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

Bingqian Zhao

South China Agricultural University

Huizi Li

South China Agricultural University

Suilan Cao

South China Agricultural University

Wenxia Zhong

South China Agricultural University

Baojian Li

South China Agricultural University

Weixin Jia

South China Agricultural University

Zhangyong Ning (✉ ningzhyong@scau.edu.cn)

South China Agricultural University <https://orcid.org/0000-0002-0591-8588>

Research Article

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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.

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 (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) [2]. HPAIV H5N1 and LPAIV H9N2 are 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, 9, 10]. Negative regulators of inflammation are evolutionarily conserved and play an important role in inhibiting the inflammatory response [11, 12, 13]. PTX3, Del-1 and GDF-15 are negative regulators of inflammation and are involved in different stages of leukocyte exudation [14, 15, 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, 22, 23]. As an inhibitor of leukocyte aggregation, PTX3 competitively interferes with P-selectin interactions. This blocks neutrophil rolling and prevents neutrophil recruitment [24, 25, 26, 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 β 2

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 has 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 and MDCK cells were maintained at 37°C 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: TTATAAGCTAGCGGCCACCATGCTGCCTGGAGGAGT (underlined is the *Nhe*I restriction site), PTX3-Rev: CAGGCAGGTACCGTAGAAACATACTGAGC TCCT (underlined is the *Kpn*I restriction site); Del-1-Fwd: TATTATCTCGAGGCCACC ATGAGCCGTGCGGTGCTG (underlined is the *Xho*I restriction site), Del-1-Rev: GTA AGAGAATTCGGTCCTGCTCCGTGCAGCCCA (underlined is the *Eco*R I restriction site); GDF-15-Fwd: AGCCATAGATCTCGCCACCATGCTGCGGGACGTG (underlined is the *Bgl* II restriction site), GDF-15-Rev: AGATCGGAATTCGGGCGCAGTGACAG CGGGTG (underlined is the *Eco*R I restriction site). The primers for MDCK cells were as follows: PTX3-Fwd: AGCCATAGATCTCGCCACCATGCACCTCCCTGCGAT (underlined is the *Bgl* II restriction site), PTX3-Rev: AGATCGGAATTCGAGAAACA TACTGGGCTCCTC (underlined is the *Eco*R I restriction site); Del-1-Fwd: AGCCAT AGATCTCGCCACCATGAAGCACTTGGTAGC, (underlined is the *Bgl* II restriction site), Del-1-Rev: AGATCGGAATTCGTTCTCCTCTGCGCAGCCCA (underlined is the *Eco*R I restriction site); GDF-15-Fwd: AGCCATAGATCTCGCCACCATGCCTGA ACAGCGACC (underlined is the *Bgl* II restriction site), GDF-15-Rev: AGATCGGAA TTCGAGCGCAGTGGCAGCCCCGG (underlined is the *Eco*R I restriction site). The amplicons were digested by the restriction enzymes and cloned into the corresponding sites of pEGFP-N1 vector. All recombinant plasmids were verified by sequencing.

DF-1 cells and MDCK cells were seeded in six-well plates at 2.5×10^5 cells/well, and transfected with plasmids 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 or H5N1 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% CO₂. For H9N2 infection, TPCK-trypsin (0.25 mg/mL) was added to the culture medium. Cells were collected at 0, 3, 6, 12 and 24 h post-infection. Three independent assays on each sample at specified time points and entire experiment three times were performed.

RNA extraction, cDNA preparation and real-time PCR

Total RNA was extracted, treated, quality determined, stored and reverse-transcribed to cDNA as described in our previous reports [34, 35]. Relative expression levels of IL-1 β , 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*™ Kit (Takara Biotech, Dalian, China). Primers used for qRT-PCR were designed by Oligo 7 software (Molecular Biology Insights Inc., Cascade, CO, USA), and the sequences for DF-1 cells are as follows: IL-1 β -Fwd: ATGACCAAAGTCTGCGGAGG, IL-1 β -Rev: GAAGGACTGTGAGCGGGTGTA; IL-6-Fwd: CAAGAAGTTCACCGTG TGCGAGA, IL-6-Rev: ATTCCAGGTAGGTCTGAAAGGCG; TNF- α -Fwd: CTCAGGA CAGCCTATGCCAACA, TNF- α -Rev: CACCACACGACAGCCAAGTCAA; β -actin- Fwd : TGAAGTCCCTGATGGTCAGGTC, β -actin-Rev: ACCACAGGACTCCATACCC AAG; Primers for MDCK cells were as follows: IL-1 β -Fwd: GTCGAGCCTCATGCCGT GTTC, IL-1 β -Rev: GGCAGGCAGCAGACTCAAAGC; IL-6-Fwd: ATGGCTACTGCT TTCCCTACC, IL-6-Rev: CAGTGCCTCTTTGCTGTCTTC; TNF- α -Fwd: ATGAATCCG CCAGCCTACTACAC, TNF- α -Rev: CAATCATCTTGTGTCAGGAAGGA-3; β -actin- Fwd: CGACGGGCAGGTCATCACTA, β -actin-Rev: TTCATGGATGCCGCAGGATT. The expression levels for these genes were normalized to that of β -actin, and relative gene expression levels were calculated using the relative standard curve $2^{-\Delta\Delta C_t}$ method.

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 were used to determine statistical significance between samples. Statistically 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-1 β , IL-6 and TNF- α increased significantly in DF-1 and MDCK cells infected by H5N1 and H9N2 AIVs. In DF-1 cells, IL-1 β , IL-6 and TNF- α increased significantly from 6 h after infection with both AIVs ($p < 0.01$), but greater 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-1 β expression increased significantly from 6 h with H9N2 and H5N1 infection ($p < 0.01$), with greater 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 greater 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 greater 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 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 cells 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-1 β , 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-1 β 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-1 β at 12 h 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-1 β 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-1 β 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-1 β 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-1 β expression from 6 to 24 h ($p < 0.01$; 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-1 β 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), 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-1 β 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-1 β and TNF- α expression at 12 and 24 h ($p < 0.01$; Fig. 4G, I), 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-1 β 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-1 β 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-1 β 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-1 β and IL-6 expression after 3 h compared to control ($p < 0.0105$; 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-1 β , 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 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-1 β , 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-1 β 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-1 β 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-1 β 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-1 β 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), TNF- α decreased at 12 h ($p < 0.01$; Fig. 6O) relative to the control. When performed in MDCK cells, we observed that IL-1 β and IL-6 expression decreased significantly at 3 and 6 h compared to the control ($p < 0.05$; Fig. 6P, Q), 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-1 β , IL-6 and TNF- α decreased significantly from 6 to 24 h compared to the control ($p < 0.01$). In MDCK 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-1 β , IL-6 and TNF- α after 6 h compared to the control ($p < 0.01$; Fig. 8A-C). When repeated in MDCK cells, IL-1 β 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).

Discussion

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 [36, 37]. 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], however their precise roles in AIV-mediated inflammation in different hosts has not been verified in detail.

Different influenza viruses cause different inflammatory reactions. Species-specific differences in inflammation between mammals and birds [37] in response to influenza may be related to negative regulatory mechanisms caused by influenza viruses. To evaluate the role of negative inflammation regulators across different species, we analyzed the responses in DF-1 and MDCK cells, which are avian and mammal cells, respectively.

Both H5N1 and H9N2 AIV infection in DF-1 and MDCK cells caused a significant increase in the expression of PTX3, Del-1 and GDF-15, implicating these factors are in regulating inflammation. A series of experiments showed that PTX3, Del-1 and GDF-15 inhibit the expression of the pro-inflammatory cytokines, IL-1 β , 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 [38, 39]. Since IL-1 β and IL-6 are specific to H9N2 AIV infection, and IL-1 β and TNF- α are specific responses to H5N1 AIV infection [5], we selected these cytokines for our study.

Single or combined overexpression of PTX3, Del-1 and GDF-15 in both DF-1 and MDCK cells inhibited H9N2 and H5N1 AIV-induced inflammatory cytokine expression. 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.

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.

Declarations

Ethics Approval and Consent to Participate

Not Applicable.

Consent for publication

All authors consent for publication.

Availability of data and materials

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

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

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.

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Authors' information

Affiliations

College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642Guangdong, China.

Bingqian Zhao, Huizi Li, Suilan Cao, Wenxia Zhong, Baojian Li, Weixin Jia and Zhangyong Ning

Maoming Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Maoming 525000, Guangdong, China.

Zhangyong Ning

Corresponding authors

Weixin Jia, E-mail: jiaweixin@scau.edu.cn

Zhangyong Ning, E-mail: ningzhyong@scau.edu.cn

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Figures

Figure 1

IL-1 β , IL-6 and TNF- α expression dynamics in DF-1 and MDCK cells infected with H5N1 and H9N2 AIVs.

* $p < 0.05$; ** $p < 0.01$. Data are presented as mean $\pm$ standard deviation (SD).

Figure 2

The expression profile for PTX3, Del-1 and GDF-15 in DF-1 and MDCK cells infected with H5N1 and H9N2 AIVs. * $p < 0.05$; ** $p < 0.01$. Data are presented as mean $\pm$ standard deviation (SD).

Figure 3

IL-1 β , IL-6 and TNF- α expression 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 $\pm$ standard deviation (SD).

Figure 4

The expression profile for IL-1 β , IL-6 and TNF- α in DF-1 and MDCK cells with pair expression of PTX3, Del-1 and GDF-15 infected with H9N2 AIV. *p<0.05; **p<0.01. Data are presented as mean $\pm$ standard deviation (SD).

Figure 5

Dynamic expression of IL-1 β , IL-6 and TNF- α in DF-1 and MDCK cells overexpressing PTX3, Del-1 and GDF-15 simultaneously infected with H9N2 AIV. * $p < 0.05$; ** $p < 0.01$. Data are presented as mean $\pm$ standard deviation (SD).

Figure 6

IL-1 β , IL-6 and TNF- α expression 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 $\pm$ standard deviation (SD).

Figure 7

Dynamic expression of IL-1 β , 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 $\pm$ standard deviation (SD).

Figure 8

Dynamic expression of IL-1 β , 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 $\pm$ standard deviation (SD).