication, which was proposed to explain elevated RV replication in asthmatic bronchial epithelial cells that are deficient in the production of type I and III IFN [24]. There are several potential reasons for the difference between these two findings, including experimental technique, viral strains, and subject selection. Furthermore, our observations suggest that the activation of caspase 3 was associated

with the progression of RV infection, which may be important for gaining insight into the specific innate responses of mast cells following viral infections.

Cytokines play an important role in the modulation of the immune response, as they are critical in directing both the innate and adaptive immune response to ensure that the host generates protective antiviral defense mechanisms. Pro-inflammatory cytokines released by mast cells may also be associated with the airway inflammation induced by RV infection [27]. The precise mechanisms responsible for the priming effects of RV16 infection on the release of cytokines by mast cells remain unknown. As expected, RV16 infection promoted the production of IL-6, IL-8, TNF- α , and IFN- α from HMC-1 cells. Our experimental results showed that the cytokine secretion peaked at 48 h following RV infection, and the elevated levels of these cytokines were unrelated to their release from dead or dying cells. These conclusions are based on the observations that RV16 titers in the culture supernatants and RV RNA in the infected cells increased with time in the HMC-1 cells. In contrast, the study by Hosoda et al. did not find that cytokines were released from HMC-1 cells following infection with RV14 [13]. This discrepancy may be related to differences in viral strains or dose used for infection.

TNF- α is involved in regulation of the immune system and host defense [28]. It has been previously shown that TNF- α is secreted by mast cells and is strongly associated with the pathogenesis of asthma [29]. Therefore, the results from our present study agree with these previous observations and further suggest that mast cells are a major source of TNF- α following RV14 infection, which may contribute to the development of RV-induced allergic disease. IFNs are critical regulators of a wide-range of protective innate responses and are known to promote acquired antiviral immunity [30]. Moreover, IFNs induce a wide variety of antiviral proteins, including antiviral protein kinase (PKR), which limits viral replication and induces apoptosis [31]. RV infection has been shown to induce the release of inflammatory mediators from PBECs, which led to a more robust antiviral IFN response associated with increased host cell apoptosis and substantially reduced the level of viral replication [32]. These results suggest that the productions of IFNs may be more important in RV infections to effectively limit spread of the virus and stimulate the inflammatory response. However, peripheral blood monocytes from asthmatic subjects have a reduced interferon- γ response [33]. In addition, previous studies reported that the induction of IFN- β and IFN- λ in

lung cells was deficient in a mouse model of asthmatic donors following RV infection [34, 35]. These findings suggest that a defective innate IFN response may be one mechanism that leads to a deficient Th1-acquired immune response and is associated with the pathogenesis of asthma or COPD. Therefore, further research is required to investigate both the innate and acquired immune responses involved in the pathogenesis and treatment of acute exacerbations of asthma or COPD induced by RV infection.

Conclusions/Key findings

The present study has shown that an RV16 infection affects mast cell function, including cellular activity, apoptosis, cytokine production, and promotes the innate immune response of mast cells to viral infection. These results suggest that mast cells to viral infection have the potential to be involved in the pathogenesis of RV-induced allergic diseases.

Disclosure statement

All authors who participated in this study have no competing interest. The authors alone are responsible for the content and writing of the article.

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