Negative Regulators of Inflammation Response to the Dynamic Expression of Cytokines in DF-1 and MDCK Cells Infected by Avian Influenza Viruses

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Received 5 June 2021; accepted 17 September 2021

Abstract—The H5N1 and H9N2 avian influenza viruses (AIVs) seriously endanger the poultry industry and threaten human health. Characteristic inflammatory responses caused by H5N1 and H9N2 AIVs in birds and mammals result in unique clinical manifestations. The role of anti-inflammatory regulators, PTX3, Del-1, and GDF-15, in H5N1 and H9N2-AIV-mediated inflammation in birds and mammals has not yet been verified. Here, the expression of PTX3, Del-1, and GDF-15 in DF-1 and MDCK cells infected with H5N1 and H9N2 AIVs and their effect on inflammatory cytokines were analyzed. Infection with both AIVs increased PTX3, Del-1, and GDF-15 expression in DF-1 and MDCK cells. Infection with H9N2 or H5N1 AIV in DF-1 and MDCK cells with overexpression of all three factors, either alone or in combination, inhibited the expression of tested inflammatory cytokines. Furthermore, co-expression of PTX3, Del-1, and GDF-15 enhanced the inhibition, irrespective of the cell line. The findings from this study offer insight into the pathogenic differences between H5N1 and H9N2 AIVs in varied hosts. Moreover, our findings can be used to help screen for host-specific anti-inflammatory agents.

KEY WORDS: avian influenza viruses; PTX3; Del-1; GDF-15; inflammatory cytokines.

INTRODUCTION

Avian influenza virus (AIV), a type A influenza virus, is a threat to both the poultry industry and human health [1]. Based on their pathogenicity in chickens, AIVs can be divided into two groups—highly pathogenic avian influenza viruses (HPAIv) and low pathogenic avian influenza viruses (LPAIVs) [2]. HPAIV H5N1 and LPAIV H9N2 are among the most important causes of avian influenza in China [3, 4]. Previous reports demonstrate that the clinical symptoms that arise due to H9N2 and H5N1 AIVs infection are unique. Specifically, H9N2 AIV usually results in a mild host response, while H5N1 AIV causes respiratory distress syndrome and can even result in death [5, 6]. These clinical differences reflect the distinctive inflammatory responses caused by different AIV subtypes.
Inflammation is one of the most important host responses against influenza virus infection, and a core indicator of virus pathogenicity [7]. Leukocyte exudation is an important hallmark of the inflammatory response, and involves leukocyte margination, adhesion, and emigration [8–10]. Negative regulators of inflammation are evolutionarily conserved and play an important role in inhibiting the inflammatory response [11–13]. PTX3, Del-1, and GDF-15 are negative regulators of inflammation and are involved in different stages of leukocyte exudation [14–16]. Developmental endothelial locus-1 (Del-1) contains three epidermal growth factor–like domain repeats and two C-terminal discoid I-like domains [12, 17]. Del-1 can bind to LFA-1 integrin and antagonize the interaction between LFA-1 on neutrophils and ICAM-1 on endothelial cells [18]. Consequently, Del-1 effectively blocks LFA-1-dependent neutrophil adhesion to the vascular endothelium [19, 20]. Pentraxin 3 (PTX3) was the first long chain pentamer to be discovered and plays an important role in humoral immunity [21–23]. As an inhibitor of leukocyte aggregation, PTX3 competitively interferes with P-selectin interactions. This blocks neutrophil rolling and prevents neutrophil recruitment [24–27]. Growth differentiation factor 15 (GDF-15) is a member of the transforming growth factor-β superfamily [28, 29]. The regulatory effect of GDF-15 on leukocyte aggregation is mediated by activating Cdc42 GTPase and inhibiting Rap1 GTPase, thereby blocking the activation of β2 integrin, which is induced by chemokines [30, 31]. Considering an inhibitory effect of these three negative regulators on the typical phase of leukocyte exudation in the inflammatory response, how they participate in the inflammatory response caused by different avian influenza virus remains to be clarified.

To date, the anti-inflammatory effects of PTX3, Del-1, and GDF-15 in the context of AIV infection have not yet been explored. Herein, how PTX3, Del-1, and GDF-15 impact inflammation in response to AIV infection was investigated. Furthermore, whether these inflammatory responses overlap between avian and mammalian cells was also examined. To accomplish this, DF-1 and MDCK cells with overexpression of PTX3, Del-1, and GDF-15, either alone or in combination, were infected by H5N1 and H9N2 AIVs, and the expression profile for inflammatory cytokines was assessed. Collectively, the results from our study provides further explanation for the pathogenic differences between AIV strains in varied hosts.

MATERIALS AND METHODS

Cells and Virus

DF-1 (UMNSAH/DF-1) and MDCK (NBL-2) cells from ATCC were maintained at 37 ºC in 5% CO2 in Dulbecco modified eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen), 100 g/mL streptomycin, and 100 IU/mL penicillin (Gibco). To passage DF-1 cells, 0.25% trypsin–EDTA (Sigma, St. Louis, MO, USA) was used for trypsinization. The two avian influenza virus strains used in this research are H5N1 influenza A virus (A/goose/Guangdong/SH7/2013) and H9N2 influenza A virus (A/chicken/Guangdong/V/2008) [5, 32, 33]. Virus propagation, maintenance, and storage were performed as previously described [5, 32, 33]. All infectious materials were handled in the Biosafety Level 3 laboratory in South China Agricultural University.

Plasmid Construction and Cell Transfection

The primer pairs used in DF-1 cells for PTX3, Del-1, and GDF-15 were as follows: PTX3-Fwd: TTA TAAGCTAGCGGCCACCATGCTGCTTGAGAGGT (underlined is the *Nhe* I restriction site), PTX3-Rev: CAGGCAAGGTACCCGTAAGACCATACTGAGC TCCT (underlined is the *Kpn* I restriction site); Del-1-Fwd: TATATCTGAGCCACC ACCATGAGCGTCTGGTGCTGCTG (underlined is the *Xho* I restriction site), Del-1-Rev: GTA AGAAGATCTCGTCTGCTCGTGCAGCGT GCTG (underlined is the *Xho* I restriction site); GDF-15-Fwd: AGCCATAGA TCTCGACACCATGCGTGCCAGCCCA (underlined is the *EcoR* I restriction site), GDF-15-Rev: AGATCGAAATTCGGGC GCGCGATGCCAGCGG (underlined is the *EcoR* I restriction site). The primers for MDCK cells were as follows: PTX3-Fwd: AGCCATAGA TCTCGACACCATGCGTGCCAGCCCA (underlined is the *EcoR* I restriction site), PTX3-Rev: AGATCGAAATTCGGGC GCGCGATGCCAGCGG (underlined is the *EcoR* I restriction site). The primers for MDCK cells were as follows: PTX3-Fwd: AGCCATAGA TCTCGACACCATGCGTGCCAGCCCA (underlined is the *EcoR* I restriction site), PTX3-Rev: AGATCGAAATTCGGGC GCGCGATGCCAGCGG (underlined is the *EcoR* I restriction site). The amplicons
were digested by the restriction enzymes and cloned into
the corresponding sites of pEGFP-N1 vector. All recom-
binant plasmids were verified by sequencing.

DF-1 and MDCK cells were seeded in six-well plates at 2.5 × 10^5 cells/well, and transfected with plas-
mids using Lipofectamine 2000 (Invitrogen) according
to the manufacturer’s instructions. At 6-hours (h) post-
transfection, fresh DMEM containing 2% FBS replaced
the transfection mixture, and cells were incubated for an
additional 48 h.

Cell transfection with PTX3, Del-1, and GDF-15
was performed in one of the following three ways: (1)
plasmids constructs were transfected individually; (2)
constructs were co-transfected at a 1:1 ratio (paired
combinations); or (3) all three constructs were co-
transfected together at a 1:1:1 ratio. Twenty-four hours post-
transfection, cells were infected with H9N2 or H5N1 AIV
as described below.

**Cell Infection**

Cells were infected with H9N2 (10^{-4.6} TCID_{50}/mL)
or H5N1 (10^{-6.8} TCID_{50}/mL) AIV at an MOI of 0.1 for
1 h at 37 °C. After incubation, the cells were washed
twice by PBS and then incubated with DMEM containing
0.2% BSA at 37 °C with 5% CO_2. For H9N2 infection,
TPCK-trypsin (0.25 mg/mL) was added to the culture
medium. Cells and supernatant were collected at 0, 3, 6,
12, and 24 h post-infection. Three independent assays on
each sample at specified time points and entire exper-
iment three times were performed.

**RNA Extraction, cDNA Preparation,
and Real-time PCR**

Total RNA was extracted, treated, quality deter-
mined, stored, and reverse-transcribed to cDNA as
described in our previous reports [34, 35]. Relative
expression levels of IL-1β, IL-6, and TNF-α were
determined by real-time PCR using DNA Engine 7500
Continuous Fluorescence Detection System (Applied
Biosystems, CA, USA) and SYBR® Premix Ex Taq TM
Kit (Takara Biotech, Dalian, China). Primers used for
qRT-PCR were designed by Oligo 7 software (Molecu-
lar Biology Insights Inc., Cascade, CO, USA), and the
sequences for DF-1 cells were as follows: IL-1β-Fwd:
ATGACCAAACTGCTGCGAGG, IL-1β-Rev: GAA
GGACTGTGAGCGGTTGTA; IL-6-Fwd: CAAGAA
GTTCCAGCGTG GCGAGA, IL-6-Rev: ATTCGAGTG
AGTCTGAAGGCGO, TNF-α-Fwd: CTCAAGGC CAG
CCTATGCGCAACA, TNF-α-Rev: CACCACACGACA
GCCAAGTCAA; β-actin-Fwd: TGAACCTCCCTGA
TGGTCAGGTC, β-actin-Rev: ACCACAGGACTC
CATACCC AAG; primers for MDCK cells were as fol-
lovs: IL-1β-Fwd: GTCGAGCCCTCATGCGGT GTTC,
IL-1β-Rev: GCCAGGCACGACTCAAGGC; IL-
6-Fwd: ATGGCTACTGCT TTCCCTACC, IL-6-Rev:
CAGTGCCCTCCTTGCTCCTTC; TNF-α-Fwd: ATG
AATCCG CCAGCCTACTACAC, TNF-α-Rev: CAA
TCATCTGTGGCAGGAGAAGA-3; β-actin-Fwd: CAG
CGGCCAGGTCACTA, β-actin-Rev: TTCATG
GATGCGCCAGGATT. The expression levels for these
genes were normalized to that of β-actin, and relative
gene expression levels were calculated using the rela-
tive standard curve 2^{−ΔΔCt} method.

**Determination of Virus Titer and the Virus
Growth Curve**

The virus supernatant was diluted tenfold from
10^{-1} to 10^{-10} by serum-free incubation solution with 8
replicates for each dilution. After incubation, the cells
were washed twice by PBS and then incubated with
diluted virus at 37 °C with 5% CO_2 for 1 h, and set
two rows as negative control. Then, the diluted virus
was discarded and 200 μL maintenance solution was
added to each well and continued to incubate. The cell
growth was observed and recorded every day, and the
cytopathic criteria were compared with control for con-
tinuous observation for 2 to 3 days. The titer of the
virus was determined by Reed-Muench method and the
growth curves of H5N1 and H9N2 AIVs were plotted
respectively.

**Statistical Analysis**

Statistical analyses were performed using SPSS
software (version 20.0), and figures were obtained using
GraphPad Prism (GraphPad Software, La Jolla, CA).
One-way ANOVA or Student’s t-test was used to deter-
mine statistical significance between samples. Statisti-
cally significant differences were obtained when p < 0.05.
RESULTS

Expression of Inflammatory Cytokines in DF-1 and MDCK Cells Infected by H9N2 and H5N1 AIVs

mRNA transcripts for inflammatory cytokines including IL-1β, IL-6, and TNF-α increased significantly in DF-1 and MDCK cells infected by H5N1 and H9N2 AIVs. In DF-1 cells, IL-1β, IL-6, and TNF-α increased significantly from 6 h after infection with both AIVs (p < 0.01), but higher expression levels were observed in response to H5N1 than H9N2 infection (Fig. 1A–C). This is consistent with the expression trend of inflammatory cytokines in DF-1 cells in our previous study [5]. In MDCK cells, IL-1β expression increased significantly from 6 h with H9N2 and H5N1 infection (p < 0.01), with higher expression in response to H9N2 AIV than H5N1 (Fig. 1D). IL-6 expression increased significantly from 3 to 24 h following both H9N2 and H5N1 infection (p < 0.05). Between 3 and 12 h post-infection, H5N1 AIV elicited significantly higher IL-6 expression than H9N2 infection (Fig. 1E). TNF-α expression increased significantly from 3 to 24 h after infection with H9N2 AIV (p < 0.01), and from 3 to 12 h after infection with H5N1 (p < 0.01). Similar to other tested cytokines, H5N1 infection caused a higher change in TNF-α expression than H9N2 infection (Fig. 1F).

Expression of PTX3, Del-1, and GDF-15 in DF-1 and MDCK Cells Infected by H9N2 and H5N1 AIVs

Upon H9N2 and H5N1 AIV infection in DF-1 cells, PTX3, Del-1, and GDF-15 expression levels increased significantly. In DF-1 and MDCK cells, PTX3 expression levels peaked at 12 h after infection by H9N2 AIV (p < 0.01), while expression levels in response to H5N1 infection steadily increased until study endpoint (p < 0.05; Fig. 2A, D). In response to H9N2 AIV infection, Del-1 expression increased and remained stable from 3 to 12 h (p < 0.05), peaking at 24 h in both cell lines (p < 0.01). H5N1 infection increased significantly Del-1 expression levels, which remained relatively stable from 3 to 24 h (p < 0.01). However, the elicited change in expression was not as robust as that observed in the H9N2 infection group (Fig. 2B, E). GDF-15 analysis revealed that

Fig. 1 Dynamic expression of IL-1β, IL-6, and TNF-α in DF-1 and MDCK cells infected with H5N1 and H9N2 AIVs. * p < 0.05; ** p < 0.01. Data are presented as mean ± standard deviation (SD).
expression levels gradually increased over 24 h in DF-1 cells irrespective of the AIV used for infection ($p < 0.01$). In contrast to DF-1 cells, MDCK cells exhibited different GDF-15 expression dynamics in response to both H9N2 and H5N1 AIVs infection. Specifically, GDF-15 slowly peaked at 12 h in MDCK cells, after which expression levels subsequently declined ($p < 0.01$; Fig. 2C, F). Altogether, these findings show that H9N2 and H5N1 AIVs infection in DF-1 and MDCK cells led to increased PTX3, Del-1, and GDF-15, and that the change in expression due to H9N2 is greater than that due to H5N1.

**Dynamic Expression of Inflammatory Cytokines in H9N2 AIV-Infected DF-1 and MDCK Cells Overexpressing PTX3, Del-1, and GDF-15 Alone**

To determine the effects of PTX3, Del-1, and GDF-15 on cytokine expression, DF-1 and MDCK cells with and without overexpression of these proteins were infected by H9N2 AIV. Cells infected by H9N2 AIV served as the control. In DF-1 cells infected with H9N2 AIV, PTX3 overexpression resulted in significantly decreased IL-1β, IL-6, and TNF-α expression at 24 h compared to the control ($p < 0.01$; Fig. 3A–C). In similarly infected MDCK cells, PTX3 overexpression resulted in significantly decreased IL-1β expression at 6 ($p < 0.05$) and 24 h ($p < 0.01$; Fig. 3D), IL-6 expression after 12 h ($p < 0.01$; Fig. 3E), and TNF-α expression at 6 h compared to the control ($p < 0.01$; Fig. 3F).

Del-1 overexpression in H9N2-infected DF-1 cells caused significantly reduced expression of IL-1β at 12 and 24 h ($p < 0.01$; Fig. 3G) and IL-6 at 6 and 12 h ($p < 0.01$; Fig. 3H) compared to the control. Unlike IL-1β and IL-6, TNF-α expression was uninhibited under these conditions (Fig. 3I). In MDCK cells infected with H9N2 AIV, Del-1 overexpression significantly reduced IL-1β and TNF-α expression from 3 to 24 h ($p < 0.05$; Fig. 3J, L), whereas IL-6 expression decreased at 3 ($p < 0.01$) and 12 h ($p < 0.05$; Fig. 3K).

H9N2 AIV infection in GDF-15-overexpressing DF-1 cells, resulted in significantly decreased IL-1β expression at 12 h ($p < 0.01$; Fig. 3M), whereas IL-6 and TNF-α expression were uninhibited (Fig. 3N, O). In GDF-15-overexpressing MDCK cells, H9N2 AIV infection significantly decreased IL-1β expression from 6 to 24 h.
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**Fig. 3** Expression of IL-1β, IL-6, and TNF-α in DF-1 and MDCK cells with overexpressing PTX3, Del-1, and GDF-15 alone infected with H9N2 AIV. *p < 0.05; †p < 0.01. Data are presented as mean ± standard deviation (SD).

(p < 0.01; Fig. 3P), while IL-6 and TNF-α expression significantly decreased from 3 to 24 h (p < 0.05, respectively; Fig. 3Q, R).

**Dynamic Expression of Inflammatory Cytokines in H9N2 AIV-Infected DF-1 and MDCK Cells Combined Overexpressing PTX3, Del-1, and GDF-15**

Similar to the above, DF-1 and MDCK cells with and without combined overexpression of PTX3, Del-1, and GDF-15 were infected by H9N2 AIV. Cells infected by H9N2 AIV served as the control. Combined overexpression of PTX3 and Del-1 in H9N2 AIV-infected DF-1 cells resulted in significantly decreased IL-1β expression between 6 and 24 h compared to the control (p < 0.01; Fig. 4A), whereas IL-6 expression was only decreased significantly at 6 h (p < 0.05; Fig. 4B), and TNF-α expression was only significantly reduced at 24 h (p < 0.05; Fig. 4C). In similarly transfected MDCK cells, H9N2 AIV infection caused decreased IL-1β expression at 24 h compared to the control (p < 0.01; Fig. 4D). Additionally, IL-6 expression decreased at 12 h (p < 0.01; Fig. 4E) and TNF-α expression reduced at 3 and 6 h post-H9N2 AIV infection (p < 0.01; Fig. 4F).

In DF-1 cells with combined PTX3 and GDF-15 overexpression, H9N2 AIV infection resulted in a significant reduction in IL-1β and TNF-α expression at 12 and 24 h (p < 0.01; Fig. 4G, I), and IL-6 expression was only decreased at 24 h (p < 0.01; Fig. 4H). In MDCK cells, PTX3 and GDF-15 co-expression followed by H9N2 AIV infection resulted in a significant reduction in IL-1β and TNF-α from 3 to 24 h (p < 0.05; Fig. 4J, L), and IL-6 at 12 h (p < 0.05; Fig. 4K).

qPCR analysis of H9N2 AIV-infected Del-1 and GDF-15-expressing DF-1 cells revealed that IL-1β expression significantly decreased from 3 to 24 h compared with the control (p < 0.01; Fig. 4M), IL-6 expression from 6 to 24 h (p < 0.01; Fig. 4N) and TNF-α expression at 12 and 24 h (p < 0.01; Fig. 4O). Similar findings for IL-1β were observed in MDCK cells (p < 0.01; Fig. 4P). However, IL-6 expression decreased significantly at 3 (p < 0.01), 6 (p < 0.05), and 24 h (p < 0.01; Fig. 4Q) and TNF-α decreased at 3 and 6 h in this cell line (p < 0.01; Fig. 4R).

Combined PTX3, Del-1, and GDF-15 overexpression prior to H9N2 AIV infection in DF-1 cells attenuated IL-1β and IL-6 expression after 3 h compared to control (p < 0.01; Fig. 5A, B) and reduced TNF-α after 6 h (p < 0.01; Fig. 5C). Similar experiments in MDCK cells showed significant inhibition of IL-1β, IL-6, and TNF-α expression levels after 6 h of H9N2 AIV infection (p < 0.01; Fig. 5D–F).

**Expression Profile of the Inflammatory Cytokine in PTX3-, Del-1-, and GDF-15-Overexpressing DF-1 and MDCK Cells Infected with H5N1 AIV**

To determine if the inflammatory responses observed were unique to H9N2 or shared with other type A influenza viruses, we performed the same experiments using H5N1 AIV. IL-1β, IL-6, and TNF-α expression decreased significantly from 6 to 24 h in PTX3-overexpressing DF-1 cells infected with H5N1 AIV compared to control (p < 0.05; Fig. 6A–C). Similar experiments in MDCK cells showed that IL-1β expression decreased significantly at 6 and 24 h (p < 0.01; Fig. 6D), IL-6 decreased from 3 to 24 h (p < 0.01; Fig. 6E), and TNF-α decreased at 6 and 12 h (p < 0.01; Fig. 6F).

In Del-1-overexpressing DF-1 cells infected with H5N1 AIV, IL-1β and IL-6 expression decreased significantly at 6 and 24 h compared with the control (p < 0.01; Fig. 6G, H), whereas TNF-α decreased at 3 and 6 h (p < 0.01; Fig. 6I). In MDCK cells, IL-1β and TNF-α decreased significantly from 6 to 24 h compared with the control (p < 0.05; Fig. 6J, L), while IL-6 expression decreased from 3 to 24 h (p < 0.01; Fig. 6K).

H5N1 AIV infection in GDF-15-overexpressing DF-1 cells led to a reduction in IL-1β at 12 and 24 h compared to the control (p < 0.01; Fig. 6M), while IL-6 decreased from 6 to 24 h (p < 0.05; Fig. 6N), and TNF-α decreased at 12 h (p < 0.01; Fig. 6O) relative to the control. When performed in MDCK cells, we observed that IL-1β and IL-6 expression decreased significantly at 3 and 6 h compared to the control (p < 0.05; Fig. 6P, Q), and TNF-α was decreased significantly at 6 and 12 h (p < 0.01; Fig. 6R).

H5N1 AIV infection in DF-1 cells with paired co-expression of PTX3, Del-1, and GDF-15, the expression of IL-1β, IL-6, and TNF-α decreased significantly from 6 to 24 h compared to the control (p < 0.01). In MDCK
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cells, expression levels for all three cytokines decreased significantly from 6 to 24 h compared to the control ($p < 0.01$; Fig. 7).

Simultaneous overexpression of PTX3, Del-1, and GDF-15 in DF-1 cells significantly attenuated H5N1 AIV–induced expression of IL-1β, IL-6, and TNF-α after 6 h compared to the control ($p < 0.01$; Fig. 8A–C). When repeated in MDCK cells, IL-1β and IL-6 expression levels decreased significantly after 6 h (Fig. 8D, E), while TNF-α decreased after 3 h ($p < 0.01$; Fig. 8F).

Effects of Overexpression of PTX3, Del-1, and GDF-15 on Proliferation of H9N2 and H5N1 AIVs

The effects of overexpression of PTX3, Del-1, and GDF-15 on proliferation of H9N2 and H5N1 AIVs in DF-1 cells were measured at different time points. For H9N2 AIV, the virus titer of PTX3, Del-1, and GDF-15 overexpression group decreased significantly from 6 to 24 h compared with the control ($p < 0.05$; Fig. 9A). For H5N1 AIV, the virus titer was significantly reduced from 6 to 12 h in PTX3 overexpression group ($p < 0.01$; Fig. 9B), and from 6 to 24 h in Del-1 and GDF-15 overexpression group ($p < 0.05$; Fig. 9B). GDF-15 showed the strongest inhibitory effect on the replication of both H9N2 and the H5N1 avian influenza viruses (Fig. 9).

DISCUSSION

It has demonstrated that inflammatory cytokines and chemokines are critical to the pathogenesis of avian influenza [36]. In the early stage of avian influenza virus infection, the pattern recognition receptors will sense the invasion of viral RNA and initiate innate immune response resulting in production of inflammatory cytokines and chemokines [37, 38]. These inflammatory factors regulate the migration and chemotaxis of a variety of leukocytes to the site of infection and leukocyte
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Fig. 6 Expression of IL-1β, IL-6, and TNF-α in DF-1 and MDCK cells overexpressing PTX3, Del-1, and GDF-15 alone that are infected with H5N1 AIV alone. *p < 0.05; **p < 0.01. Data are presented as mean ± standard deviation (SD).

Exudation causes severe inflammatory pathological damage [39, 40].

Previous studies have shown that different clinical manifestations arise upon infection of different influenza viruses in the same host, or identical influenza viruses in different hosts [41, 42]. The underlying reason for this is differences in host inflammatory responses. The inhibitory effects of negative regulatory factors, PTX3, Del-1, and GDF-15, play an important role in inflammation development [8]. PTX3, also known as the “antibody precursor,” is a humoral pattern recognition receptor that recognizes foreign microorganisms and acts as an opsonin to regulate inflammatory responses [43]. PTX3 is produced by phagocytes and stored by neutrophil granulocytes. After stimulation, PTX3 can bind to antigens and be recognized by macrophages or neutrophil Fcγ receptor IIa (FcγRIIa/CD32) and complement receptor 3 (CD11b/CD18), thus enhancing its phagocytosis activity and promoting innate immunity [44]. PTX3-deficient mice showed higher levels of neutrophil accumulation in pleural inflammation and acid-induced acute lung injury; bone marrow chimerism experiments showed that PTX3, a hematopoietic source, mediated the inhibitory effect of leukocyte rolling [45]. Consistent with the above findings, PTX3 knockout mice showed increased neutrophil infiltration and more severe myocardial necrosis injury in the cardiac ischemia–reperfusion injury model [45]. In addition, PTX3 has been proven to be an effective inhibitor of influenza virus. The surface of PTX3 structure has salivated ligand, which can mimic the structure of cell receptor used by influenza virus, thus blocking the receptor binding site of HA, and playing a series of anti-influenza virus responses, including neutralization of virus, inhibition of hemagglutination, and inhibition of viral neuraminidase [46]. Del-1 is a secreted multi-domain protein that interacts with integrins and phospholipids and modulates different phases of the host inflammatory response depending on its expression location [47]. Due to its anti-inflammatory properties, Del-1 can prevent many of inflammation-related diseases, such as autoimmune encephalitis, inflammatory bone loss, lung inflammation and fibrosis, and inflammation associated with islet transplantation [47]. Del-1 has been shown to be an inhibitor of IL-17 which could mediate the recruitment of neutrophils. In periodontitis models, Del-1 inhibits the recruitment of neutrophils and the associated inflammatory response to mediate the pathological response by inhibiting the expression of IL-17 [48]. Interestingly, there is a reciprocal relationship between Del-1 and IL-17 in inflammatory tissues. IL-17 can down-regulate the expression of Del-1 in endothelial cells by regulating an important transcription factor C/EBPβ [48]. GDF-15 is a stress response protein belonging to the transforming growth factor-β superfamily. Under normal physiological conditions, GDF-15 is not expressed in many tissues, but under stress conditions such as hypoxia, inflammation, or pressure load, its expression in tissues, especially the heart, is significantly increased [49]. GDF-15 improves the inflammatory response of the heart after myocardial infarction by inhibiting leukocyte recruitment, thereby reducing infarct size; in contrast, mortality increased after myocardial rupture and infarction in GDF-15 deficient mice [50]. In addition, GDF-15 can inhibit the transendothelial migration of leukocytes and protect the myocardium from ischemia–reperfusion injury [13]. In addition to its anti-inflammatory effects, GDF-15 is closely related to the occurrence and development of tumors [51]. However, the precise roles of PTX3, Del-1, and GDF-15 in AIV-mediated inflammation in different hosts have not been verified in detail.

Different influenza viruses cause different inflammatory reactions. Species-specific differences in inflammation between mammals and birds in response to influenza viruses may be related to negative regulatory mechanisms [42]. To evaluate the role of negative inflammation regulators across different species, the response was analyzed in DF-1 and MDCK cells, which are avian and mammal cells, respectively.

Both H5N1 and H9N2 AIVs infection in DF-1 and MDCK cells caused a significant increase in the expression of PTX3, Del-1, and GDF-15, implicating these factors in regulating inflammation. A series of experiments showed that PTX3, Del-1, and GDF-15 inhibit the expression of the pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, which are notably also increased in response to infection by various AIV subtypes. Moreover, many of the damaging pathological effects in the acute inflammation phase are caused by the combined actions of these cytokines [52, 53]. Since IL-1β and IL-6 are specific to H9N2 AIV infection, and IL-1β and TNF-α are specific responses to H5N1 AIV infection [5], these cytokines were selected in this study.
For DF-1 cells, when PTX3, Del-1, and GDF-15 were individually overexpressed, the inhibitory effect on the expression of H9N2-induced inflammatory factors was strong, while, when GDF-15 was overexpressed, there was no inhibitory effect on the expression of H9N2-induced inflammatory factors. The overexpression of PTX3, Del-1, and GDF-15 all induced strong inhibition of IL-1β and IL-6 in the H5N1 infected group, while the inhibition of TNF-α was not obvious in the group with Del-1 overexpression, GDF-15 overexpression, and Del-1 and GDF-15 combined overexpression. Therefore, it is speculated that PTX3 may play the most important role in inhibiting inflammation during H5N1 infection. For MDCK cells, the overexpression of PTX3, Del-1, and GDF-15 can cause the inhibition of inflammatory cytokines in the H5N1 and H9N2 infected groups starting from 6 h. Whether it is MDCK cells or DF-1 cells, co-expression of PTX3, Del-1, and GDF-15 enhanced the inhibition. These findings suggest that initiating PTX3, Del-1, or GDF-15 could effectively inhibit the inflammatory response. Therefore, activators for the above genes would be plausible candidates to suppress AIV-mediated inflammatory responses. Overexpression of PTX3, Del-1, and GDF-15 in DF-1 cells inhibited the proliferation of H9N2 and H5N1 AIVs. The influence of PTX-3, Del-1, or GDF-15 on the proliferation of influenza virus and its possible mechanism need further research.

In summary, PTX3, Del-1, and GDF-15 attenuated the expression of inflammatory cytokines in DF-1 and MDCK cells infected H9N2 or H5N1 AIV. This partly explains the species-specific differences in clinical symptoms in response to infection by different AIV subtypes. The findings presented here contribute to the foundational knowledge that explains differences in pathogenesis by different AIV subtypes in identical or unique hosts. Additionally, given the unique cytokine expression profiles observed due to H9N2 and H5N1, the results from our study have clinical implication, as PTX3, Del-1, and GDF-15 may be used to screen for host-specific anti-inflammatory agents.

![Fig. 7 Dynamic expression of IL-1β, IL-6, and TNF-α in DF-1 and MDCK cells with pair expression of PTX3, Del-1, and GDF-15 infected with H5N1 AIV. *p < 0.05; **p < 0.01. Data are presented as mean ± standard deviation (SD).](image)

![Fig. 8 Dynamic expression of IL-1β, IL-6, and TNF-α in DF-1 and MDCK cells overexpressing PTX3, Del-1, and GDF-15 simultaneously infected with H5N1 AIV. *p < 0.05; **p < 0.01. Data are presented as mean ± standard deviation (SD).](image)
ACKNOWLEDGEMENTS

We thank all the members in our team for the excellent work.

AUTHOR CONTRIBUTION

BZ, WJ, and ZN designed the experiment; BZ, HL, SC, WZ, and BL did the experiment; BZ and HL and SC analyzed the data; BZ, WJ, and ZN wrote and revised the manuscript.

FUNDING

This work was supported by the Key Research and Development Program of Guangdong Province (2019B020218004).

AVAILABILITY OF DATA AND MATERIALS

All data and materials included in this study are available on request to the corresponding author.

Fig. 9  Inhibitory effect of overexpression of PTX3, Del-1, and GDF-15 on proliferation of H9N2 and H5N1 AIVs. * $p < 0.05$; ** $p < 0.01$. Data are presented as mean ± standard deviation (SD).
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