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Original Article

Adenovirus replication and host innate response in primary human airway epithelial cells

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Abstract *Background:* Adenovirus infections are very common in children and sometimes fatal. Immune responses and hypercytokinemia are related to disease severity in patients with adenovirus infection. Understanding of viral replication and immune responses could help elucidate the immunopathogenesis of severe adenovirus infections.
Methods: Polarized human airway epithelial cells (hAECs) were set up to mimic human airway, and we conducted high (1 the multiplicity of infection, MOI) and low dosage (0.5 MOI) of wild-type adenovirus serotype 3 infection in hAECs from both apical (AP) and basolateral (BL) compartments, compared the viral replication kinetics and measured 25 cytokine and 9 chemokine levels by multiplex immunoassay to evaluate the host immune response.
Results: Virus titer was the highest in the apical compartment in low dose apical infection. BL infection showed a relative steady viral titer in different doses and infection sites. Responses of several cytokines such as IL-1RA, IL-21 and all of the chemokines were found after adenovirus infection. Besides, the responses detected in the BL compartment were generally higher than those in the apical compartment, especially IL-1RA, IL-21, GM-CSF, GRO-α, SDF-1α and IL-8.
Conclusion: During the infections of hAECs by adenovirus, higher viral replication was found in the apical compartment but cytokine and chemokine responses were higher in the basolateral compartment. This indicated viral entrance and replication occurred more in the apical part and major innate response took place in the basolateral part,

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which may make adenovirus infect human airway efficiently and cause different degree of severity.

Introduction

Adenoviruses (AdV) are non-enveloped double-stranded DNA viruses containing 35000-37000 base pairs. It was classified to 7 species from A to G based on DNA homology including more than 60 types. It causes significant human disease morbidity of upper and lower airway infections year around,[1] but also induces keratoconjuntivitis, acute gastroenteritits and even leads to severe fatal diseases in immunocompromised patients.[2] It was epidemically reported infection in children and military recruits.[3] Adenovirus also becomes a popular vehicle for gene therapy applications recently, and the initial innate immune response plays a role in acute toxicity from adenovirus vector exposure.[4] The immune response influences both the course of adenovirus disease and the success of the applied vectors. To date, adenoviruses are also the most frequently used vectors for gene therapy and the research of adenovirus emerges.

There are different receptors for adenovirus in polarized human airway epithelial cells. Coxsackievirus and adenovirus receptor (CAR), a single-pass transmembrane protein, was found over tight junction of human airway epithelial cells (hAEC), and CAREx8 was found at the apical surface of hAEC for all species except group B. IL-8 could stimulates the protein synthesis and apical localization of CAREx8 that interferes infiltrating neutrophils at the apical surface of epithelial cells. CD46, intergrins, DSG2, liver tropism, sialic acid-containing glycoproteins are also characterized adenovirus receptors for different species.[5] The virus binds to the receptors through interaction with the knob structure of the fiber. After intranuclear replication, there are also report of genetic and environmental influence of adenovirus spreading from cells.[6,7]

Some factors predispose to adenovirus infection but the detailed mechanism is still under study. The virus itself, is regarded as a proinflammatory virus that can stimulate the secretion of cytokines involving host innate immune response that makes facilitation of viral entry.[8,9] Innate response was initiated by the infected cell to release chemokines to attract white blood cells and activate interferon to block the replication of virus,[10] but the innate response became a major hurdle to adenoviral-mediated gene therapy. Polarized secretion of cytokines and chemokines is important for establishing a microenvironment for airway infection.[11] However, data about polarized secretion of proinflammatory cytokines and its signaling pathway in adenovirus-infected human airway epithelial cell is limited.

Immune responses and hypercytokinemia are related to disease severity in patients with adenovirus infection. Studying virus adaption and immune responses in hAECs may help to understand copious important issues concerning adenovirus infection in humans. Knowing viral entry to human epithelial cells and the immune response of host cell are critical and also potential for developing therapies of preventing adenovirus infection. In this study, we used primary hAECs as a model to evaluate viral replication kinetics after different ways of infection and also investigated the elicitation of cytokines and inflammatory mediators following adenovirus infection in hAECs to evaluate the host innate immune response.

Materials and methods

Human airway epithelial cell (hAEC) cultures

Primary hAECs were isolated from lung cancer patients who underwent lobectomy surgery at the Department of Surgery, National Taiwan University Hospital (NTUH), Taipei, Taiwan. Informed consent was obtained from all patients. Isolated tissues were digested with protease XIV-DNase I (Sigma—Aldrich, St. Louis, MO, US) and the following additives (Sigma): penicillin G sulfate (100 units/ml), streptomycin sulfate (100 μg/ml), amphotericin B (1.25 μg/ml), gentamicin (50 μg/ml), and nystatin (100 units/ml), and immersed in minimal essential medium (MEM; Invitrogen, Carlsbad, CA, US) at 4 °C for 24—48 h. After cell dissociation, the hAECs were maintained for one or two serial passages as a monolayer in bronchial epithelial cell serum-free growth medium, which is Laboratory of Human Carcinogenesis (LHC) basal medium (Invitrogen) supplemented with the required additives (Sigma Aldrich). BEGM was refreshed at 2- or 3-day intervals. Upon reaching 80% confluence, hAECs were passaged to form pseudostratified hAEC cultures as described elsewhere. Cultures were maintained at air-liquid interface for 4—6 weeks for cellular differentiation. Prior to the experiments, all cultures were maintained at 37 °C in a 5% CO2 incubator. The process of cell culture was approved by the institutional review board of NTUH by the serial of 201309062RINB.

Immunofluorescence assay

hAEcs was fixed with 4% paraformaldehyde at room temperature for 20 min. Fixed cells were then washed with PBS, permeabilized with 0.3% of tritonX-100 for 5 min and blocked with 5% BSA-PBS at room temperature for one hour. Cells were stained with mouse anti β-tubulin IV (ciliated cell marker, 1:200, BioGenex, Fremont, CA, US) and rabbit anti cytokeratin5 (basal cell marker, 1:200, Abcam, Cambridge, UK) or FITC conjugated Sambucus nigra lectin (SNA, substrate of α2,6-sialic acid, 1:100, Vector Lab, Burlingame, CA, US) and Biotin labeled Maackia Amurensis lectin

II (MALII, substrate of α2,3-sialic acid. 1:50, Vector Lab) on 37 °C for 1.5 h. Cells were washed with PBS and stained with fluorescence labeled secondary antibodies, including Alex568 conjugated goat anti-mouse IgG (Invitrogen, 1:1000) and Alexa488 conjugated goat anti rabbit IgG (1:500, Biotium, Fremont, CA, US) or Cy5 conjugated Streptavidin (Invitrogrn, 1:1000) on 37 °C for 1 h. Cells were washed with PBS and covered with mounting medium with DPAI (Biotium). Stained cells were preserved on cover slide and observed under ZEISS, LSM 510 META confocal microscope.

Adenovirus infection on hAECs and virus replication

Four groups of infection way were designed by 0.5 or 1 moi (multiplicity of infection) of the amplified adenovirus serotype 3 inoculated in the apical or basolateral compartment. Virus was diluted in 200 μl HBSS and was directly used to inoculate the surface of pseudostratified hAECs. After incubation at 37 °C in a 5% CO2 incubator for 1 h, the inoculated virus supernatant was isolated and washed with HBSS to remove the attached viruses. Inoculated cultures were maintained at 37 °C in a 5% CO2 incubator. After 24, 48 and 72 h post infection (hpi), 300 μl of the virus supernatants from apical and basolateral compartment were collected. Supernatant harvest was performed by adding 300 μl HBSS to the surface and incubating for 30 min at 37 °C under 5% CO2. 200 μl virus supernatant was transferred to 300 μl lysis buffer for total nucleic acid extraction.

Virus titer determination

Viral DNA was isolated from the collected samples of the apical and basolateral harvests by using MagNA Pure LC 2.0 System and MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science, Roche Diagnostics, Indianapolis, IN, USA). Virus titer was determined by real-time PCR on the hAdV conserved hexon gene region, which was established in previous studies.[11–13] The primer and probe sequences were described as following, Forward primer 5′-GCC ACG GTG GGG TTT CTA AAC TT-3′, Reverse primer 5′-GCC CCA GTG GTC TTA CAT GCA CAT C-3′, Probe (TaqMan) 6FAM-TGC ACC AGA CCC GGG CTC AGG TAC TCC GA –TMR. The PCR cycles consist of denaturing samples at 95 °C for 10 min, followed by 45 cycles, each consisting of 95 °C for 10 s, 55 °C for 10 s, and 65 °C for 40 s with 1-s increments between each cycle. Roche Light cycler 1.5 system was used for real-time PCR.

Cytokine and inflammatory mediator analysis

The supernatants from the group "0.5 moi-AP infection" were used for inflammatory mediator analysis. The supernatants from apical and basolateral compartments were inactivated by 0.1% paraformaldehyde at room temperature for 3 h and later submitted to immune response determination. Cytokines and chemokines concentrations were analyzed by ProcartaPlex Multiplex Immunoassay (Affymetrix, eBioscience, Santa Clara, CA, US). Time course cytokine analysis (24, 48 and 72 hpi) was done, and a total of 34 cytokines and chemokines were analyzed, including 25 cytokines: IL-1α, IL-1β, IL-2, IL-1RA, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-γ, IFN-α, TNF-α, TNF-β, GM-CSF and 9 chemokines: eotaxin, GRO-α, IL-8, IP-10, RANTES, MIP-1α, MIP-1β, SDF-1α, MCP-1. Mann–Whitney U test was performed to compare apical and basolateral response of the cytokines and chemokines after infection. P value less than 0.05 was considered statistically significant.

Results

Cell differentiation and immunofluorescence assay

Before adenovirus infection, immunofluorescence assay was performed to determine the cell differentiation. The primary hAECs were maintained as a monolayer in bronchial epithelial cell serum-free growth medium and had passaged to form pseudostratified hAEC in 4–6 weeks in this study. The Immunofluorescence assay (Fig. 1) revealed the hAECs finally differentiated to two cell layers of ciliated cells and basal cells detected by different antibody markers. The upper or apical layer was mainly formed by ciliated cells with the red marker of mouse anti β-tubulin IV and α2,3-sialic acid. From the lateral view of cells, the lower layer was mainly formed by basal cells with the green marker of rabbit anti cytokeratin 5 and α2,6-sialic acid.

Virus replication in different sites of infection with different inoculation doses

After we successfully cultured cells with pseudostratified differentiation, we innoculated adenovirus serotype 3 to the hAECs and detected the viral replication by quantitative PCR in 24, 48 and 72 h. The titer of adenovirus in the supernatants of hAECs showed difference from different infection sites, infection dosage and the time after infection. In the supernatants of apical compartment post infection, higher viral titer was found in the apical infection group, especially by the dose of 0.5 moi, and the titer increased with the time post infection (Fig. 2). In that group of 0.5 moi AP infection, 8.9×10^9 copies/ml was detected 72 hrs post infection (hpi), which was the highest titer in the study. The viral titers in the basolateral infection group were 2.3×10^7 to 5×10^8 copies/ml 72 hpi in the supernatants of apical compartment, which were lower than the titers in the apical infection group.

In the supernatants of the basolateral compartment post infection, the highest viral titer was found in the basolateral infection group, especially with high dose infection. The titer was 2×10^7 copies/ml in the basolateral supernatant of 1 moi basolateral infection 72 hpi, but this was only 1% of the viral titer in the apical supernatant (data not shown). There was no cytopathic effect or cell mortality observed in every time point (24, 48 and 72 hpi) in either 0.5 or 1 MOI infection in the study.

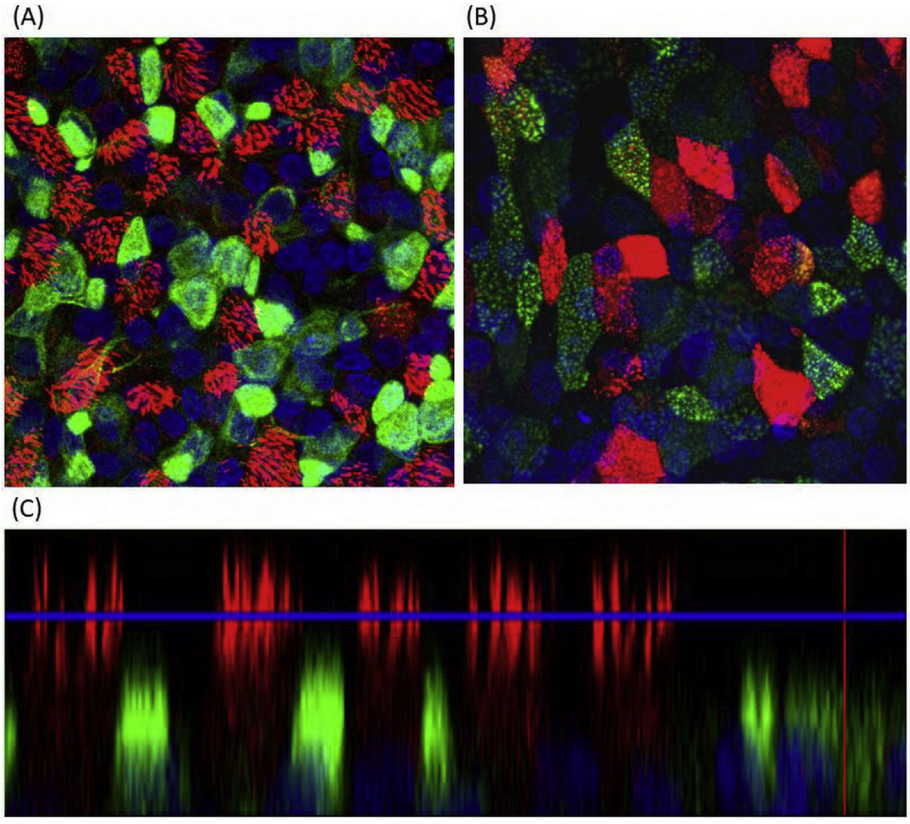

Figure 1. Human airway epithelial cell morphology after culture. (A) hAEC (human airway epithelial cell) was stained with antibodies to show ciliated cells by mouse anti β-tubulin IV (red) and basal cells by rabbit anti cytokeratin5 (green) from the apical view. (B) hAEC was stained by α2,3-sialic acid (red) and α2,6-sialic acid (green) from the apical view. (C) From the lateral view of cultured hAEC, ciliated cells were stained by mouse anti β-tubulin IV (red) over the apical area and basal cells were stained by rabbit anti cytokeratin5 (green) over the basal area, and the hAECs finally differentiated to two cell layers of ciliated cells and basal cells.

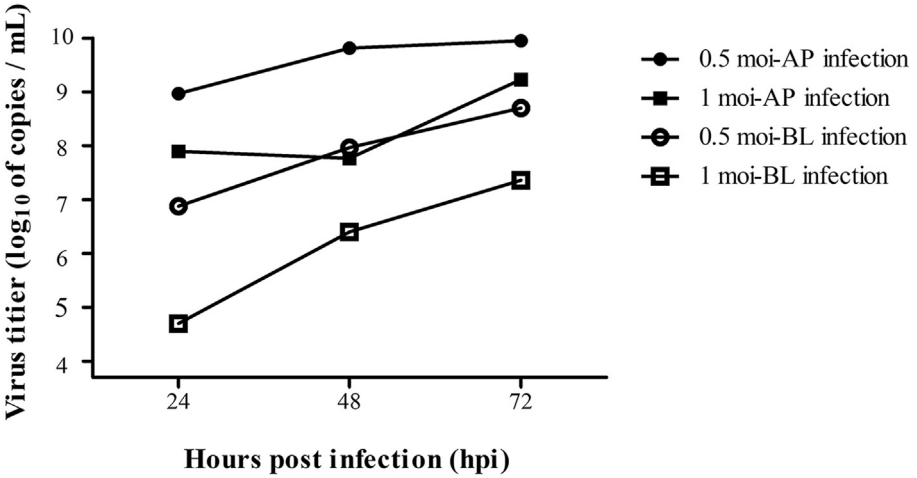

Figure 2. Time course of adenovirus viral titer after adenovirus infection of pseudostratified hAECs with different doses in different sites. Viral titer was the highest in the group of 0.5 moi AP infection. Viral titer increased with the time post infection. BL denotes basolateral, AP apical and moi the multiplicity of infection.

Cytokine and chemokine responses

Since the highest viral titer was detected in the group of 0.5 moi apical infection (Fig. 3), we measured the immune response in this group only, cytokine and chemokine responses were detected in both basolateral and apical compartments by multiplex immunoassay at 24, 48, 72 hpi. Sixteen of 25 cytokines and all nine chemokine responses were detected (Table 1). The responses in the BL compartment were generally higher than those in the

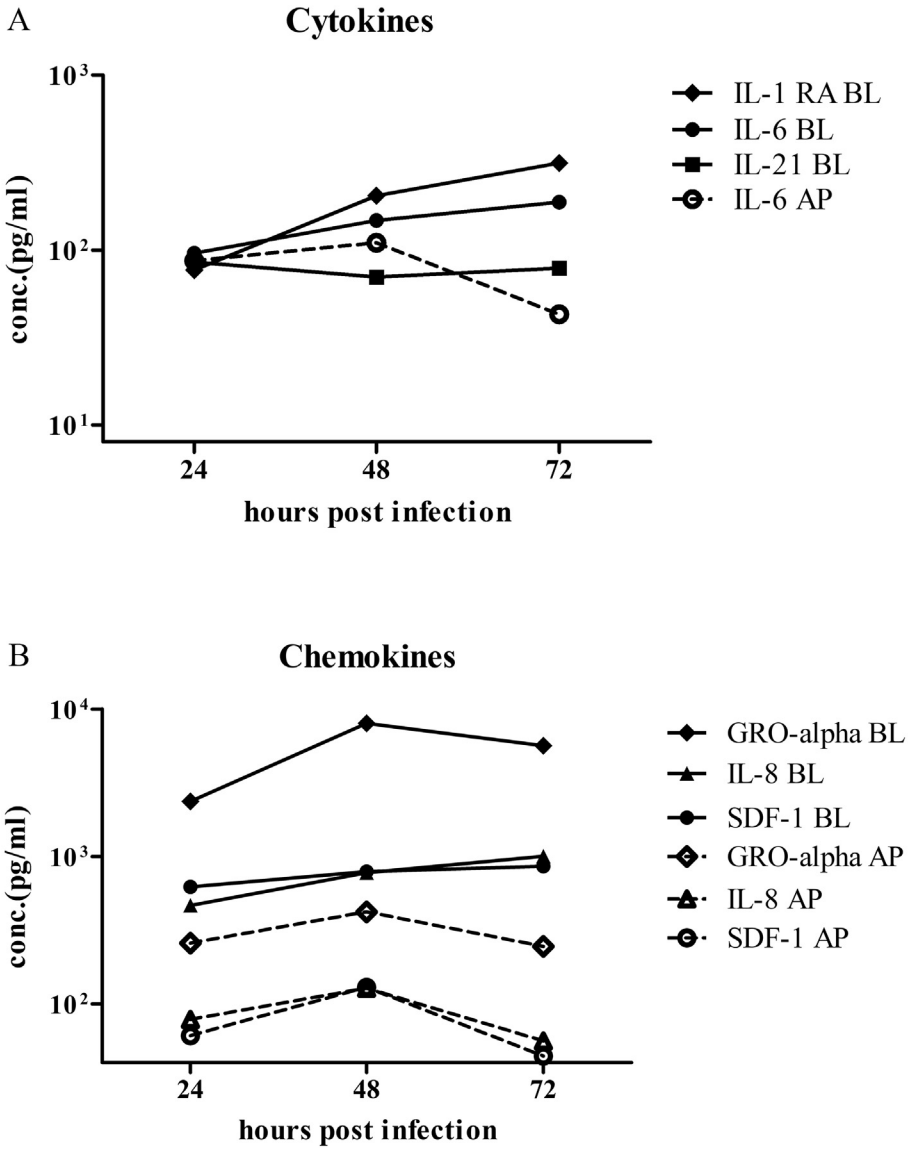

Figure 3. Times course of cytokines and chemokine responses after adenovirus infection on polarized hAECs. (A) After 0.5 moi apical infection, the elevated cytokines are shown including IL-1 RA, IL-6, Il-21 and GM-CSF in the basolateral supernatant and IL-6 in the apical supernatant. (B). After 0.5 moi apical infection, the elevated chemokines, GRO-α, IL-8 and SDF-1α, in both BL and AP supernatants are shown. All the levels in the BL supernatant were higher than those in the AP supernatant. BL denotes basolateral, and AP apical.

apical compartment, including IL-1RA, IL-21, IL-31, GM-CSF, IL-1α, IL-23, IL-4, IL-1β, IL-12p70,GRO-α, SDF-1α, IL-8, MIP-1β, MCP-1, IP-10, RANTES, MIP-1α, and eotaxin.

The elevated levels also diverged among different cytokines. The 3 highest elevated cytokines detected in the supernatant are IL-1RA, IL-6, and IL-21 (Fig. 3A). Among all the cytokines tested, IL-1RA, IL-21, GM-CSF, IL-1α, IL-10, IL-4, IL-18, IL-12p70, TNF-β, IFN-γ,IL-13, were only detected in the basolateral supernatant. IFN-α, TNF-α, IL-2, IL-5, IL-7, IL-9, IL-15,IL-17A, IL-22 and IL-27 were not detected in this study. All the nine chemokines in the immunoassay were all detected (Table 1B). The 3 highest elevatedchemokines were GRO-α, SDF-1α,IL-8 (Fig. 3B). Only one chemokine, MIP-1α, was not detected in the apical compartment.

Discussion

This study elucidated the viral replication kinetics after adenovirus infection of human epithelial cells and the innate immune response from human airway epithelial cells. Cells entry of adenovirus involves attachment to a primary receptor and subsequently interaction with a secondary receptor for internalization. Earlier studies found the relative resistance to adenovirus infection from the apical surface due to lack of coxsackievirus and adenovirus receptor (CAR),[14] but there was a study showed that adenovirus could still infect polarized epithelial cell through apical receptors.[15] In our results, higher viral titer detected from apical site infection indicated that different sites of polarized cells involved different adenovirus

Table 1 Cytokines and chemokines response of 0.5 MOI adenovirus apical infection of hAECs.

	AP-24 hr	AP-48 hr	AP-72 hr	mean	BL-24 hr	BL-48 hr	BL-72 hr	mean	p
A. Cytokines									
IL-1RA	0	0	0	0	76.9	204.5	313.9	198.4	0.037*
IL-6	86.7	110.2	43	80	95.9	147.8	187.8	143.8	0.127
IL-21	0	0	0	0	85.5	70.1	78.7	78.1	0.037*
IL-31	24.9	15.7	39.6	26.8	54.3	57.9	47.1	53.1	0.050*
GM-CSF	0	0	0	0	12.6	37.8	60.3	36.9	0.037*
IL-1alpha	0	0.6	0	0.2	13.3	8.6	10.3	10.8	0.046*
IL-10	0	2.4	2.9	2.6	8.7	9.1	7.7	8.5	0.083
IL-23	5.9	2.7	5.4	4.6	10.2	6.8	7.3	8.1	0.050*
IL-4	0	0	0	0	4.5	11.1	7	7.5	0.037*
IL-1beta	0	1.2	1.2	0.8	1.3	3.6	4.8	3.2	0.050*
IL-18	0	0	0	0	0	3.9	1.1	1.6	0.121
IL-12p70	0	0	0	0	1.3	1.3	1.1	1.2	0.034*
TNF-beta	0	0	0	0	3.6	0	0	1.2	0.317
IFN-gamma	0	0	0	0	0	2.5	0	0.8	0.317
IL-13	0	0	0	0	0	0.3	0	0.1	0.317
B. Chemokines									
GRO-alpha	257.9	421.4	246.1	308.5	2359.1	7994	5631.2	5328.	0.050*
SDF-1alpha	60.9	129.4	44.3	78.2	621.9	792	860.8	758.2	0.050*
IL-8	78.5	127.9	56	87.5	466.8	775.1	1004.1	748.6	0.050*
MIP-1beta	58.7	108.4	53.3	73.4	507.7	625.9	640.5	591.4	0.050*
MCP-1	0	5.5	0	1.8	59.9	125.9	135.5	107.1	0.046*
IP-10	1.7	5.7	4	3.8	23.1	35.4	46.6	35	0.050*
RANTES	1.4	3	2.2	2.2	12.4	15.4	14.7	14.2	0.050*
MIP-1alpha	0	0	0	0	7.8	11.6	13	10.8	0.037*
Eotaxin	0	3.34	0	1.1	8.9	9.9	11.9	10.2	0.046*

MOI denotes multiplicity of infection, AP apical compartment, and BL basolateral compartment. 24hr denotes for 24hr after infection, 48hr denotes for 48hr after infection, 72hr denotes for 72hr after infection. The cytokines and chemokines were listed as the order of mean levels of the basolateral compartment. Cytokine of IFN-alpha, TNF-alpha, IL-2, IL-5, IL-7, IL-9, IL-15, IL-17A, IL-22, IL-27 were not detected in all the panels. * Denotes for $p \leq 0.05$ in Mann–Whitney U test.

receptors and mechanism of cell entry. The primary receptor for most adenoviruses is CAR that is normally localized at the basolateral surface of polarized epithelial cell. However, CAREx8 receptor was found in the apical surface and allowed the initial infection of adenovirus that interacted with innate immune response to stimulate the expression of its primary receptor, CAREx8.[8] Higher viral load from the apical site may be a result of facilitation by the interaction of host innate immune response. In our study, the viral load of 0.5 moi apical infection was higher than 1 moi infection in both apical and basolateral supernatants. In a previous study, adenovirus traveled between basolateral and apical surface in epithelial cells. The adenovirus fiber protein binds to CAR and disrupted junctional integrity, that facilitated apical escape.[16] The mechanism that infection dose disassociated with post-infection viral load needs further investigation. The viral load is higher in the AP supernatant of the AP infection and in the BL supernatant of the BL infection. This illustrates the polarization of cell-free transmission of adenovirus in human epithelial cells.

The airway epithelium participates in inflammation process by responding to a variety of inflammatory mediators and cytokines that play a major role in the pathogenesis of tissue damage. The innate immune system consists of the local immediate response to infection and results in the acute inflammation during wild-type infection and in the case of adenovirus gene therapy toxicity.[4] The surface epithelium itself is also responsible for the synthesis and release of cytokines that recruit and accumulate of various inflammatory cells.[11] In our result, human epithelial cell responses to adenovirus infection with several innate immune response. Immunoarray data showed that 25 cytokines and chemokines were elevated out of the 34 tested, including marked elevation in IL-1 RA, IL-21, GRO-α, SDF-1α and IL-8, secretion. The elevation of proinflammatory cytokines and mediators secreted from human epithelial cells were significantly higher in basolateral secretion than secretion into the apical medium. The results demonstrate that infection of human airway epithelial cell stimulates polarized secretion of varieties of cytokines and chemokines. It is important for establishing a specific microenvironment for airway inflammation. This basolaterally secretion of cytokines may play an important role in epithelial cell responses to infection and inflammation.

Elevation of proinflammatory cytokine effect was found in IL-6, IL-1β and GM-CSF that will induce T, B cell, eosinophil and neutrophil activation and chemoattractant in vivo. Increased concentrations of IL-6 were associated with hypoperfusion, febrile peaks, tonic-clonic seizures, and septic shock of adenovirus infection in children.[17] Elevation of Th2 cytokines such as IL-4 and MIP-1α could

induce Th2 related activation response and T-cell recruitment. Inhibitory cytokines including IL-10, IL-18 and IL-1 RA were elevated in the basolateral compartment after infection in this study. These cytokines would decrease eosinophil survival, Th2 cell proliferation, macrophage activation, mast cell growth and release of pro-inflammatory cytokines.

Some cytokines, such as like IL-1α, TNF-α and IFNs, involving inflammatory response of adenovirus infection, were low in our study. These cytokines are potent inducers for the production of chemokines such as IL-8, and previous studies had already shown elevation of these cytokines after adenovirus infection.[18] It may be due to the lack of other cellular response for innate immunity in this in-vitro study. There were only human airway epithelial cells in the study that cannot fully represent the complicated immune response in human body. However, one of the explanation may attribute to the mechanisms down-regulating nonspecific and specific inflammatory responses, which had been demonstrated in adenoviruses.[19] The E3 region of adenovirus encodes several proteins that modulate host functions in response to viral infection.[19] It protects cells from apoptosis-inducing cytokines, such as tumor necrosis factor.[20]

Several chemokines were also elevated in the basolateral supernatant, such as IL-8, IP-10, RANTES, eotaxin, MCP-1, GRO-α and MIP-1β. They are chemoattractants for Th1 cells, mast cells, monocytes, neutrophil, eosinophil, basophils, memory T-cells and NK T-cells. Eotaxin could activate eosinophils from bone marrow and adhesion of eosinophils to endothelium.[21] IL-8 is also the major neutrophil chemotactic factor in the lung and its level correlates with clinical outcome.[22] Previous studies reported there was type-specific induction of IL-8 by adenovirus type 7,[23,24] however, adenovirus serotype 3 infection in our study also induces IL-8 production.

There are some limitations in this study. First, there is lack of a control group for this study. Further experiment may be performed to reconfirm the results of this study. Second, the viral replication and immune response in vitro cannot represent the reaction in vivo. Host defense in the respiratory tract is complicated and the interaction of cytokines and chemokines needs to be assessed for in vivo host response. Finally, different adenovirus species entry the cell through different receptors. That could influence the condition of viral replication and host immune response. A profounder understanding of the nature of the immune response to adenovirus will likely benefit both fields as we search for novel methods to defend the host against this pathogen or to use a modified pathogen to restore normal function of the host.

Protection against adenovirus infection by the innate immune system involves complex factors through recruitment or activation of inflammatory cells. We found there are different viral replication load and different host cell response from different sites of infection of human airway epithelial cells by adenovirus. The viral load was higher in the apical site but the cytokines were detected higher from the basolateral compartment of hAECs. This indicated a possible mechanism that host cell innate response to adenovirus infection may interfere the entrance and trafficking of the virus. The process could make the virus infect and transmit human more efficiently. The result could also provide information for adenovirus vector immunity of gene delivery platform for therapeutic and vaccine purpose.

In conclusion, higher viral replication was found in the apical compartment but cytokine and chemokine responses were higher in the basolateral compartment during the infections of hAECs by adenovirus. This indicated viral entrance and replication occurred more in the apical part and major innate response took place in the basolateral part, which may make adenovirus infect human airway efficiently and cause different degree of severity during adenovirus infection.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

TIY and LYC conceived and designed the study, performed analyses, interpreted the data, drafted and revised the manuscript. WLL carried out the experiments. WLL, CYL, JMC, PIL, LMH and LYC revised the manuscript. All authors read and approved the final version of this manuscript.

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